

**CENTER FOR DRUG EVALUATION AND
RESEARCH**

APPLICATION NUMBER:

125509Orig1s000

MICROBIOLOGY / VIROLOGY REVIEW(S)

BLA 125509 Standard Submission**Recommendation: Approval.****BLA125509
Addendum (1)
Review #2, February 22, 2016**

Drug Name/Dosage Form	Anthim® (obiltoxaximab) / Injection
Strength/Potency	600mg/Vial (100 mg/mL)
Route of Administration	Intravenous
Rx/OTC Dispensed	Rx
Indication	Treatment of adult and pediatric patients with inhalational anthrax due to <i>Bacillus anthracis</i> in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate.
Applicant/Sponsor	Elusys Therapeutics, Inc.
US agent, if applicable	N/A

A. Names

- a. **Proprietary Name:** Anthim
- b. **Trade Name:** Anthim®
- c. **Non-Proprietary/USAN:** Obiltoxaximab
- d. **CAS name:** CAS#1351337-07-9
- e. **Common name:** ETI-204
- f. **INN Name:** Obiltoxaximab
- g. **OPB systematic name:** MAB CHIMERIC [ETI204] (b) (4)

- B. Pharmacologic category:** Therapeutic recombinant chimeric DeImmunized™ monoclonal antibody (IgG1) binds to *Bacillus anthracis* protective antigen

Product Overview

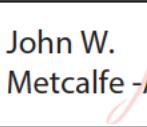
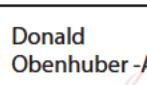
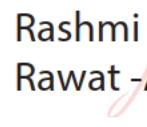
Obiltoxaximab (Anthim®) is a chimeric (mouse/human) affinity-enhanced monoclonal antibody of the IgG1κ isotype. Obiltoxaximab binds the free protective antigen domain 4 (PAD4) component of *B. anthracis* toxin and inhibits the binding of protective antigen (PA) to its cellular

receptors, and prevents the intracellular entry of the anthrax lethal factor and edema factor; the enzymatic toxin components responsible for the pathogenic effects of anthrax toxin.

Quality Review Team

DISCIPLINE	REVIEWER	BRANCH/DIVISION
Drug Substance, Drug Product and Immunogenicity	Tao Xie	Division of Biotechnology Review Research II
Facilities	Donald Obenhuber	Division of Inspectional Assessment
Microbiology Drug Substance	Bo Chi	Division of Microbiology Assessment
Microbiology Drug Product	John Metcalfe	Division of Microbiology Assessment
Business Regulatory Process Manager	Melinda Bauerlien	OPRO/OPQ
Application Technical Lead	Rashmi Rawat	Division of Biotechnology Review Research II

Quality Review Team – Signature Page

DISCIPLINE	REVIEWER	DIVISION/OFFICE	e-SIGNATURE
Drug Substance Drug Product	Tao Xie	DBRR II/OPB/OPQ	 Digitally signed by Tao Xie -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Tao Xie -S, 0.9.2342.19200300.100.1.1-0011466376 Date: 2016.02.22 14:57:17 -05'00'
Microbiology Drug Substance	Bo Chi	DMA/OPF/OPQ	 Digitally signed by Bo Chi -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Bo Chi -S, 0.9.2342.19200300.100.1.1-1300104820 Date: 2016.02.22 15:42:28 -05'00'
Microbiology Drug Product	John Metcalfe	DMA/OPF/OPQ	 Digitally signed by John W. Metcalfe -A DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=John W. Metcalfe -A, 0.9.2342.19200300.100.1.1-1300198103, cn=John W. Metcalfe -A Date: 2016.02.22 15:13:59 -05'00'
Microbiology Team Leader	Patricia Hughes	DMA/OPF/OPQ	 Digitally signed by Patricia F. Hughestrost -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Patricia F. Hughestrost, 0.9.2342.19200300.100.1.1-13000096547, cn=Patricia F. Hughestrost -S Date: 2016.02.23 07:17:35 -05'00'
Facilities	Don Obenhuber	DIA/OPF/OPQ	 Digitally signed by Donald Obenhuber -A DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Donald Obenhuber, 0.9.2342.19200300.100.1.1-2000330000, cn=Donald Obenhuber -A Date: 2016.02.23 11:26:51 -05'00'
Application Technical Lead and Team Leader	Rashmi Rawat	DBRRII/OPB/OPQ	 Digitally signed by Rashmi Rawat -A DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Rashmi Rawat -A, 0.9.2342.19200300.100.1.1-0014137532 Date: 2016.02.22 14:53:51 -05'00'

Acting Review Chief Product Quality	Juhong Liu	DBRRII/OPB/OPQ	Juhong Liu -S
			Digital signature details: Digitally signed by Juhong Liu -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, cn=Juhong Liu -S, 09224219200300100.1.1-00104 01517 Date: 2016.02.22 21:47:29-05'00'

Quality Review Data Sheet

1. LEGAL BASIS FOR SUBMISSION: 351(a)

2. RELATED/SUPPORTING DOCUMENTS:

A. Submission Reviewed Under This Addendum

SUBMISSION(S) REVIEWED	DOCUMENT DATE
Response to quality questions (IR-11, dated 13Nov2015), eCTD seq#0045	3 February. 2016

B. Other Documents: *The sponsor's response to the issues identified in 483 form during pre-licensing inspection of Lonza Plc, Portsmouth, NH were received and reviewed by the facility reviewer.*

3. CONSULTS: *None*

Executive Summary

I. Recommendations

A Recommendation and Conclusion on Approvability

a. Recommendation

The Office of Pharmaceutical Quality, CDER, recommends approval of STN 125509 Anthim (obiltoxaximab) manufactured by Elusys Therapeutic, Inc. The data submitted in this application are adequate to support the conclusion that the manufacture of Anthim is well controlled and leads to a product that is pure and potent. It is recommended that this product be approved for human use under conditions specified in the package insert.

b. Approval action letter language

This section remains the same as in the original Executive Summary and is therefore not repeated.

c. Review Summary and Benefit/Risk Considerations

The obiltoxaximab is a chimeric antibody that targets anthrax protective antigen (PA). The mechanism of action is based on its ability to neutralize the lethal toxin mediated cytotoxicity. In the primary review of the BLA, the microbiology product quality reviewer identified the DS and DP endotoxin testing issue related to the endotoxin recovery assessment in the product formulation. The sponsor was asked to perform a study demonstrating that the DS and DP formulation does not affect the endotoxin recovery in the proposed LAL endotoxin test. The sponsor submitted their response on February 3, 2006. The product quality microbiology reviewers have evaluated the sponsor's response and found it adequate in addressing the issue of endotoxin recovery in LAL assay. Additionally the drug substance manufacturing facility issues, identified in Form FDA 483 during pre-licensing inspection of the Lonza facility are also adequately addressed.

Therefore, we conclude that obiltoxaximab DS and DP manufacturing processes are well controlled and capable of producing product of consistent quality.

There are some minor product quality issues identified during the BLA review, which do not preclude approval of the BLA and will be addressed as post marketing commitments.

B Recommendation on Phase 4 (Post-Marketing) Commitments, Agreements, and/or Risk Management Steps, if Approvable

1. Develop reduced and non-reduced SDS-based assays capable of providing quantitative data for the evaluation of size related product impurities and implement these assays in the release and stability program for obiltoxaximab drug substance and drug product after sufficient data have been acquired to set appropriate acceptance criteria. Provide the analytical procedure, validation report, proposed acceptance criteria, and data used to set the proposed acceptance criteria.

2. Conduct validation studies to confirm the shipper is suitable for maintaining critical quality attributes during shipping of obiltoxaximab drug product. This should include consideration for worst case shipping routes. The study will include monitoring of temperature during the shipment, as well as testing of pre- and post- shipping samples of obiltoxaximab drug product quality (e.g., appearance, protein concentration, purity by SEC-HPLC, reduced and non-reduced SDS-PAGE, icIEF, visible and sub-visible particulates and potency).
3. Conduct a study to confirm compatibility of the drug product with syringe infusion components used for administration. These studies will include monitoring samples for protein concentration, purity by SEC-HPLC, icIEF, visible and sub-visible particulates; and potency.
4. Conduct a study to support the worst case cumulative hold times in obiltoxaximab drug substance manufacturing process to demonstrate that the worst case cumulative hold time will not adversely affect the product quality of obiltoxaximab drug substance. These data are expected to demonstrate that there is no adverse impact to product quality when the manufacturing of a drug substance batch involves [REDACTED] (b) (4)
5. Re-evaluate obiltoxaximab drug substance lot release and stability specifications after 20 lots have been manufactured using the commercial manufacturing process. Provide the final report, the corresponding data, the analysis, and the statistical plan used to evaluate the specifications.
6. Re-evaluate obiltoxaximab drug product lot release and stability specifications after 20 lots have been manufactured using the commercial manufacturing process. Provide the final report, the corresponding data, the analysis, and the statistical plan used to evaluate the specifications.
7. Establish a permanent control limit for [REDACTED] (b) (4), of production [REDACTED] (b) (4) and [REDACTED] (b) (4) of [REDACTED] (b) (4) unit operations after [REDACTED] (b) (4) control points have been analyzed. The [REDACTED] (b) (4) limits and supportive data should be submitted to the BLA.
8. To conduct drug substance specific leachable and extractable studies on the [REDACTED] (b) (4). The drug substance manufacturing processes will be optimized, as needed, based on the results.
9. Conduct a study to qualify the bioburden test for the primary recovery samples using the increased sample volume (10 mL).
10. Re-evaluate and establish final [REDACTED] (b) (4) bioburden and endotoxin limits for all the sampling points after ten commercial lots have been manufactured.



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II. Summary of Quality Assessments

A. CQA Identification, Risk and Lifecycle Knowledge Management

Table 1 below provides a summary of product related critical quality attributes that are relevant to both drug substance and drug product. The table includes the identification of the various attributes along with their risk management. For additional information see Appendix A for the OBP product quality review and Appendix B for the product quality microbiology review.

Identification of other CQAs specific to drug substance (e.g., process related impurities, adventitious agents, pH, appearance, etc.) or drug product are described in separate risk tables in section B, Drug Substance Quality Summary and section C, Drug Product Quality Summary.

Table 1: Drug Substance API CQA Identification, Risk and Lifecycle Knowledge Management

CQA	Risk	Origin	Control Strategy	Other (b) (4)



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(b) (4)

B. Drug Substance [obiltoxaximab] Quality Summary

Table 2 provides a summary of the identification, risk, and lifecycle knowledge management for drug substance CQAs that derive from the drug substance manufacturing process and general drug substance attributes.

Table 2: Drug Substance CQA Identification, Risk, and Lifecycle Knowledge Management

CQA	Risk	Origin	Control Strategy	Other (b) (4)
(b) (4)	Safety and immunogenicity	Process-related impurity from the (b) (4) [redacted]		
(b) (4)	Safety	Process related impurity from the (b) (4) [redacted]		
(b) (4)	Safety, PK and immunogenicity	Process related impurity from the (b) (4) [redacted]		



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(b) (4)	Safety	Process related impurity	(b) (4)
Bioburden	Safety	Contaminant. Bioburden can be introduced throughout the manufacturing process.	
(b) (4)	Safety	Contaminant (b) (4) can be introduced throughout the manufacturing process.	
(b) (4)	Safety	Contaminant (b) (4) would most likely be introduced during cell culture operations.	
(b) (4)	Safety	Contaminant (b) (4) would most likely be introduced during cell culture operations.	
pH	General CQA: Safety and efficacy	General CQA. Formulation	(b) (4)
Protein Concentration	General CQA: Safety and efficacy		



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(b) (4)

Appearance: color	General CQA: Safety and efficacy	The color of the DS can be affected by all steps of the manufacturing process, storage condition and exposure of light	
Appearance, clarity	General CQA: Safety and efficacy	The clarity of the DS can be affected by all steps of the manufacturing process. Study shows that deamidation and oxidation of the product result in change of color	
Identity	Safety and efficacy	N/A	
Leachables/extractables	Safety and efficacy (indirectly through effects on stability)	Process-related impurity. Leachables and extractables could potentially be introduced by any product contact equipment, containers and consumables.	

- a. Description: Obiltoxaximab is a chimeric affinity-enhanced monoclonal antibody (mAb) of the IgG1κ isotype that binds the PA component of *B. anthracis* toxin. Obiltoxaximab has a molecular weight of approximately 148 kDa. Obiltoxaximab is produced via cultures of [REDACTED] (b)(4) murine GS-NS0 myeloma cells.
- b. Mechanism of action: Obiltoxaximab binds free PA with an affinity equilibrium dissociation constant (Kd) of 0.33 nM, and inhibits the binding of PA to its cellular receptors, preventing the intracellular entry of the anthrax lethal factor and edema factor, the enzymatic toxin components responsible for the pathogenic effects of anthrax toxin.
- c. Potency Assay: The biological activity of obiltoxaximab is determined by two methods. An ELISA based antigen binding assay PAA3 that measures the functional binding activity of obiltoxaximab and a cell-based bioassay Lethal Neutralization Assay (LNA) that measures neutralization of lethal-toxin mediated cytotoxicity.
- d. Reference material(s): The current reference material [REDACTED] (b)(4) was produced from [REDACTED] (b)(4). The sponsor commits to [REDACTED] (b)(4)
- e. Critical starting materials or intermediates: [REDACTED] (b)(4)

Cell Banks: Obiltoxaximab is produced from a recombinant DNA-derived murine myeloma cell line (NS0) using standard cell culture techniques. The obiltoxaximab antibody was engineered from the murine monoclonal antibody (14B7) gene sequences (H and L chain sequences) originally developed by the United States Army Medical Research Institute for Infectious Diseases (USAMRIID). Elusys obtained the original 14B7 hybridoma from NIH under a Cooperative Research and Development Agreement (CRADA) with USAMRIID. The manufacturing cell line (b)(4) for the production of ETI-204 was developed by Elusys under contract with [REDACTED] (b)(4)
[REDACTED] (b)(4) DNA sequence in variable regions on both the heavy and light chains of obiltoxaximab gene have been manipulated to eliminate potential immunogenic murine sequences and enhance the affinity of the antibody for its target. The sequence coding Fc region is from human. The recombinant obiltoxaximab gene is expressed using Glutamine Synthase (GS) Expression System.
- f. Manufacturing process summary: Obiltoxaximab is manufactured at Lonza Biologics NH, USA using standard monoclonal antibody manufacturing process. [REDACTED] (b)(4)

(b) (4)



- g. Container closure: The container closure system (CCS) for obiltoxaximab BDS is a (b) (4) [REDACTED] . A proprietary extractable study has been conducted on the DS container closure system to demonstrate that the (b) (4) for obiltoxaximab is safe and acceptable for storage of the BDS at the recommended storage conditions.
- h. Dating period and storage conditions: The data provided in the BLA support a shelf of (b) (4) months for obiltoxaximab drug substance when stored at (b) (4) C. The post-approval stability protocols and commitment to place (b) (4) DS (b) (4) each year (with production) on stability protocols are provided and found to be acceptable.

C. Drug Product [Anthim] Quality Summary

Table 3 provides a summary of the identification, risk, and lifecycle knowledge management for drug product CQAs:



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Table 3: Drug Product CQA Identification, Risk, and Lifecycle Knowledge Management

CQA	Risk	Origin	Control Strategy	Other (b) (4)
Sterility	Safety	Contaminants could be introduced throughout DP manufacturing or through a container closure integrity failure.		
Endotoxin	Safety	Contaminants could be introduced throughout DP manufacturing or due to integrity failure of the container closure.		
Particulate matter (sub-visible)	Safety and Immunogenicity	Sub-visible particulates could form throughout manufacturing or on storage. Might contain product- and process- related impurities		
Appearance - Visible particulates	Safety and efficacy	Visible particulate might be impacted by the manufacturing process and storage conditions		
Appearance – Color, Clarity and degree of opalascence	Safety and efficacy	Manufacturing process, formulation, and storage conditions		
pH	Safety and efficacy	pH is most likely impacted by formulation and compounding. No change on stability is expected.		
Osmolality	Safety	Osmolality is most likely impacted by formulation and compounding. No change is		

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		expected on stability.		
Polysorbate 80	Safety and efficacy	Level of Polysorbate 80 excipient is impacted by formulation and compounding. No changes are expected on stability.	(b) (4)	
Protein Concentration	Safety and efficacy	Protein concentration would be impacted by formulation and compounding. Might be impacted by storage conditions		
Identity	Safety and efficacy	General CQA		
Container closure integrity	Safety	CQA, Might be impacted by storage conditions		
Leachables/extractables	Safety	Process-related leachables and extractables could potentially be introduced by any product contact equipment, containers and consumables.		

- a. Potency and Strength: Anthim is supplied as 600 mg/6 mL (100 mg/mL) solution in single-dose vials for intravenous injection.
- b. Description/Commercial Image: Anthim is a clear to opalescent, colorless to pale yellow to pale brownish-yellow solution and may contain few translucent-to-white proteinaceous particulates. Anthim is provided in sterile, [REDACTED] (b)(4) mL type 1 glass vial with [REDACTED] (b)(4) rubber stopper (gray) and an aluminum seal with [REDACTED] (b)(4) flip-off cap [REDACTED] (b)(4)
- c. List of Excipients: Each mL of Anthim contains 6.2 mg of L-histidine, [REDACTED] (b)(4) mg of polysorbate 80, 36 mg of sorbitol. [REDACTED] (b)(4)
[REDACTED] pH to 5.5.
- d. Reference material(s): Same as drug substance
- e. Manufacturing Process: [REDACTED] (b)(4)
- f. Container Closure: The container closure system for Anthim drug product includes vial, stopper and seal. The vial is a [REDACTED] (b)(4) mL Type 1 glass [REDACTED] (b)(4)
- g. Expiration Date & Storage Conditions: The stability data in the BLA support a shelf life of 18 months for Anthim drug products when stored at 5 ±3°C.
- h. List of co-packaged components: N/A

D. Novel Approaches/Precedents

None

E. Any Special Product Quality Labeling Recommendations

Store in a refrigerator at 2-8°C

Do not freeze

Protect from light

Do not shake

**QUALITY REVIEW BLA 125509 (Obiltoxaximab)****F. Establishment Information**

OVERALL RECOMMENDATION:					
DRUG SUBSTANCE					
Function	Site Information	Duns/FEI Number	Preliminary Assessment	Inspectional Observations	Final Recommendation
Manufacture of ETI-204 BDS (b) (4)	Lonza Biologics, Inc. Portsmouth, NH 03801	3001451441	Approve Facility: Based on PLI inspection		Approved
(b) (4)	(b) (4)	(b) (4)	Approve Facility: Based on Profile	Inspection of this control testing laboratory was on (b) (4) and covered a nonclinical laboratory study focused on biocompatibility testing for the (b) (4)	Approved



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(b) (4)	(b) (4)	(b) (4)	(b) (4)	The current inspection did not result in the issuance of a FDA 483 and no verbal observations were discussed.	(b) (4)	
(b) (4)	(b) (4)	(b) (4)	Approve Facility: Based on Profile	(b) (4)	Approved	



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



(b) (4)	(b) (4)	(b) (4)	(b) (4)	(b) (4)
(b) (4)	Approve			Approved
Approve Facility: based on profile	Current inspection (b) (4) covered the firm's quality, laboratory and facility and equipment systems. No FDA 483 was issued and there were no significant discussion items addressed with management.	(b) (4)		Approved



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



					(b) (4)
					(b) (4)
			(b) (4)	Approve facility: based on profile	Approved
DRUG PRODUCT					
Function	Site Information	Duns/Fei Number		Inspectional Observations	Final Recommendation
			(b) (4)	Approve Facility: district recommendation	Approved
		Approved facility		(b) (4)	Approved



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



(b) (4)		
Approved		Approved
Approve Facility based on profile	(b) (4)	
Approve Facility Based on Profile		Approved



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



(b) (4)	(b) (4)	(b) (4)	(b) (4)	(b) (4)
(b) (4)	Approve facility: based on profile	(b) (4)	(b) (4)	Approved
Approve	Abbreviated cGMP inspection of this	Approved		



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



(b) (4)	Facility: District Recommen- dation	contact testing laboratory specializing in (b) (4) Inspection is classified NAI.	
		Previous inspection of (b) (4) was classified NAI.	
(b) (4)	Approved facility		Approved



QUALITY REVIEW BLA 125509 (Obiltoxaximab)



G. Lifecycle Knowledge Management

B. Drug Substance

- a. Protocols approved: Annual GMP stability protocol, stability protocols for the [REDACTED] (b) (4) for bulk drug substance, concurrent [REDACTED] (b) (4) protocols
- b. Outstanding review issues/residual risk: None
- c. Future inspection points to consider: Follow up on 483 citations

C. Drug Product

- a. Protocols approved: Annual GMP stability protocol and Stability protocols for the [REDACTED] (b) (4)
- b. Outstanding review issues/residual risk: See PMCs
- c. Future inspection points to consider: Follow up on 483 citations

Quality Assessment Summary Tables

Table 1: Noteworthy Elements of the Application

#	Checklist	Yes	No	N/A
Product Type				
1.	Recombinant Product	X		
2.	Naturally Derived Product		X	
3.	Botanical		X	
4.	Human Cell Substrate/Source Material		X	
5.	Non-Human Primate Cell Substrate/Source Material		X	
6.	Non- Primate Mammalian Cell Substrate/Source Material	X		
7.	Non-Mammalian Cell Substrate/Source Material		X	

**QUALITY REVIEW BLA 125509 (Obiltoxaximab)**

8.	Transgenic Animal Sourced		X	
9.	Transgenic Plant Sourced		X	
10.	New Molecular Entity	X		
11.	PEPFAR Drug		X	
12.	PET Drug		X	
13.	Sterile Drug Product	X		
14.	Other _____			
Regulatory Considerations				
15.	Citizen Petition and/or Controlled Correspondence Linked to the Application (#_____)		X	
16.	Comparability Protocol(s)		X	
17.	End of Phase II/Pre-NDA Agreements item)			
18.	SPOTS (Special Products On-line Tracking System)		X	
19.	USAN Name Assigned	X		
20.	Other _____			
Quality Considerations				
21.	Drug Substance Overage		X	
22.	Design Space	Formulation	X	
23.		Process	X	
24.		Analytical Methods	X	
25.		Other	X	
26.	Other QbD Elements			
27.	Real Time Release Testing (RTRT)		X	
28.	Parametric Release in lieu of Sterility Testing		X	
29.	Alternative Microbiological Test Methods		X	

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30.	Process Analytical Technology in Commercial Production		X	
31.	Non-compendial Analytical Procedures	Drug Product	X	
32.		Excipients		X
33.		Drug Substance	X	
34.	Excipients	Human or Animal Origin		X
35.		Novel		X
36.	Nanomaterials		X	
37.	Genotoxic Impurities or Structural Alerts		X	
38.	Continuous Manufacturing		X	
39.	Use of Models for Release		X	
40.	Other _____			



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Center for Drug Evaluation and Research
WO Bldg 51
10903 New Hampshire Ave.
Silver Spring, MD 20993

Date: 2/1/2016
To: Administrative File, STN 125509/0
From: Bo Chi, Ph.D., CDER/OPQ/OPF/DMA/Branch IV
Endorsement: Patricia Hughes, Ph.D., Acting Branch Chief, CDER/OPQ/OPF/DMA/Branch IV
Subject: Addendum to review memo for New Biologic License Applications (BLA)
STN125509/0 dated 11/20/2015
Applicant: Elusys Therapeutics, Inc.
US License: 1907
Facility: Lonza Biologics, Incorporated
Portsmouth, NH
FEI: 3001451441
Product: Anthim (Obiltoxaximab)
Dosage: 600mg/6 mL single use vial, Intravenous Infusion
Indication: Adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis* in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate
PDUFA date: March 20, 2016

Recommendation: The drug substance section of this BLA, as amended, is recommended for approval from product quality microbiology perspective with the following post-market commitments:

- Re-evaluate and establish final [REDACTED] (b) (4) bioburden and endotoxin limits for all the sampling points.
 - Conduct a study to qualify the bioburden test for the primary recovery samples using the increased sample volume (10 mL).
-

Review Summary

This review amends the drug substance microbiology product quality review memo for Elusys' BLA STN125509/0 dated 11/20/2015 with new information and data submitted by the applicant (amendments dated 2/3/2016, sequence 45) pertaining to endotoxin [REDACTED] (b) (4) data for drug substance and drug product.

Endotoxin [REDACTED] (b) (4)

2 Page(s) have been Withheld in Full as b4 (CCI/TS) immediately following this page

Reviewer comment: The DS and DP samples met the acceptance criteria of endotoxin (b)(4) for up to 14 days. The current DS and DP endotoxin release tests are acceptable.

Satisfactory

The sponsor sent an email to the OND RPM Jane Dean on 2/4/2016 and requested (b)(4)

Conclusion

The drug substance section of this BLA, as amended, is recommended for approval from product quality microbiology perspective with the following post-market commitments:

- Re-evaluate and establish final (b)(4) bioburden and endotoxin limits for all the sampling points.
- Conduct a study to qualify the bioburden test for the primary recovery samples using the increased sample volume (10 mL).

Cc: Chi
Hughes
Dean

Primary reviewer signature

Bo Chi -A
Digitally signed by Bo Chi -A
DN: c=US, o=U.S. Government, ou=HHS,
ou=FDA, ou=People, cn=Bo Chi -A,
0.9.2342.19200300.100.1.1=1300194820
Date: 2016.02.18 16:31:41 -05'00'

Secondary reviewer signature

Patricia F.
Hughestroost -S
Digitally signed by Patricia F. Hughestroost -
S
DN: c=US, o=U.S. Government, ou=HHS,
ou=FDA, ou=People,
0.9.2342.19200300.100.1.1=1300096547,
cn=Patricia F. Hughestroost -S
Date: 2016.02.19 06:55:37 -05'00'



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Center for Drug Evaluation and Research
10903 New Hampshire Avenue
Silver Spring, MD 20993

Date: 10 February 2016
To: Administrative File, STN 125509/0
From: John W. Metcalfe, Ph.D., CDER/OPQ/OPF/DMA
Endorsed: Patricia Hughes, Ph.D. CDER/OPQ/OPF/DMA
Subject: Original Biologic License Application
US License: 1907
Applicant: Elusys Therapeutics, Inc.
Facility: DP: [REDACTED] (b) (4)
Product: Anthim (Obiltoxaximab)
Dosage: Sterile solution (600 mg/6 mL) in a single dose vial for IV infusion
Indication: The drug product is indicated for the treatment of adult and pediatric patients with inhalation anthrax in combination with antibacterials
Sub Dates: Original Application: 20 MAR 2015; Micro Info Amendments: 15 SEP 2015, 27 OCT 2015, 13 NOV 2015, 03 DEC 2015 and 03 FEB 2016
Goal date: 20 March 2016

Recommendation: BLA 125509, as amended, is recommended for approval from a microbiology product quality perspective.

INTRODUCTION

The subject BLA was submitted on 20 March 2015 for Anthim (obiltoxoaximab); an affinity enhanced IgG1 monoclonal antibody produced in GS-NSO myeloma cells that targets *B. anthracis* protective antigen. The application is submitted in eCTD format. The drug substance is manufactured at Lonza Biologics (101 International Dr., Portsmouth, NH).

This review covers issues pertaining to the microbiological quality of the drug product. The microbiological quality review of the drug substance was performed by Dr. Bo Chi in a separate review memorandum.

Microbiology Information Requests were forwarded to the applicant on 27 August 2015, 06 October 2015, 05 November 2015 and 23 November 2015. The applicant responses to these requests were submitted on 15 September 2015, 27 October 2015, 13 November 2015 and 03 December 2015. The questions and applicant responses are summarized in appropriate sections of this review.

ASSESSMENT

P DRUG PRODUCT

P.1 Description of the Composition of the Drug Product

- Description of drug product

The applicant describes the subject drug product as a “clear to opalescent, colorless to pale yellow-pale brownish-yellow solution” (module 3.2.P.1 of the subject submission).

- Drug product composition

The drug product composition is provided in table 1 which is copied from table 1 of module 3.2.P.1.

Table 1. Drug Product Composition

Component	Function	Quality Standard	Concentration	Amount per Vial (b) (4) nL)
ETI-204	Active ingredient (b) (4)	Elusys (see 3.2.S.4.1)	100 mg/mL	(b) (4)
L-Histidine		USP/NF	40 mM	
Sorbitol		USP/NF	200 mM	
Polysorbate 80		USP/NF	0.01%	
		USP/NF	As needed	
		USP/NF	As needed	

- Description of container closure system

The container closure system is comprised of a (b) (4)mL Type 1 glass vial

a grey (b) (4) rubber stopper

and an aluminum

flip-off seal

Satisfactory

Reviewer's Comment

The applicant has provided an adequate description of the drug product for the reviewer to assess the manufacturing process, controls and testing with regard to the microbiological quality of the drug product.

P.2 Pharmaceutical Development

P.2.5 Microbiological Attributes

- Container-Closure and Package integrity

The applicant demonstrated container closure integrity by performing a microbial immersion test in media filled vials. The applicant states that they have determined the worst case capping limits to be compression forces of (b) (4)

The units used for container closure

integrity testing were filled with 2.5 mL of TSB. A total of 1377 units were filled, half of which were capped using each of the above worst case compression forces.

A contract testing facility [REDACTED] (b)(4) performed the CCI testing. [REDACTED] (b)(4)

[REDACTED] To demonstrate growth promotion of the media, 2 units each were inoculated with:

- *Aspergillus brasiliensis*
- *Bacillus subtilis* subsp. *spizizenii*
- *Candida albicans*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*

(b)(4)



Data from this container closure integrity test are described in section 5.8.2 of module 3.2.P.3. Growth was observed in all of the positive controls and growth promotion controls. No microbial growth was observed in either the challenge units or the negative controls.

- Preservative Effectiveness

The subject drug product is labeled as a single dose product.

Satisfactory

Reviewer's Comments

1. The application also describes a helium leak test performed on 40 filled units by [REDACTED] (b)(4). This information is not reviewed here since the microbial ingress data are acceptable.
2. The applicant's verification of container closure integrity is consistent with regulatory expectations for a sterile pharmaceutical product.
3. Demonstration of preservative effectiveness is not necessary for a single dose product.

P.3 Manufacture

P.3.1 Manufacturers

(b) (4)

P.3.3 Description of the Manufacturing Process and Process Controls

The drug product manufacturing process begins by

(b) (4)

(b) (4)

IR for Applicant on 23 November 2015

1. Reference is made to table 4 of module 3.2.P.3.4 which identifies the

(b) (4)

for manufacture of the drug product. We note that the volume of the

(b) (4)

bioburden sample is not provided.

- Amend table 4 of module 3.2.P.3.4 with the bioburden sample volume. If the volume is less than 100 mL, provide a rationale for the size of the sample.

Summary of Response Submitted on 03 December 2015

The applicant states that the bioburden sample volume is

(b) (4)

mL. This

(b) (4)

volume was chosen based on the

The bioburden sample volume allows for some excess to ensure the

(b) (4)

mL of sample is available for this testing.

Satisfactory

Reviewer's Comment

The rationale for the bioburden sample volume is acceptable. In addition, the applicant updated table 4 of module 3.2.P.3.4 of the application with this information.

(b) (4) MANUFACTURING PROCESS

22 Page(s) have been Withheld in Full as b4 (CCI/TS) immediately following this page

(b) (4)



END

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

JOHN W METCALFE
02/10/2016

COLLEEN THOMAS
02/10/2016

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BLA #125509 (Original; SDN-001)
Date Company Submitted: 03/20/2015
Date Received by CDER: 03/20/2015
Date Assigned: 05/13/2015
Date Review Completed: 11/16/2015
Reviewer: Lynette Y. Berkeley

APPLICANT

Elusys Therapeutics, Inc.
25 Riverside Drive, Unit 1'
Pinebrook,
New Jersey

CONTACT PERSON

Elusys Therapeutics, Inc.
25 Riverside Drive, Unit 1'
Pinebrook, New Jersey

DRUG PRODUCT NAME

- a. Proprietary: Anthim
- b. Nonproprietary name: Obiltoxaximab; ETI-204
- c. Antibody Class Type: IgG1κ humanized monoclonal antibody

STRUCTURAL FORMULA:

None

Molecular weight:

(b) (4)

Emperical Formula:

(b) (4)

PROPOSED INDICATION

Treatment of adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis*, in combination with appropriate anti-bacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate.

**PROPOSED DOSAGE FORM, STRENGTH, ROUTE OF ADMINISTRATION
AND DURATION OF TREATMENT**

Dosage form: Liquid solution (100 mg/mL)
Route of administration: Intravenous infusion over 90 minutes /intramuscular
Dosage: 16 mg/kg

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DISPENSED

Rx.

RELATED DOCUMENTS

IND 12,285; PreIND [REDACTED] (b) (4) [REDACTED] DMF [REDACTED] (b) (4)
[National Institutes of Allergy and Infectious Disease (NIAID)], and DMF [REDACTED] (b) (4) (Type
V) – Lonza.

APPEARS THIS WAY ON ORIGINAL

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1. EXECUTIVE SUMMARY

The details of the method and performance characteristics for five different types of assays are presented in this report. The assays relate to analyses involving the presence of the protective antigen (PA) in *Bacillus anthracis*, bacteremia a symptom of anthrax, and therapy with ETI-204. PA is a toxin produced by *Bacillus anthracis* in infected subjects, and ETI-204 is a humanized, deimmunized, monoclonal antibody that binds PA with high affinity, and neutralizes its toxic effect. ETI-204 is being proposed for use in the treatment of anthrax.

The assays are: (i) Electrochemiluminescent (ECL) assay (screening) for detection of the PA in the sera of New Zealand White Rabbits and Cynomolgus monkeys, and the ECL assay for quantitation of ETI-204. (ii) The direct and (iii) indirect Enzyme-linked Immunosorbent Assay (ELISA) for detection of PA, and anti-PA IgG antibodies respectively, in the sera of monkeys and rabbits. (iv) The toxin neutralization (TNA) assay for the detection of neutralizing antibodies in monkeys and rabbits. (v) Culture for isolation, and differentiation of *Bacillus anthracis* from contaminating bacteria.

All of the assays have been validated. However, there were shortcomings in some of the parameters. One of the major weaknesses that cuts across all assays was the wide limits that were specified for the acceptance criteria. The second major shortcoming was the omission of some critical interferents that can be found in the testing of blood samples in general, this included the testing of different concentrations of some of the components that might interfere with the sample results. The most common interferent that lacked sufficient definition, was hemolysis which might interfere with the expression of an analyte at a high concentration, but not at low concentrations. Additionally, some components specific to infection with *Bacillus anthracis* that may cross-react with PA or inhibit its detection, were omitted.

Temperature stability of PA was evaluated in a few assays. In general, specimens were stable for as many as 100 days at freezing temperature of -60°C to – 80°C. In one study, it was advised that specimens not be held at room temperature for more than 15 minutes, another study suggested that the specimens could be held for as long as 4 hours at room temperature without decreased activity.

The ECL assay used for testing of monkey and rabbit sera for PA in infected animals was generally acceptable. The rabbit ECL assay emphasized the fact that animals should be pre-screened for the presence of anti – PA IgG. In the study where it was found that 12% of the aerosol challenged rabbits were falsely negative, anti-PA IgG was detected in 56% of the PA spiked hemolyzed specimens that were false-negative for PA. Some of the samples that were subsequently found to be positive for PA were incorrectly flagged as negative 6 to 12 hours earlier. This observation is important because of the urgency required for life saving treatment. The applicant stated that the bacterial count at the time of negativity was ≤ 5 colony forming units. The applicant did not state the volume of blood that was used to inoculate the culture plate.

Two types of ELISA assays were used in the study. The direct ELISA assay was used to quantitate PA in the blood of monkeys and rabbits. The indirect ELISA was used to quantitate antibody production in the same animals. The direct ELISA quantitated both total and free PA. The results of testing to support the accuracy, precision, specificity of assays for total PA by ELISA in cynomolgus monkeys were adequate, and met the criteria set forth for acceptability by the applicant. However, 27% of the high level QC values were above 300 ng/mL, the nominal

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value was 248 ng/mL. Additionally, 37% of the QC values were above 2 standard deviations. This indicates a problem at the high end of the curve. This problem might be related to the instability of the ULOQ cut-off. The precision of the test, as indicated by the percent coefficient of variation and percent relative error, was appropriate. Overall, the assay supported the LOD of 9.68 ng/mL and ULOD of 309 ng/mL.

The results of testing to demonstrate the accuracy, precision, and specificity of testing for free PA in rabbits using this ELISA assay met the criteria set forth for acceptability by the applicant. The assay showed acceptable linearity but only between 10.5 ng/mL and 410 ng/mL. Specificity tests showed that PA was not affected by most of the interferents at low or moderate levels that are expected to be found in specimens of recently infected animals. Ciprofloxacin was included in the group of agents that may possibly mask the detection of PA in a sample, this is a drug with which the infected animal may be treated. Ciprofloxacin did not affect the recovery of PA. PA is not consistently affected by the other two major components, edema factor and lethal factor, of the tri-partite toxin, except at elevated concentrations of each. The assay demonstrated a high level of sensitivity with regard to the minute quantity of PA that could be detected. The LOD for PA in both monkeys and rabbits was 4.2 ng/mL.

The indirect ELISA assay was used to measure the specific IgG antibody to PA in the serum of cynomolgus monkeys and rabbits infected with, or exposed to, *Bacillus anthracis*. All parameters passed the validation criteria.

The toxin neutralization test, for the detection of neutralizing antibodies was conducted in two laboratories at [REDACTED] ^{(b) (4)} The tests were evaluated using endpoints of effective dilution₅₀ (ED₅₀) and neutralization factor₅₀ (NF₅₀). This report focused primarily on the results of ED₅₀, which were more independent than NF₅₀, because the calculation of NF₅₀ depended on the value of ED₅₀. The applicant first compared the results obtained from both laboratories to ascertain that the results fell within an acceptable limit, in order to be generalized. The results obtained from the two laboratories were comparable.

All of the parameters tested in the TNA assay were validated. One of the unique characteristics of this assay was that the dilution results were not reported as the direct values obtained, but were calculated according to the initial dilution factor of the specific sample. The accuracy of the results tended to decrease with increasing initial factors of dilutions e.g. 1:50 versus 1:100. When the sample was tested more than once the error rate decreased, this is normally expected. The purpose of this assay in real life would be to determine the antibody response to infection or vaccination. The assay presented three types of curves for qualitatively describing sample results - full/reactive (positive), partial reactive (positive) and non – reactive (negative). The neutralizing antibody titers were higher in rabbit plasma than in rabbit sera.

The ECL assay for the quantitation of ETI-204. The results of this assay were accurate and precise, as indicated by the low percentages obtained for the bias and coefficient of variation where at least 75% of the values fell below 5%. None of the interferents affected the quantitation of ETI-204. One of the unique features of this assay was the fact that hemolysis was measured quantitatively at two levels 140 mg/dl and 550 mg/dl. Table 1 shows the value of selected parameters.

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Table 1. Cutoff limits and variability ranges for Monkey and Rabbit ECL, ELISA and TNA assays

Parameters Assay	LOD	LOQ	ULOQ	Comments
Rabbit Screening ECL*(Serum) for detection of PA	4 ng/mL	NA	NA	LOD confirmed by NIAID. Applicant stated that LOD = 1 ng/mL
Monkey Screening ECL (Serum) for detection of PA	4 ng/mL	NA	NA	LOD confirmed by NIAID. Applicant stated that LOD = 2 ng/mL
Monkey ELISA (Serum) for quantitation of Total PA in the serum	4.2 ng/mL	0.87 ng/mL	6,400 ng/mL	No comment
Monkey ELISA (Serum) for quantitation of Free PA in the serum	9.68 ng/mL	9.68 ng/mL	309 ng/mL	No comment
Rabbit ELISA (Serum) for quantitation of Total PA in the serum	4.2 ng/mL	10.5ng/mL	410 ng/mL	No comment
Rabbit ELISA (Serum) for quantitation of Free PA in the serum	9.68 ng/mL	9.68 ng/mL	309 ng/mL	No comment
Monkey ELISA (Serum) for quantitation of anti-PA IgG antibodies	1.6 µg/mL	5.42 µg/mL	256 µg/mL	No comment
Rabbit ELISA (Serum) for quantitation of anti-PA IgG antibodies	1 µg/mL	5µg/mL	NA	No comment
Monkeys TNA	36.6	55	NA	No comment
Rabbit TNA	23	39	24,000	No comment

LOD = lower limit of detection, LOQ = lower limit of quantitation, ULOQ = upper limit of quantitation,

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2. REMARKS AND CONCLUSIONS

Acceptance Criteria

In general the acceptance criteria of the assays were too wide. A wide range causes a decrease in accuracy. For example, the percent coefficient of variation (%CV) in the electrochemiluminescence (ECL) assay for detection of the protective antigen, the %CV for comparing results was $\leq 35\%$, a difference of more than one-third of the value. However, in the actual tests the %CV was generally less than 10%. This provided a greater degree of accuracy than the applicant-specified criteria.

Interferents

The applicant stated in the anti-PA monoclonal antibody assay, that there were anti-PA IgG antibodies present in the hemolyzed samples where the results were negative. These antibodies would bind PA found in the serum resulting in negative values, because the PA is then not available to react with the putative interferent. This is important information when assessing results of ECL since as much as 56% of the hemolyzed samples contained anti-PA IgG. It was not stated if the other negative results were true negatives.

As was noted previously, there is a wide spectrum of concentrations associated with hemolysis, not all will affect the results of an assay. The TNA and ECL for the quantitation of ETI-204 were the only assays in this report in which there was an attempt to describe the level of hemolysis. The applicant described the level of hemolysis in interferent testing, as severe in the TNA assay. In the ECL assay for the quantitation of ETI-204, the applicant measured the amount of hemoglobin quantitatively in milligrams /deciliter. The most accurate description would be to measure the optical density for hemoglobin, spectrophotometrically.

Only one assay measured lipemia and none measured icterus, both of these conditions in blood can affect the quantitation of an analyte. Most assays examined the effect of the various levels of lethal factor and edema factor on the PA. None of the assays checked the effect of cross-reactivity with other species of *Bacillus* e.g. *Bacillus cereus*. Drugs can modify chemical reactions; drug interference was measured in one assay that tested the effect of ciprofloxacin interference.

Anticoagulants play a significant role in keeping whole blood from clotting, as is necessary in the culture of blood. In the assay that was reviewed in this document, the anticoagulant used was ethylenediaminetetraacetic acid (EDTA) which has antimicrobial properties. An anticoagulant that is recommended for blood cultures in humans is sodium polyanethol sulfonate (SPS), which also inhibits the growth of some bacteria. The effect of SPS on *Bacillus anthracis*, to the exclusion of other natural inhibitory substances, superiority over EDTA, and dilution of whole blood, was not investigated. It was felt in the study reviewed in this document, that EDTA did not play a significant role in decreasing the bacterial load.

Plates

There are advantages to the optimum positioning of samples on the microtiter plates. Placing samples in specific positions attempts to minimize the effects carry-over of samples and reagents

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from one sample well to another. Many of the samples were tested in duplicate, this ensures greater accuracy. The value of testing samples on the same plates e.g. spiked and unspiked samples is that this partially eliminated the effect of the test environment, as a reason for variation of results. The environment and placement are especially important because the volumes of liquid used in these tests are small and are more subject to the influence of environmental conditions, than are large test volumes.

Infected animals

A serious cause for concern is the fact that, in the rabbit study 'Real World Sample Assessment' that comprised animals that had been aerosol challenged with spores of *Bacillus anthracis*, there was a 12% false negative result. The applicant stated that some of the samples were incorrectly flagged as negative at 6 to 12 hours prior to treatment. Another reason for the negative flag could be a result of the LOD, which might not have been able to detect the limited amount of PA that would be present at that time. This is critical because this difference in time to treatment may determine the period between life and death of a subject. The applicant also stated that some of the negative results occurred in samples in which the blood culture bacterial count was ≤ 5 colonies. The adequacy of this response can be determined when the volume of blood in which these 5 colonies were found, is known. If the volume of the inoculum is small, 5 colonies will represent a significant number of bacteria in the blood circulation of the animal. The number of *Bacillus anthracis* cells in the blood circulation of a rabbit at that time, could range from 1,400 to 7,000.

The correlation between the ECL assay and bacterial culture in the 'real world' animal infected samples showed that the false negative rate for monkeys was 5% and for rabbits 12%. This indicated an unacceptable false negative rate. It is being suggested that when ECL tests are run there should be back-up blood cultures.

Operator variability

The results for ELISA quantitation of total PA, in general, were just accurate enough to cause the assay to be acceptable. Operator variability played a leading role in the percentage of total errors. Additionally, values at the ULOQ were, at times, inaccurate. Therefore, although the results at this level may fit within the standard curve, the samples close to the ULOQ should be diluted. The recovery rate posed a problem in a specimen with PA at a concentration of 1,024 ng/mL. The recovery rate of this spike was 80% of the expected concentration. But, inaccuracy also was evident in values that were higher than the expected concentration such as 4.2 ng/mL and 10.5 ng/mL which were too high at 117% and 120 % of PA recovery respectively, of the expected concentration.

Specific Assays

The ECL assay for detection of PA can produce results in approximately one hour. It is also easy to perform, which makes it ideal for a screening test. The most important problem with this assay was that it did not detect PA in low concentrations, when a bacterial load in the blood was high enough to grow 5 colonies in culture. For the assay to be effective for screening, the LOD needs to be lowered.

The results of the ELISA for the presence of anti-PA IgG show that it will accurately determine the amount of anti-PA antibodies in a monkey serum sample. Additionally, it is easy to perform

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and will provide results within three hours. However, since antibodies take as much as ten days to develop to a detectable level, this test would not be useful in a situation that requires early diagnosis. Perhaps it would be more useful in a therapeutic monitoring situation or evaluating immune response.

This ELISA assay was validated for the testing of monkey serum samples for the presence of anti-PA IgG. It cannot be used for early identification because the antibody is formed approximately 7-10 days after infection, but the test can be used for follow-up. The secondary antibody will have to be changed to a species specific antibody conjugate e.g., goat anti-rabbit IgG horseradish peroxidase conjugate for IgG detection in rabbit serum, or goat anti-human IgG horseradish peroxidase conjugate for antibody IgG detection in human serum. Cross-reactivity with antibodies against other organisms was not examined.

The toxin neutralization test for the detection of neutralizing antibodies appears to have mediocre utility with regard to anthim testing. It is unclear whether the assay will differentiate between anthim and the antibodies induced *in vivo*. TNA assay should not be used as a stand-alone test for measuring efficacy of an anti-toxin agent. The length of time taken to run the test is a disadvantage in a situation that requires 'stat' results. Another disadvantage is that studies have shown that 3-(4,5-Dimethylthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide (MTT) can be reduced by agents other than mitochondrial reductase; however, many studies have shown the utility of this tetrazolium salt in cell viability studies. Another factor of great importance in this assay, as with the ECL assay, is the location of samples in the plates. Additionally, the culture of cells takes a significant amount of time and great care must be exercised in order to prevent contamination of the cells.

Controls

The choice of controls has also been problematic for all of the assays. A control that is comprised only of diluent or matrix is a blank sample/control; a negative control is comprised of a specimen that gives negative results. The control sample should be added to the same diluent or be dissolved in a matrix that is similar to that of the test samples. Additionally, there were instances in the assays when the calibrators were used as either the positive or negative control. In most of the assays in this protocol, the test/control samples were run more than once – this provided an additional quality assurance.

Overall, there was an effort to maintain a reasonable standard of quality assurance by the use of more than one analyst to prepare some of the controls, mixtures and aliquots. Diligence was demonstrated in the temperature stability testing for storage of specimens and reagents. Assays were repeated on more than one day, and also in some assays, two laboratories were employed for test performance. Perhaps one of the greatest short comings, was the fact that many assays were run in too many laboratories leading to reduced consistency in methodologies and result reporting.

Table 1 (above) shows the Limits of Detection of the validation assays.

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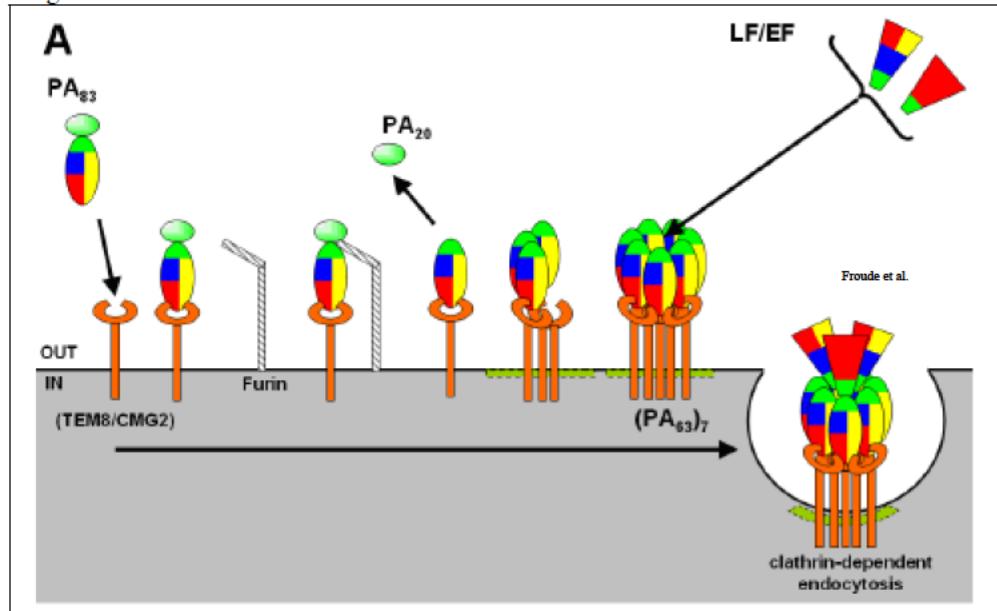
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3. INTRODUCTION AND BACKGROUND

The subject of this BLA is ETI-204 (Anthim) a deimmunized IgG1κ monoclonal antibody, for the treatment of anthrax. This antibody neutralizes the protective antigen (PA) produced by *Bacillus anthracis* and prevents PA from binding to its receptor. In the disease process, PA binds the lethal factor (LF) and the edema factor (EF) both of which are also produced by *Bacillus anthracis*. Binding of PA to either LF or EF causes the secretion of an A/B toxin which leads to a rapid progression of hemorrhagic pathology and death of the infected animal, if intervention is not administered immediately. The detection of PA in addition to bacteremia may, therefore, be used as a marker of infection.

PA is an 83 kDa protein secreted by *Bacillus anthracis* that is cleaved into two fragments – a 20 kDa component at the amino terminal and PA63 on the host cell surface, by the proteolytic action of a furin-like protease, after the 83 kDa molecule binds two receptors, the tumor endothelial marker 8, and the morphogenesis protein 2. After the release of PA20, the binding sites for EF and LF on PA63 are exposed. PA63 which is bound to the host cell surface by the two receptors, forms a heptameric ring-shaped structure that binds to molecules of the edema factor and lethal factor. The combined heptameric structures are then translocated into the cell by endocytosis. Inhibition of the binding of PA to its cell surface receptor is critical to the prevention of lethality by *Bacillus anthracis*. Table 1 shows the process of toxin formation. The monoclonal antibody ETI-204, is directed against the protective antigen (PA). This antibody neutralizes PA produced by *Bacillus anthracis* and prevents PA from binding to its receptor. If PA does not bind to its receptor it will not be able to bind to either the edema factor or the lethal factor to produce toxins.

Figure 1: Formation of Anthrax Toxin¹



¹ Jeffrey W. Froude II, Philippe Thullier, Thibaut Pelat. Antibodies Against Anthrax: Mechanisms of Action and Clinical Applications. *Toxins* 2011, 3, 1433-1452

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In animal efficacy studies in New Zealand White rabbits and Cynomolgus monkeys, the applicant has used (1) electrochemiluminescence (ECL) immunoassay for detection of PA, (2) enzyme-linked immunosorbent assay (ELISA) for quantitation of PA, (3) ELISA for detection of anti-PA antibodies and (4) toxin neutralization assays (TNA) for detection of anti-toxin antibodies (5) culture. However, the assays used are experimental. The methods used and the performance characteristics of the assays are discussed in this section.

Important major characteristics in an analytical assay are that the assay can detect the analyte for which it is designed, to the exclusion of other components in a sample (specificity). The other important characteristic of an assay is how minute is the amount of the analyte in the test sample that the assay can accurately detect (sensitivity). The degree to which the assay can determine these two characteristics is important in determining its utility. The following test parameters were evaluated for the assays used in this study:

- Sensitivity
- Accuracy
- Precision – Reproducibility, Repeatability
- Dilutional Linearity
- Limits of Detection
- Limits of Quantitation
- Specificity

4. REVIEW OF METHODS AND PERFORMANCE CHARACTERISTICS OF THE ASSAYS

4.1. Electrochemiluminescence assay for detection of Protective Antigen (PA) (SOP ^{(b)(4)} V-061.04; Validation Protocol. VP2013-266)

This was a screening assay designed to detect PA (83 KDa) in circulation.

The ECL assay utilized 96 well Meso Scale Discovery (MSD) microplates that contain electrodes which are integrated into the bottom of the wells. *Bacillus anthracis* anti-PA polyclonal antibody, the capture antibody, was coated on to the electrodes. Then appropriate, monkey or rabbit, spiked matrix matched recombinant PA (rPA) serum, or test serum, or plasma samples from rabbits or monkey was added. The same polyclonal anti-PA antibody tagged with the SULFO-TAG, the detection molecule, was added to each well. Test plates were incubated, washed and read. The PA in the specimen binds to the capture anti-PA antibody on the electrode while the detection anti-PA SULFO-TAG binds the captured PA (Figure 1). Light detection, read by an imager, occurs when the electrodes at the bottom of the plate become stimulated with the SULFO-TAG, and emit light at a wavelength of 620 nm. The emitted light, further enhanced by the addition of a read buffer, is converted to read out values called counts, that are proportional to the light intensity. The amount of emitted light is proportional to the amount of PA in the serum. Fluorescent labeling is usually very sensitive allowing for the measurement of low concentrations of the analyte. The assay can be completed within a few hours and is a

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useful tool for screening. This is a qualitative assay and results are expressed as either positive or negative.

Figure 1: Mechanism of ECL Assay



MesoScale

Briefly, the aliquots of 25 μ L of sample/control were added to each well in 96 well microtiter plates that were coated with anti-PA antibody (capture). Twenty-five microliters of 0.4X detection antibody solution (50X SULFO-TAG™ anti-PA antibody) prepared fresh each day of use, were added. Each sample was run in triplicate. The plates were covered with adhesive paper and incubated with shaking (200 ± 10 rpm) for approximately 1 hour at room temperature. The supernatants were removed, the well with samples washed 3 times, read buffer was added and the plates read within 6 minutes. Figure 2 shows the layout of samples on a 96 well ECL plate. All samples were run in triplicate. The researcher advised that wells B1 through B12 not be used for testing of specimens so that contamination of the specimens would be prevented.

This protocol covers both monkey and rabbit sera.

The assay was performed at [REDACTED] ^{(b)(4)} by 4 operators.

The negative control (NC) comprised naive monkey or rabbit serum as appropriate to the animal being tested. Spikes of rPA in pooled naive rabbit or monkey sera were used as the positive control (PC). The serum samples were obtained from 12 rabbits and 6 non-human primates. Hemolyzed samples were obtained from 3 rabbits and 3 monkeys, 83 KDa recombinant protective antigen (rPA), lethal factor (LF), and edema factor (EF) were obtained from [REDACTED] ^{(b)(4)}

[REDACTED] for each animal species. All samples were tested fresh except for those that were tested for robustness.

Briefly, the aliquots of 25 μ L of sample/control were added to each well in 96 well microtiter plates that were coated with anti-PA antibody (capture). Twenty-five microliters of 0.4X detection antibody solution (50X SULFO-TAG™ anti-PA antibody) prepared fresh each day of use, were added. Each sample was run in triplicate. The plates were covered with adhesive paper and incubated with shaking (200 ± 10 rpm) for approximately 1 hour at room temperature. The supernatants were removed, the well with samples washed 3 times, read buffer was added and the plates read within 6 minutes. Figure 2 shows the layout of samples on a 96 well ECL

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plate. All samples were run in triplicate. The researcher advised that wells B1 through B12 not be used for testing of specimens so that contamination of the specimens would be prevented.

Figure 2: The layout of a plate for ECL testing

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
A.	NC	NC	NC	PC	PC	PC	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦
B.	§	§	§	§	§	§	§	§	§	§	§	§
C.	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
D.	TS5	TS5	TS5	TS6	TS6	TS6	TS7	TS7	TS7	TS8	TS8	TS8
E.	TS9	TS9	TS9	TS10	TS10	TS10	TS11	TS11	TS11	TS12	TS12	TS12
F.	TS13	TS13	TS13	TS14	TS14	TS14	TS15	TS15	TS15	TS16	TS16	TS16
G.	TS17	TS17	TS17	TS18	TS18	TS18	TS19	TS19	TS19	TS20	TS20	TS20
H.	TS21	TS21	TS21	TS22	TS22	TS22	TS23	TS23	TS23	TS24	TS24	TS24

♦♦ = Wells dedicated to stability or qualification testing of Quality Control lots

§ = Unused wells. If Row B is used for sample analysis, then all test samples will shift up and TS25 to TS27 will be added to Row H.

(b) (4) OP

Acceptance Criteria

Quality Control – Rabbits and Monkeys

- Reagents:
The mean ECL of a new lot of reagents must be > zero .
- Positive (PC) and negative (NC) controls and individual samples:
(1) The mean ECL value of the NC and PC must be >zero but \leq 100 ECL units. There was no %CV criterion (2) If the mean ECL value of the NC and PC was \geq 100 ECL units then the %CV must be \leq 35%.
An individual sample was rejected when (1) The mean ECL value of the NC and PC was \leq zero and (2) The mean ECL value was \geq 100 ECL units and the %CV was > 35%.
- Signal/Noise ratio
Rabbits: \leq 1.54
Monkeys: \leq 1.31

The value of the NC was 0.00 ng/mL rPA and was prepared from pooled naïve serum (Aleken Biologics Lot No. RB 1003 or RB 1004 for rabbit and Aleken Biologics Lot No. CY3010 for monkeys) treated with 100X protease inhibitor cocktail (PIC).

The PC for rabbits was 1 ng/mL rPA (BEI, NR-164, Lot No.5051797) prepared in 1X protease inhibitor cocktail (PIC) pooled naïve rabbit serum.

The PC for monkeys was 2 ng/mL rPA in 1X PIC- treated pooled naïve monkey serum.

Reconstituted controls were aliquoted into 100 μ L/per aliquot and stored at \leq -70°C. The signal noise (S/N) ratio i.e. mean positive control divided by the mean negative control had to be greater than 2 ng/mL for the rabbits but >2.5 ng/mL for the monkeys.

Definition of test results as positive or negative.

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Negative : ECL unit value of the sample is < the mean ECL unit value of the positive control (PC).

Positive : ECL unit value of the sample is \geq the mean ECL unit value of the positive control (PC).

Storage

The effect of long term storage was examined to determine the effect on results from samples stored at $\leq -60^{\circ}\text{C}$; the acceptance level for the results was that at least 95% of the results after storage, would concur with the results before storage. The number of rabbit serum samples tested was 443, of these samples 431 (98%) concurred with the original results after 64 days of storage. For monkeys 245 samples were tested and 240 (98%) concurred with the original results up to 95 days of storage.

Evaluation Parameters

The parameters used for the valuation of the ECL assay were as follows:

1. Sensitivity
 - Limit of Detection
2. Specificity
 - False negative rate
 - False positive rate
 - Matrix effect
3. Precision
 - Composite of all tests listed above, including
 - Short term stability
 - Long term stability
4. Robustness

4.1.1. ECL Screening Assay for the Detection of Protective Antigen in Monkey Serum

4.1.1.1. Plate suitability

Fifty of the 51 plates used for the monkey studies achieved the plate suitability test acceptance criteria (see section on acceptance criteria). One plate failed, and this plate resulted in a NC that was less than 0 (mean ECL value -12 units). The acceptance criterion for the NC was 0 ng/mL.

4.1.1.2. Specificity

False Negative Rate

The individual sera from 6 monkeys were spiked with 10 ng/mL of rPA. These occupied 9 plates with a total of 62 data points. Each sample was tested in 2 to 4 batches (replicates). The sample in each batch was tested in triplicate. The % CV from each triplicate ranged from 2% to 21%. All of the samples except 4 recorded %CV $< 10\%$ in each triplicate. Table 1 shows that all 62 results samples were positive i.e. there were no false negative results. The false negative rate for monkey serum was 0%. The false negative rate was calculated as in equation 1.

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Equation 1

$$1 - \frac{\text{Number of Observed True Positives}}{\text{Number of Observed True Positives} + \text{Number of False Negatives}}$$

Table 1: Results of 62 Samples of Monkey Serum Spiked with Recombinant Protective Antigen.

VTS	Mean ECL	% CV	Result									
	Replicate 1			Replicate 2			Replicate 3			Replicate 4		
Plate ID: NHP -1												
VTS #N-25	1,085	9%	P	2,007	2%	P						
VTS #N-26	2,023	3%	P	3,614	2%	P						
VTS #N-27	1,368	10%	P	2,208	11%	P						
VTS #N-28	959	6%	P	1,114	5%	P						
VTS #N-29	1,459	9%	P	4,016	8%	P						
VTS #N-30	2,165	9%	P	3,983	5%	P						
Plate ID: NHP -2 (NHP-1&2 Repeat)												
VTS #N-25	1,005	8%	P	1,821	3%	P						
VTS #N-26	1,972	3%	P	3,553	6%	P						
VTS #N-27	545	4%	P	2,121	4%	P						
VTS #N-28	914	3%	P	1,134	10%	P						
VTS #N-29	1,453	10%	P	3,524	3%	P						
VTS #N-30	2,038	2%	P	3,906	4%	P						
Plate ID: NHP -3												
VTS #N-29	1,753	11%	P	1,771	2%	P	1,785	2%	P			
VTS #N-30	2,492	4%	P	2,344	9%	P	2,249	2%	P			
Plate ID: NHP -4												
VTS #N-29	1,684	5%	P	1,860	1%	P	1,689	1%	P			
VTS #N-30	2,340	1%	P	2,368	4%	P	2,550	1%	P			
Plate ID: NHP -5												
VTS #N-25	1,357	4%	P	1,492	1%	P	1,472	3%	P	1,439	7%	P
VTS #N-28	735	4%	P	712	1%	P	738	4%	P			
Plate ID: NHP -6												
VTS #N-25	1,141	21%	P	1,412	0%	P	1,351	1%	P	1,313	11%	P
VTS #N-28	757	17%	P	851	6%	P	876	5%	P			
Plate ID: NHP -7												
VTS #N-26	1,902	5%	P	1,991	1%	P	2,180	8%	P			
VTS #N-27	1,318	5%	P	1,265	6%	P	1,281	2%	P			
Plate ID: NHP -8												
VTS #N-26	1,706	3%	P	1,782	5%	P	2,066	7%	P			
VTS #N-27	1,141	3%	P	1,146	2%	P	1,150	2%	P			

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False Positive Rate

The individual serum samples from 6 naïve monkeys were tested by ECL to determine the false positive rate. The samples were tested in 20 plates and generated 182 data points. Ten samples were repeated which increased the total number of data points to 192. The samples were run in 4 batches and each sample was run in triplicate. The variability of test results even in the same sample was wide e.g., VTS#N-22 had a %CV of 350. The tests were repeated, and all data points were reported as negative, which indicates that there were no false positive results. The equation 2 used to calculate the false positive rate was as follows:

Equation 2

$$1 - \frac{\text{Number of Observed True Negatives}}{\text{Number of Observed True Negatives} + \text{Number of False Positives}}$$

Matrix effect

The matrix effect was performed using monkey serum spiked with 10 ng/mL of rPA, and serum that was not spiked (naïve). To both the rPA spiked serum and unspiked serum, were added either 10 ng/mL of rEF or 10 ng/mL of rLF or hemolytic serum. The results are shown in Table 2. The mean ECL value ratios of spiked to unspiked sera recorded values such as

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209:50 or 205:32. The results were determined to be negative. All of the naïve sera were negative so there were no false positive results. However, all of the 10 ng/mL rPA spiked samples to which 10 ng/ml rEF or 10 ng/mL rLF were added gave positive results (no false negative) i.e., this means that rEF and rLF did not mask the presence of rPA. The acceptability criteria for false negatives were that no more than 10% of the samples should give a false negative result. Therefore the matrix effect of rEF and rLF failed.

For hemolysis, the mean ECL values represented by paired hemolyzed samples that were either rPA spiked or unspiked naïve samples, showed that hemolysis did not quench the signal of the rPA in some samples, for example in the 39:40 and 41:42 pairs in Table 2, but did in other samples such as the 37:38 pair. Acceptance criteria for false negatives were that <10% of the samples should give a false negative result. Test of the matrix of hemolysis failed because 33% (1/3) of the samples gave a false negative result. This means that hemolysis may mask the presence of PA.

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Table 2: Matrix using monkey rPA spiked and naïve serum

rPA Spike Status	VTS	Mean ECL	% CV	Result	Mean ECL	% CV	Result
		Replicate 1		Replicate 2			
Plate ID: NHP-9							
Spiked	VTS #N-31	209	6%	N	197	8%	N
Naïve	VTS #N-32	50	39%	N	58	54%	N
Plate ID: NHP-10							
Spiked	VTS #N-31	205	11%	N	185	7%	N
Naïve	VTS #N-32	32	38%	N	13	127%	N
Plate ID: NHP-11							
Naïve	VTS #N-34	48	27%	N	21	71%	N
Spiked	VTS #N-35	230	3%	N	229	13%	N
Naïve	VTS #N-36	39	78%	N	28	47%	N
Spiked	VTS #N-37	16	131%	N	26	44%	N
Naïve	VTS #N-38	26	58%	N	36	45%	N
Spiked	VTS #N-39	2,187	12%	P	2,185	3%	P
Plate ID: NHP-12							
Naïve	VTS #N-34	36	30%	N	36	49%	N
Spiked	VTS #N-35	207	18%	N	219	5%	N
Naïve	VTS #N-36	44	41%	N	43	20%	N
Spiked	VTS #N-37	31	41%	N	26	18%	N
Naïve	VTS #N-38	49	39%	N	50	8%	N
Spiked	VTS #N-39	2,020	6%	P	2,251	2%	P
Plate ID: NHP-15							
Spiked	VTS #N-33	268	2%	N	278	1%	N
Naïve	VTS #N-40	37	2%	N	36	46%	N
Spiked	VTS #N-41	1,680	1%	P	1,674	1%	P
Naïve	VTS #N-42	42	40%	N	47	39%	N
Plate ID: NHP-16							
Spiked	VTS #N-33	211	22%	N	216	7%	N
Naïve	VTS #N-40	21	99%	N	26	58%	N
Spiked	VTS #N-41	1,457	9%	P	1,439	5%	P
Naïve	VTS #N-42	28	29%	N	47	4%	N

N= Negative for rPA; P = Positive for rPA

 Samples 31, 33 and 35 are naïve samples spiked with either 10 ng/mL of EF or 10 ng/mL of LF (b) (4) VP 2013-266

The tests were repeated in an unblinded protocol. The results showed that rPA (in samples 31, 33, 35) + rEF+rLF or hemolyzed serum, gave the results shown in Table 3. Both the false negative and false positive rates were 0% and were acceptable. The applicant states that the previous results reported under the validation study above (Table 2) may have been caused by dilution errors. NIH recommended that the entire validation study be repeated with the previously specified blind.

The hemolyzed protocol was repeated with samples 37, 39 and 41, and the false negative rate was 55.56% above the acceptable rate of 10%. There were no false positive results. The matrix failed again. Reasons for failure such as lot variation, or expiration of rPA₈₃ that was used to spike the samples were ruled out. It was suggested that the negative findings resulted from the presence of anti-PA IgG antibodies in animal ID 13691 but the origin of this immunity could not be verified. Concurrence with bacteremia and circulating PA ELISA results was investigated by the researcher. The researcher stated that the results of ECL and ELISA matched and that bacteremia and ECL results were similar in 32/35 samples. One of

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the three samples was a false negative and the other two were false positive. Hemolysis as a matrix failed.

Table 3: Repeated tests for the effect of selected matrices on monkey serum samples.

rPA Spike Status	VTS	Mean ECL	% CV	Result	Mean ECL	% CV	Result
		Replicate 1		Replicate 2			
Plate ID: NHP-1-R2							
Spiked	VTS #N-31	1,997	3%	P	1,691	8%	P
Naïve	VTS #N-32	9	263%	N	39	17%	N
Spiked	VTS #N-33	4,969	12%	P	51	7%	P
Naïve	VTS #N-34	27	39%	N	32	25%	N
Spiked	VTS #N-35	3,468	4%	P	2,420	16%	P
Naïve	VTS #N-36	29	25%	N	29	78%	N
Spiked	VTS #N-37	20	183%	N	34	73%	N
Naïve	VTS #N-38	16	119%	N	37	43%	N
Spiked	VTS #N-39	3,621	3%	P	2,431	4%	P
Naïve	VTS #N-40	26	36%	N	26	63%	N
Spiked	VTS #N-41	3,127	9%	P	3,192	5%	P
Naïve	VTS #N-42	30	71%	N	47	24%	N
Plate ID: NHP-2-R2							
Spiked	VTS #N-31	1,911	1%	P	1,554	7%	P
Naïve	VTS #N-32	20	50%	N	31	52%	N
Spiked	VTS #N-33	4,505	16%	P	5,049	3%	P
Naïve	VTS #N-34	21	109%	N	23	26%	N
Spiked	VTS #N-35	3,263	2%	P	2,477	11%	P
Naïve	VTS #N-36	-18	-131%	NR	19	57%	N
Spiked	VTS #N-37	3	400%	N	39	17%	N
Naïve	VTS #N-38	8	188%	N	23	95%	N
Spiked	VTS #N-39	3	9%	P	3	4%	P
Naïve	VTS #N-40	18	20%	N	2,626	79%	N
Spiked	VTS #N-41	3,365	6%	P	3,228	3%	P

N = Negative for rPA; P = Positive for rPA

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4.1.1.3. Stability

Short-Term Stability

Short-term stability was assessed by the use of six 10 ng/mL rPA spiked and 6 naïve serum specimens which were tested in pairs. The total number of samples resulting from this combination was 172. There was 100% concurrence in the results. The monkey serum samples were validated for accurate sampling after a delay of up to 28 minutes from the paired (backup) sample.

Long-Term Stability

Long-Term stability was tested by the use of freeze/thaw (F/T) cycles after 1, 2 and 3 cycles. The specimens were tested on 3 days after being allowed to thaw at room temperature for varying amounts of time, alternating with storage of the sample at $\leq -70^{\circ}\text{C}$.

Table 4 shows the results of long term stability tests. The overall results between the replicates 1 and 2 were appropriate. Table 5 shows the false positive and false negative rates; there were no false positive or negative results for F/T cycles 1 and 2. F/T cycle 3, the false negative rate was 17% which does not meet the acceptability criteria of 10%. Therefore long-term stability for monkey samples was validated for 2 F/T cycles. The equation used for concurrence was as follows:

$$\% \text{ Concurrence} = \frac{\text{Total number of identical Results} \times 100\%}{\text{Total Number of Results}}$$

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Table 4: Long-Term Stability tests for specimens obtained from monkeys

VTS	Mean ECL	% CV	Result	Mean ECL	% CV	Result
	Replicate 1		Replicate 2			
Plate ID: NHP -19; Freeze/Thaw 1						
VTS #N-19	37	81%	N	38	28%	N
VTS #N-20	33	27%	N	29	58%	N
VTS #N-21	41	29%	N	37	36%	N
VTS #N-22	39	22%	N	46	7%	N
VTS #N-23	21	30%	N	58	23%	N
VTS #N-24	58	3%	N	52	40%	N
VTS #N-25	1,001	7%	P	994	2%	P
VTS #N-26	1,340	8%	P	1,369	4%	P
VTS #N-27	846	9%	P	870	2%	P
VTS #N-28	685	4%	P	706	2%	P
VTS #N-29	1,244	6%	P	1,313	6%	P
VTS #N-30	1,787	3%	P	1,948	3%	P
Plate ID: NHP -21; Freeze/Thaw 2						
VTS #N-19	4	603%	N	47	33%	N
VTS #N-20	20	95%	N	56	18%	N
VTS #N-21	19	114%	N	32	69%	N
VTS #N-22	36	50%	N	46	10%	N
VTS #N-23	-27	-64%	NR	34	8%	N
Plate ID: NHP -21; Freeze/Thaw 2						
VTS #N-24	26	8%	N	67	28%	N
VTS #N-25	915	5%	P	1,027	3%	P
VTS #N-26	1,208	7%	P	1,412	6%	P
VTS #N-27	940	5%	P	936	5%	P
VTS #N-28	672	7%	P	691	2%	P
VTS #N-29	1,067	6%	P	1,044	2%	P
VTS #N-30	1,553	8%	P	1,547	2%	P
Plate ID: NHP-22; Freeze/Thaw 2						
VTS #N-23	-25	-92%	NR			
Plate ID: NHP -17; Freeze/Thaw 3						
VTS #N-19	40	28%	N	27	37%	N
VTS #N-20	26	37%	N	43	34%	N
VTS #N-21	26	76%	N	39	56%	N
VTS #N-22	34	28%	N	60	6%	N
VTS #N-23	44	53%	N	32	22%	N
VTS #N-24	61	32%	N	62	6%	N
VTS #N-25	816	4%	P	835	5%	P
VTS #N-26	949	9%	P	939	8%	P
VTS #N-27	746	7%	P	738	4%	P
VTS #N-28	593	4%	N	588	4%	N
VTS #N-29	933	1%	P	880	4%	P
VTS #N-30	1,343	7%	P	1,360	6%	P

N= Negative for rPA; P = Positive for rPA; NR = Not Reportable and considered negative for rPA per IR-628 and DR-14248-BBRC

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Table 5: False Positive and False Negative rates of Thawed Monkey Specimens

	F/T 1	F/T 2	F/T 3
False Positive Rates (VTS #N-19 to VTS #N-24)	0%	0%	0%
False Negative Rates (VTS #N-25 to VTS #N-30)	0%	0%	17%

(b) (4) VP2013-266

Long-Term Stability of Real-World Test Samples and Robustness

Monkeys were aerosol challenged with 200X the median lethal dose of spores of the Ames strain of *Bacillus anthracis*. Sera from the infected animals were tested by the ECL assay and then stored at $\leq -60^{\circ}\text{C}$ for a length of time ranging from 100 to 112 days, and retested. The results from the initial sample were used as the standard value. There was a 97.96 % (240/245) concurrence of the results between the initial ECL value and the ECL value of the stored specimen.

Test of specimen robustness was determined by the length of time a blood specimen could be held after collection before it is tested, without affecting the results. Robustness is important because many specimens are tested in batches and since specimens do not all arrive at the laboratory at the same time, some must be held back in order to fill the batch. Determination of robustness is also important for this PA assay because it might be used as a trigger-to-treat for anthrax.

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In order to evaluate the effect of delay in sampling, duplicates of blood samples from monkeys were tested after storing them for specific periods of time. Two of three replicates of a specimen were held for up to 2 hours and 39 minutes after collection and blindly tested at the same time as freshly prepared samples, by the same (one) operator. Acceptance required 100% concurrence of both the negative – naïve samples, and 100% of the rPA spiked samples. All of the negative samples were negative and all of the rPA spiked samples were positive. The values of three samples were not reportable. Table 6 shows the results. Samples could therefore be held up to 2 hours and 39 minutes after collection without affecting the results.

Table 6: Results of tests for robustness of monkey samples.

VTS	Mean ECL	%CV	Result	Mean ECL	%CV	Result
	Plate ID: NHP -1			Plate ID: NHP-1&2 Repeat		
VTS #N-19	-17	-132%	NR	60	40%	N
VTS #N-20	24	136%	N			
VTS #N-21	-51	-24%	NR	78	15%	N
VTS #N-22	10	26%	N			
VTS #N-23	-8	-308%	NR	31	52%	N
VTS #N-24	23	50%	N			
VTS #N-25	1,085	9%	P			
VTS #N-26	2,023	3%	P			
VTS #N-27	1,368	10%	P			
VTS #N-28	959	6%	P			
VTS #N-29	1,459	9%	P			
VTS #N-30	2,165	9%	P			
	Plate ID: NHP -2			Plate ID: NHP-1&2 Repeat		
VTS #N-19	28	55%	N			
VTS #N-20	27	76%	N			
VTS #N-21	3	945%	N			
VTS #N-22	26	41%	N			
VTS #N-23	13	115%	N			
VTS #N-24	26	67%	N			
VTS #N-25	1,005	8%	P			
VTS #N-26	1,972	3%	P			
VTS #N-27	Sample failed CV criteria			545	4%	P
VTS #N-28	914	3%	P			
VTS #N-29	1,453	10%	P			
VTS #N-30	2,038	2%	P			

N= Negative for rPA; P = Positive for rPA; NR = Not Reportable and considered negative for rPA per IR-628 and DR-14248-BBRC

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4.1.1.4. Precision

Precision was assessed by using a comparison of the results obtained for the same analysis by more than one operator (2 to 4), on different days, using different lots of reagents. The effect of the type of matrix on false negative and false positive rate, and (robustness) were evaluated by a single operator, on a single day, and therefore was not used in the determination of precision. There were four classes of analyses:

- Test Plan 1A. False Negative Rate compared multiple operators and multiple plate lots
- Test Plan 2A. False Positive Rate compared multiple operators and plate lots on multiple days
- Test plan 4A. Short Term Stability compared multiple operators and plate lots on multiple days
- Test Plan 5A. Long Term Stability compared multiple operators on multiple days

Table 7 shows the results of the assessment and shows that the assessment was acceptable. The results suggested that the data could be replicated with little variation.

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Table 7: Assay Precision Across Days, Operators, and ECL Plate Lots

Test Plan Number	Test Plan Passed Validation Criteria	Multiple Operators	Multiple Testing Days	Multiple Plate Lots
1B	PASS	4	N/A	3
2B	PASS	4	2	3
3B	PASS ^a	N/A	2	N/A
4B	PASS	4	2	3
5B	PASS	3	N/A	N/A
7B	PASS	3	N/A	2
8B	PASS	N/A	N/A	N/A

^a Excluding the hemolyzed sample containing anti-PA IgG

4.1.1.5. Limit of Detection

Previous tests with spiked monkey serum of 10 ng/mL showed that this concentration was always detected. Initially, a sample from the same monkey was spiked to concentrations of 2 ng/mL, 4 ng/mL and 5 ng/mL of rPA. The lowest concentration that could have been 100% detected was 4 ng/mL. Concentrations of rPA of 4 ng/mL(VTS#43), 6 ng/mL (VTS#44), 8 ng/mL(VTS#45) and 10 ng/mL(VTS#28) were re-tested blind and evaluated for LOD. Table 8 shows that all concentrations resulted in positive ECL values but the lowest concentration that passed validation was 4 ng/mL.

Table 8: Results of Samples tested for the Limit of Detection of PA in Monkey serum

VTS	Mean ECL	%CV	Result									
	Replicate 1			Replicate 2			Replicate 3			Replicate 4		
Plate ID: NHP -1												
VTS #N-28	959	6%	P	1,114	5%	P						
Plate ID: NHP -2												
VTS #N-28	914	3%	P	1,134	10%	P						
Plate ID: NHP -5												
VTS #N-28	735	4%	P	712	1%	P	738	4%	P			
Plate ID: NHP -6												
VTS #N-28	757	17%	P	851	6%	P	876	5%	P			
Plate ID: NHP -13												
VTS #N-43	434	11%	P	440	4%	P	425	3%	P	436	6%	P
	429	1%	P	432	4%	P	427	8%	P	411	8%	P
VTS #N-44	649	4%	P	672	4%	P	665	3%	P	659	4%	P
	615	7%	P	722	5%	P	686	2%	P	666	2%	P
Plate ID: NHP -14												
VTS #N-43	440	3%	P	433	3%	P	454	14%	P	456	7%	P
	430	7%	P	447	2%	P	464	8%	P	419	5%	P
VTS #N-44	627	4%	P	642	4%	P	639	6%	P	675	5%	P
	655	5%	P	658	6%	P	675	2%	P	697	2%	P
Plate ID: NHP-15												
VTS #N-45	1,320	3%	P	1,388	0%	P	1,331	1%	P	1,271	5%	P
	1,447	1%	P	1,423	3%	P	1,345	5%	P	1,336	2%	P
Plate ID: NHP-16												
VTS #N-45	1,140	2%	P	1,140	2%	P	1,109	2%	P	1,115	3%	P
	1,182	4%	P	1,120	5%	P	1,126	5%	P	1,206	2%	P

P = Positive for rPA

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4.1.1.6. Testing of sera from infected animals.

The applicant tested sera from one animal study which was designed to assess the efficacy of two anti-toxins against *Bacillus anthracis* PA. These anti-toxins were administered in a combination with 10 mg/kg of ciprofloxacin, 24 hours after a positive ECL result was obtained. Also the study was planned to demonstrate the reproducibility of the added benefit of the anti-toxin in treatment of the diseased animal. Samples were obtained from monkeys that had been aerosol challenged with spores 200X the median lethal dose (LD₅₀) (b) (4)

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spores) of *Bacillus anthracis* (Ames strain). Blood culture plates were inoculated pre-challenge, to rule out false positives. Plates were also inoculated with blood samples post-challenge, but pre-treatment, to verify positive results and to rule out false negative ECL results. Another blood culture was inoculated at the time of treatment. Of the 229 samples that resulted in growth, 208 were also positive by ECL, 11(5%) were false negative. Forty-seven samples were negative by both ECL and culture. The acceptable rate for false negatives was 10%. Therefore, in tests from infected animals the ECL assay was shown to provide an acceptable level of results.

Overall, the ECL assay used for testing of monkey sera from infected animals is appropriate (Table 9). Some of the limitations include possibility of false negative findings in the presence of EF, LF, or anti-PA IgG. Also, the LOD should be 4 ng/mL and not 2 ng/mL as proposed by the applicant.

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Table 9: Summary of Validation Results of the ECL assay for Monkeys

Parameter	Validation Acceptance Criteria	Test results of Parameter	Validation Decision	Comment
Limit of Detection	Minimum concentration of rPA in which there is 100% detection PA	4 ng/mL	Acceptable	None
Precision	Composite of all tests individually validated. False negatives, False positives, Short & Long term stability, LOD	False neg = Pass False pos = Pass Short-term= Pass Long term = Pass LOD = Pass	Acceptable	Long term Stability : validated up to 2 Freeze/Thaw cycle
Matrix effect	False negative rate = <10% False positive rate = <5%	rPA + Hemolytic sera = Neg rPA +rLF or rEF = Neg Specimens without PA IgG =0%.	Failed	Hemolytic samples found to have anti-PA IgG therefore false negative results (55.56%) rEF, rLF mask rPA
Specificity for detection of rPA	False Negatives PA = <2 results/ batch and rate <10% rate ----- False Positives PA = <4 Results/batch and rate < 5% ----- Matrix effect: False negative = <10% False positive = <5%	0% ----- 0% ----- EF & LF False negative = 0 % False positive = 0% Hemolytic serum: False Negative = 0% False positive = 0%	Acceptable	None
Stability PA63	Short-Term - Concurrence > 95% ----- Long-Term – False Negative rate =<10% False positive rate =<5%	100% validated up 28 minutes ----- False Negative 0% False positive 0%	Acceptable	Validated up to 28 minutes Validated up to 2 freeze/thaw cycles
Robustness	Samples incubated at room temperature for 1.5 hours: rPA spiked samples = 100% positive Naïve samples = 100% negative	rPA = Positive 100% Naïve samples = Negative 100%	Acceptable	Validated up to 2 hours 39 minutes

Neg = negative, pos = positive, EF = edema factor, , LF = lethal factor, rPA = recombinant protective antigen

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4.1.2. ECL Screening Assay for the Detection of Protective Antigen in Rabbit Serum

4.1.2.1. Specificity

False Negative Rate

The false negative rate was determined by testing 6 individual naïve rabbit sera that were spiked with 10 ng/mL rPA. Sixty two positive samples were obtained and were analyzed in 8 plates.

The acceptable validation criteria for false negatives were specified to be ≤ 2 false negative results in the test group and a false negative rate of $< 10\%$. The false negative rate of the test group was 0%.

False Positive Rate

The percentage of false positive rate was examined using known samples that were PA negative. The acceptability criterion was stated to be $< 5\%$. Specimens were obtained from 6 naïve rabbits and 325 results were obtained. No false positive results were identified.

Matrix Effect on False Negative and False Positive Rates

The matrices tested were rabbit sera with and without rPA that were spiked with 10 ng/mL of rEF or 10 ng/mL rLF or hemolytic serum. The matrix effect was tested by 4 operators on one day. To be acceptable the ECL result should have false negatives $\leq 10\%$, and false positives $< 5\%$. The tests resulted in no false positives or false negatives. Table 1 shows the results of rabbit sera when tested in various matrices. VTS#R 13 - 18 tested matrices in which 10% rEF and rLF were added, VTS#19 - 24 evaluated the effect of hemolysis.

Tests were run to determine whether hemolysis would give false positive results. Rabbit hemolytic samples had no effect on the results. However, it was observed that 55.56% of the samples that resulted in false negative results, when tested, contained anti-PA IgG antibody.

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Table 1: Results of tests for Rabbit Matrices of Edema and Lethal factors and Hemolysis

VTS	Mean ECL	% CV	Result	Mean ECL	% CV	Result
	Replicate 1			Replicate 2		
Plate ID: Rabbit -1-R2						
VTS #R-13	1,199	2%	P	1,038	7%	P
VTS #R-14	42	43%	N	40	29%	N
VTS #R-15	875	4%	P	1,118	5%	P
VTS #R-16	19	48%	N	22	34%	N
VTS #R-17	1,185	4%	P	1,003	10%	P
VTS #R-18	27	38%	N	32	63%	N
VTS #R-19	1,076	5%	P	1,077	11%	P
VTS #R-20	19	116%	N	41	74%	N
VTS #R-21	1,172	3%	P	892	8%	P
VTS #R-22	26	73%	N	29	79%	N
VTS #R-23	1,887	5%	P	1,854	5%	P
VTS #R-24	23	152%	N	28	28%	N
Plate ID: Rabbit-2-R2						
VTS #R-13	1,185	6%	P	1,177	10%	P
VTS #R-14	38	21%	N	25	92%	N
VTS #R-15	932	5%	P	1,034	8%	P
VTS #R-16	31	72%	N	12	200%	N
VTS #R-17	1,226	3%	P	1,009	7%	P
VTS #R-18	23	80%	N	29	87%	N
VTS #R-19	1,056	1%	P	1,106	5%	P
VTS #R-20	19	86%	N	51	36%	N
VTS #R-21	1,132	7%	P	910	7%	P
VTS #R-22	8	404%	N	47	13%	N
VTS #R-23	1,784	4%	P	1,637	10%	P
VTS #R-24	11	48%	N	40	30%	N

N= Negative for rPA; P = Positive for rPA; NR = Not Reportable and considered negative for rPA per IR-628 and DR-14248 (b) (4)

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Comment:

The applicant does not state the level of hemolysis that was tested. Slightly hemolytic samples may yield a different result from samples that are grossly hemolytic. The presence of anti-PA antibodies suggest that the animal was in previous contact with PA or that the samples were contaminated with the antibody. The possibility of cross-reactivity with other organisms was not examined. To exclude an animal that had produced anti-PA antibodies from inclusion in the assay, screening for the presence of this antibody should have been a part of the selection process for animals in this study.

4.1.2.2. Stability

Short-Term Stability

Short-term stability was tested by a comparison of the results of two separate plates of rabbit rPA spiked specimens and experimentally naïve rabbit serum specimens. This assay assessed the length of time a ‘back-up’ sample could be incubated beyond the time designated for testing, in the SOP to avoid compromising the accuracy of the results. The timing of the start of testing of the primary and ‘back-up’ specimens was the same, but the length of incubation phase during the conduct of the test, differed. In the 170 pairs of samples that were tested for the short term stability, there was 100% concurrence in the paired results after an extended incubation period of 37 minutes. Therefore, for the ECL assay, an extended incubation period of up to 37 minutes made no difference in the test results. Thirty-seven minutes was the longest time that was tested.

Long -Term Stability

Long-term stability was tested by the use of freeze/thaw (F/T) cycles after 1, 2 and 3 cycles, which were tested on 3 days. Both naïve and rPA spiked samples were used. The stored samples were allowed to thaw at room temperature for varying lengths of time, tested and returned to storage at $\leq -70^{\circ}\text{C}$. The cycle was repeated for a designated number of times. The

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acceptance criteria for false negative rate was <10% and for the false positive result the rate must be < 5%. The freeze/thaw schedule is shown in Table 2. The rabbit serum samples remained stable for up to 3 freeze-thaw cycles. Table 3 shows the long term stability testing results for rabbit serum. There were no false negative or false positive results.

Table 2: Freeze/Thaw Schedule for Long-Term Stability Testing of Rabbit serum

VTS	Aliquot ID	Testing Day 1	Testing Day 2	Testing Day 3	Testing Day 4	Testing Day 5	Testing Day 6
VTS #R-1 to VTS #R-12	F/T 1 Aliquot	Prepared			Tested (F/T=1)		
	F/T 2 Aliquot	Prepared		Thawed (F/T=1)	Tested (F/T=2)		
	F/T 3 Aliquot	Prepared	Thawed (F/T=1)	Thawed (F/T=2)	Tested (F/T=3)		
VTS #N-1 to VTS #N-12	F/T 1 Aliquot		Prepared				Tested (F/T=1)
	F/T 2 Aliquot		Prepared			Thawed (F/T=1)	Tested (F/T=2)
	F/T 3 Aliquot		Prepared		Thawed (F/T=1)	Thawed (F/T=2)	Tested (F/T=3)

Prepared = The VTS was created, tested, then aliquots were prepared and frozen at ≤-70°C.

Thawed = The aliquot was removed from the freezer at ≤-70°C, thawed at RT, held for at least 1 hr at RT, and then returned to the freezer.

Tested = The aliquot was removed from the freezer at ≤-70°C, thawed at RT, and te

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Table 3: Long Term Stability Results for Rabbit Validation Test Samples

VTS	Mean ECL	%CV	Result	Mean ECL	%CV	Result
				Replicate 1		
Plate ID: Rabbit -21; Freeze/Thaw 1						
VTS #R-1	26	96%	N	6	209%	N
VTS #R-2	39	32%	N	32	58%	N
VTS #R-3	47	3%	N	29	75%	N
VTS #R-4	40	23%	N	36	77%	N
VTS #R-5	27	52%	N	4	847%	N
VTS #R-6	41	24%	N	12	303%	N
VTS #R-7	871	3%	P	840	9%	P
VTS #R-8	1,112	2%	P	1,134	3%	P
VTS #R-9	938	4%	P	1,014	4%	P
VTS #R-10	1,040	3%	P	1,134	3%	P
VTS #R-11	872	6%	P	929	2%	P
VTS #R-12	1,045	3%	P	1,096	6%	P
Plate ID: Rabbit -19; Freeze/Thaw 2						
VTS #R-1	20	23%	N	22	214%	N
VTS #R-2	36	19%	N	17	122%	N
VTS #R-3	25	114%	N	36	47%	N
VTS #R-4	33	28%	N	23	45%	N
VTS #R-5	36	58%	N	52	39%	N
VTS #R-6	31	35%	N	37	34%	N
VTS #R-7	706	2%	P	724	1%	P
VTS #R-8	857	5%	P	873	2%	P
VTS #R-9	765	2%	P	845	2%	P
VTS #R-10	932	3%	P	997	5%	P
VTS #R-11	714	5%	P	765	6%	P
VTS #R-12	817	3%	P	868	2%	P
Plate ID: Rabbit -17; Freeze/Thaw 3						
VTS #R-1	45	12%	N	13	227%	N
VTS #R-2	43	24%	N	11	39%	N
VTS #R-3	22	77%	N	20	23%	N
VTS #R-4	58	3%	N	33	44%	N
VTS #R-5	49	21%	N	9	124%	N
VTS #R-6	43	45%	N	45	47%	N
VTS #R-7	902	5%	P	860	3%	P
VTS #R-8	1,175	4%	P	1,152	2%	P
VTS #R-9	889	9%	P	977	1%	P
VTS #R-10	1,041	0%	P	1,041	3%	P
VTS #R-11	922	4%	P	931	5%	P
VTS #R-12	1,091	3%	P	1,179	5%	P

N= Negative for rPA; P = Positive for rPA.

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4.1.2.3. Robustness

The effect of delay in sampling was measured by testing of duplicates samples after storing for 2 hours and 20 minutes longer than the other. Table 4 shows the results of the test for

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robustness. The acceptability criterion was 0% false negative or false positive. Robustness was validated for 2 hours 20 minutes of delay in testing after a specimen is drawn.

Table 4: Results of Robustness of ECL test for the presence of Protective Antigen in Rabbit Serum

VTS	Mean ECL	% CV	Result	Mean ECL	% CV	Result
	Plate ID: Rabbit -1-R			Plate ID: Rabbit -2-R		
VTS #R-1	41	17%	N	27	147%	N
VTS #R-2	28	60%	N	26	35%	N
VTS #R-3	25	84%	N	32	39%	N
VTS #R-4	27	44%	N	35	57%	N
VTS #R-5	18	85%	N	16	86%	N
VTS #R-6	27	72%	N	27	12%	N
VTS #R-7	1,573	2%	P	1,534	9%	P
VTS #R-8	1,534	6%	P	1,557	4%	P
VTS #R-9	1,303	1%	P	1,341	2%	P
VTS #R-10	1,809	4%	P	1,848	0%	P
VTS #R-11	1,552	1%	P	1,517	11%	P
VTS #R-12	1,650	2%	P	1,644	2%	P

N= Negative for rPA; P = Positive for rPA

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4.1.2.4. Precision

The protocol for testing precision in rabbits is the same as that presented for monkeys.

Table 5 shows the criteria that were used for assessment of precision for rabbit serum ECL tests. All of the tests passed the acceptance criteria.

Table 5: Rabbit Assay Precision Across Days, Operators, and ECL Plate Lots.

Test Plan Number	Test Plan Passed Validation Criteria	Multiple Operators	Multiple Testing Days	Multiple Plate Lots
1A	PASS	4	N/A	3
2A	PASS	4	2	3
3A	PASS	N/A	N/A	N/A
4A	PASS	4	2	3
5A	PASS	3	N/A	N/A
7A	PASS	2	N/A	2
8A	PASS	N/A	N/A	N/A

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4.1.2.5. Limit of Detection – Rabbit Serum

The Limit of Detection was calculated on the basis of three rPA spiked (2 ng/mL, 4 ng/mL, and 5 ng/mL) samples using serum from the same animal. The concentration of 2 ng/mL was selected as the lowest starting concentration to be tested because earlier tests in a different study (QD-157) suggested that the lowest concentration of PA that could be detected was 2 ng/mL. The samples were tested 4 times by this ECL assay and the lowest concentration in which there was 100% positive detection of PA was selected as the limit of detection. Table 6 shows the results of such testing. As can be noted, none of the replicates for the 2 ng/mL rPA sample was positive but all of the 4 ng/mL and 5 ng/mL samples were positive. The LOD for test results for rabbit sera by ECL was therefore chosen to be 4 ng/mL.

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Table 6: Limit of Detection for the Rabbit serum ECL test

VTS	Mean ECL	% C V	Result	Mean ECL	% CV	Result	Mean ECL	% C V	Result	Mean ECL	% CV	Result
	Replicate 1				Replicate 2			Replicate 3			Replicate 4	
Plate ID: Rabbit -13												
VTS #R- 25	271	7%	N	281	6%	N	306	8%	N	0	NA	NA
	268	20%	N	292	5%	N	313	7%	N	276	3%	N
VTS #R- 26	552	10%	P	576	1%	P	568	4%	P	609	4%	P
	502	11%	P	579	4%	P	587	1%	P	605	4%	P
Plate ID: Rabbit -14												
VTS #R- 25	270	5%	N	272	5%	N	294	2%	N	241	13%	N
	250	10%	N	283	8%	N	283	4%	N	244	21%	N
VTS #R- 26	496	6%	P	524	6%	P	519	3%	P	488	11%	P
	481	11%	P	539	7%	P	577	4%	P	553	9%	P
Plate ID: Rabbit -15												
VTS #R- 27	848	2%	P	810	2%	P	844	4%	P	822	11%	P
	812	6%	P	806	3%	P	831	4%	P	808	10%	P
Plate ID: Rabbit -16												
VTS #R- 27	761	4%	P	772	3%	P	807	4%	P	753	4%	P
	738	7%	P	802	4%	P	791	5%	P	783	2%	P

N= Negative for iPA; P = Positive for iPA; NA = Not Appli

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4.1.2.6. Real World Sample Assessment - Data from Infected animals

New Zealand White rabbits were aerosol challenged with 200X the median lethal dose of spores of the Ames strain of *Bacillus anthracis*. Blood specimens from unchallenged naïve rabbits were tested by ECL and also cultured. Results from blood cultures of these naïve rabbits were used to confirm, if necessary, if animals were positive prior to challenge (false positive rate). Positive results of tests that were performed after the animal was challenged, but prior to treatment, were used to assess the false negative rate. The applicant stated that all of the false negative results occurred at one or two time points immediately prior to treatment. This early stage of the infection appeared to be too weak to be detected by this assay.

Bacteremia was identified for 168 samples; of these, 148 were positive by ECL assay. Culture is the 'gold standard' for detecting infection by *Bacillus anthracis*. Therefore, there was an 88% true positive rate and a 12% false negative rate. The applicant stated that 12 of the false negative ECL samples had culture positive results of <5 colonies. All of 80 true negative samples that were obtained prior to challenge were negative by both ECL and culture.

In another animal study, sera from 56 recently infected animals were tested post exposure by ECL. When the samples became positive the animals were treated with one of four antibiotics. The specimens were tested fresh then stored at $\leq -60^{\circ}\text{C}$ for 64 to 118 days, and retested by the ECL assays. The concurrence between the fresh and stored samples was 97.9% (431/443). Of the 77 samples that were negative at baseline 48 were positive post challenge but prior to treatment, 34 % of the samples were positive at 24 hours, 29% were positive at 30 hours, the other samples had insufficient quantity and could not be tested. One sample was negative at baseline and at subsequent time points tested.

Comment:

The data from infected animals raises some concern in terms of accuracy. A blood culture that grows 5 colonies of *Bacillus anthracis* is positive for infection with this pathogen. The applicant also stated that the false negative ECL results occurred 1 or 2 time points prior to treatment; if the time points were 6 hours apart that means a 12 hour delay in diagnosis by ECL. The culture results will take 18 to 24 hours for recognizable growth to occur. The false negative rate is cause for concern in this deadly disease.

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Table 7: Summary of Validation Parameters for Electrochemiluminescence assay for Rabbit studies

Parameter	Validation Acceptance Criteria	Test results of Parameter	Validation Decision	Comment
Limit of Detection	Minimum concentration of rPA in which there is 100% detection PA	4 ng/mL	Acceptable	None
Precision	Composite of all tests individually False negatives, False positives, Short & Long term stability, LOD, Robustness	False negatives =Pass False positives = Pass Short-term stability =Pass Long term stability = Pass LOD = Pass Robustness = Pass	Acceptable Acceptable	None
Matrix effect	False negative rate = <10% False positive rate = <5%	Hemolytic sera = Pass 0% false negative , 0% false positive rPA +rLF or rEF = Failed False positive = 0% False negative = 0% Specimens without anti-PA IgG = 0%.	Failed	rEF, rLF mask rPA Applicant stated that error resulted from dilution. When repeated the false negative rate = 0% (Pass)
Specificity for detection of rPA	False Negatives PA = <2 results/ batch and Rate <10% rate ----- False Positives PA = <4 Results/batch and Rate < 5% ----- Matrix effect = False negative = <10% False positive = <5%	0% ----- 0% ----- EF & LF False negative = 0 % False positive = 0% Hemolytic serum False Negative = 0% False positive = 0%	Acceptable	None
Stability PA63	Short-Term - Concurrence > 95% ----- Long-Term – False Negative rate =<10% False positive rate =<5%	100% validated up 37 minutes ----- False Negative 0% False positive 0%	Acceptable	Validated up to 3 freeze/thaw cycles
Robustness	Samples incubated at room temperature for 1.5 hours: rPA spiked samples = 100% positive Naïve samples = 100% negative	rPA = Positive 100% Naïve samples = Negative 100%	Acceptable	Validated up to 2hrs 20 minutes

Neg = negative, pos = positive, EF = edema factor , LF = lethal factor, rPA = recombinant protective antigen

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Comment:

Overall, the ECL assay used for testing of rabbit sera from infected animals is appropriate (Table 7). Some of the limitations include possibility of false negative findings in the presence of anti-PA IgG. A possibility of false positive findings with organisms other than Bacillus anthracis was not evaluated

Also, the LOD for both rabbits and monkeys should be 4 ng/mL and not 1 ng/mL and 2 ng/mL respectively, as proposed by the applicant.

Specificity was measured by the rate of false negative and false positive results. Measurement of the matrix effect involved the assessment interference of test results by components that could likely be found in serum of Bacillus anthracis infected animals and by extension, humans. These were edema and lethal factors, hemolysis, and anti-PA IgG antibodies. All of these components adversely affected the results giving rise to false negatives in both species of animals. Testing of the hemolytic matrix in monkeys failed because one-third of the rPA spiked samples gave false negative results. In the rabbit serum there were problems when rEF and rLF were added to sera spiked with rPA. The applicant attributed the hemolysis results to a dilution error. When the assay was repeated the results was acceptable. It should be noted that the level of hemolysis was not specified. There may be no interference with a slightly hemolytic sample. But with a grossly hemolytic sample significant interference might occur. Alternatively, hemolysis may have variable effect on different animals.

The applicant stated that there was anti-PA IgG antibodies present in the hemolyzed samples where the results were negative. These antibodies would bind PA that was found in the serum resulting in negative values. This is important information when assessing results of ECL. However, only 56% of the hemolyzed samples contained anti-PA IgG.

A serious cause for concern is the fact that in the rabbit study 'Real World Sample Assessment', in comprised animals that had been aerosol challenged with Bacillus anthracis spores, there was a 12% false negative result. The applicant stated that some of the samples were incorrectly flagged as negative at 6 to 12 hours prior to treatment. Another reason for the negative flag could be a result of the LOD, which might not have been able to detect the limited amount of PA that would be present at that time. This is critical because this difference in time to treatment may determine the period between life and death of a subject. The applicant also stated that some of the negative results occurred in samples in which the blood culture bacterial count was ≤5 colonies. This is a reasonable observation. The correlation between the ECL assay and bacterial culture in the 'real world' animal infected samples showed that the false negative rate for monkeys was 5% and for rabbits 12%. This indicates an unacceptable false negative rate. It is being suggested that when ECL tests are run there should be a back-up blood culture.

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4.2. Enzyme linked Immunosorbent Assay (ELISA)

ELISA is one of the common tests used for assessing immunological responses. There are two basic types of ELISAs the direct and the indirect (Figure 1). The direct is used for detecting an antigen and the indirect used for detecting antibodies in a sample. In the latter test, the antibody is sandwiched between the antigen on the plate and the tagged anti-species antibody, thus the name ‘sandwich’ ELISA (Figure 2). The assay in this study is a ‘sandwich’ ELISA designed to measure PA levels in the serum of New Zealand White rabbits and Cynomolgus monkeys infected with *Bacillus anthracis*.

A specific concentration of purified polyclonal anti-PA IgG antibody (rabbit anti-PA IgG) was immobilized in the wells of a 96 well microtiter plate, incubated, and washed. The capture antibody was blocked with skim milk. The diluted standard samples, quality control samples and test samples were added to the plate, incubated, and washed. The primary detection agent goat anti-PA serum was added to the washed plate and re-incubated. After incubation and washing, bovine anti-goat horseradish peroxidase (HRP)-conjugated anti-gamma chain antibody (conjugate) which was the secondary antibody used as the signal, was added. This combination was incubated with a stop solution and then read spectrophotometrically. The endpoint of the assay was the concentration of PA in nanograms/milliliter (ng/mL). The tests were read on a 96 well microplate reader (SpectraMax 190 or SpectraMax Plus) and analyzed using SOFTmax-PRO.

Figure 1: Two Basic types of ELISA tests used in this Validation study

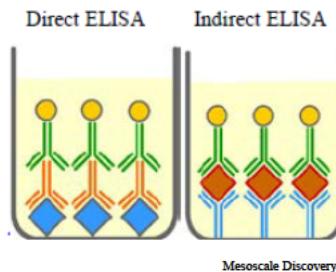
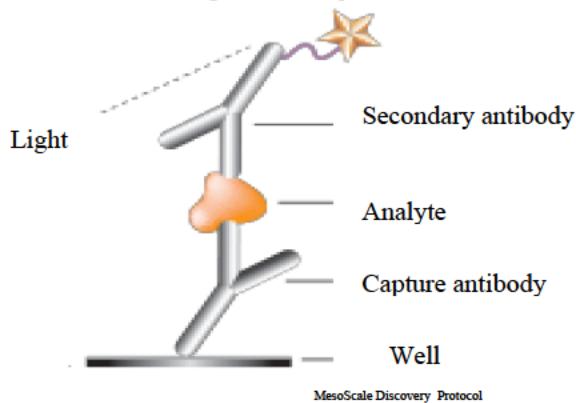


Figure 2: Principle for Sandwich ELISA



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4.2.1. Enzyme Linked Immunosorbent Assay for Detection of *Bacillus anthracis* Protective Antigen in Monkeys

Testing of monkey sera was performed using three different ELISAs at two laboratories:

- [REDACTED] (b) (4)
- [REDACTED] (b) (4)

Test performed at [REDACTED] (b) (4)

Two different assays were performed at [REDACTED] (b) (4). One was designed to measure total PA i.e., both complexed PA (PA bound to ETI-204, LF, and EF) and uncomplexed (or “free” not bound to ETI-204) PA (PA83, PA63). The free PA assay would not detect PA bound to ETI-204 or PA 20, but will detect PA complexed to other proteins such as LF or EF.

4.2.1.1. Quantitation of total PA

The assay was designed to detect bound and free PA from *Bacillus anthracis* in the serum of cynomolgus monkeys (SOP- [REDACTED] (b) (4) X-180 03, Validation Protocol VP2008-200, Appendix A²). Briefly, purified polyclonal anti-PA IgG (rabbit anti-PA IgG) was used as the solid phase antibody to capture the antigen PA in the serum of infected monkeys, as well as recombinant PA (rPA63) in spiked normal monkey serum. A 2 µg/mL concentration of the rabbit anti-PA IgG was immobilized in the wells of a 96 well microtiter plate, incubated, and washed; skim milk was used as the blocking agent. A 16,000 ng/mL standard of rPA spiked monkey serum was diluted 4-fold up to seven times to 0.244 ng/mL concentration, in order to generate a standard curve. The diluted standard samples, quality control samples (high = 1000 ng/mL, medium =100 ng/mL, and low =10 ng/mL) diluted 5-fold, and test samples, were added to the plate, incubated, and washed. The primary detection agent goat anti-PA serum was added to the washed plate and re-incubated. After incubation and washing, bovine anti-goat HRP-conjugated anti-gamma chain antibody (conjugate) which was the secondary antibody used as the signal, was added. This combination was incubated with a stop solution and then read spectrophotometrically.

4.2.1.2. Acceptance Criteria

Plate Acceptability

The primary criteria required that specimens in the plates meet collectively the minimum criteria. The criteria used to assess the plate acceptance were:

Quality Control (QC) -high, medium, low and negative, and reference standards (RS). The acceptability criteria for each of these parameters are summarized in the respective sections addressed below.

Figure 1 shows the arrangement of the samples on the microtiter plate. The samples were diluted vertically down the plate. The two reference standards were each diluted 7 times. The relative standard deviation (RSD) between equal concentrations of the same substance must be <20 to be valid.

² Source: DMF [REDACTED] (b) (4)

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4.2.1.3. Standards and Controls

Calibration Standards

The reference standards (RS) were allowed to be censored for as many as 4 dilution points.

Quality Control Samples

The three QC samples used in this assay were the high QC (HQC) with a concentration of 1000 ng/mL, the medium QC (MQC) 100 ng/mL, and the low QC (LQC) 10 ng/mL. Each QC sample was diluted 1:5 for three successive times. To be accepted the following were required:

- The HQC and MQC fell within $\pm 30\%$ of their nominal value.
- The Low QC fell within $\pm 50\%$ of its nominal value.
- The value of the negative control had an optical density of ≤ 0.200 .
- At least two of the three QC samples fell within the acceptable value range described above.

QC was allowed to be censored one dilution at a time in order to have a percent coefficient of variation (%CV) of $\leq 30\%$.

Test Samples

Stability of Samples

The stated conditions under which the samples were to be handled in order to prevent degradation of the PA₆₃ molecule were as follows:

The samples were to be tested within 72 hours of collection and held at 2 to 8°C during this period. After 72 hours the samples had to be stored at $\leq -60^{\circ}\text{C}$. For long term storage the samples were held at $\leq -60^{\circ}\text{C}$ and could last for as much as 3 months without losing activity.

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Sample Testing Criteria

For samples tested two independent times the %CV must be $\leq 35\%$. If greater than 35% the samples were to be repeated. If the quantity was insufficient and the %CV was <35 , then the median value of the test results was used.

Figure 1 : The layout of samples in the ELISA plate for Monkey studies

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS-1 4000 ng/mL	RS-1 4000 ng/mL	TS-1 neat	TS-2 neat	TS-3 neat	TS-4 neat	TS-5 neat	TS-6 neat	TS-7 neat	TS-8 neat	QC-Mid neat	QC-Low neat
B	RS-2 1000 ng/mL	RS-2 1000 ng/mL	TS-1 1:5	TS-2 1:5	TS-3 1:5	TS-4 1:5	TS-5 1:5	TS-6 1:5	TS-7 1:5	TS-8 1:5	QC-Mid 1:5	QC-Low 1:5
C	RS-3 250 ng/mL	RS-3 250 ng/mL	TS-1 1:25	TS-2 1:25	TS-3 1:25	TS-4 1:25	TS-5 1:25	TS-6 1:25	TS-7 1:25	TS-8 1:25	QC-Mid 1:25	QC-Low 1:25
D	RS-4 62.5 ng/mL	RS-4 62.5 ng/mL	TS-1 1:125	TS-2 1:125	TS-3 1:125	TS-4 1:125	TS-5 1:125	TS-6 1:125	TS-7 1:125	TS-8 1:125	QC-Mid 1:125	QC-Low 1:125
E	RS-5 15.6 ng/mL	RS-5 15.6 ng/mL	TS-9 neat	TS-10 neat	TS-11 neat	TS-12 neat	TS-13 neat	TS-14 neat	TS-15 neat	TS-16 neat	NC neat	QC-High neat
F	RS-6 3.9 ng/mL	RS-6 3.9 ng/mL	TS-9 1:5	TS-10 1:5	TS-11 1:5	TS-12 1:5	TS-13 1:5	TS-14 1:5	TS-15 1:5	TS-16 1:5	NC 1:5	QC-High 1:5
G	RS-7 0.98 ng/mL	RS-7 0.98 ng/mL	TS-9 1:25	TS-10 1:25	TS-11 1:25	TS-12 1:25	TS-13 1:25	TS-14 1:25	TS-15 1:25	TS-16 1:25	NC 1:25	QC-High 1:25
H	RS-8 0.24 ng/mL	RS-8 0.24 ng/mL	TS-9 1:125	TS-10 1:125	TS-11 1:125	TS-12 1:125	TS-13 1:125	TS-14 1:125	TS-15 1:125	TS-16 1:125	NC 1:125	QC-High 1:125

RS = reference standard

TS = test sample

NC = negative control

QC= quality control

Validation SOP this submission

Comment:

Overall, the applicant's acceptable criteria are very wide and do not contribute to a high level of accuracy or precision.

4.2.1.4. Sensitivity

Eight concentrations of PA63 spikes were used to compute the standard curve. The concentrations ranged from 16,000 ng/mL to 2.44 ng/mL. Each standard was diluted 4-fold to obtain the next lower dilution. The Lower Limit of Quantitation (LLOQ) and the Upper Limit of Quantitation (ULOQ) were determined from this standard calibration curve. Three hundred and six values were used to determine the Limit of Quantitation. The spikes for the standard curve were prepared on each day of use from certified, frozen monkey serum. The frozen serum was thawed and diluted in a mixture of ELISA Wash buffer (Phosphate buffered saline and Tween 20) and Recovery buffer (Wash Buffer +3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate- CHAPS + EDTA). CHAPS is used for the dissociation of proteins; in this study, it caused the dissociation of aggregates of rPA63 and EDTA making the solution easier to read, spectrophotometrically.

The %CVs ranged from 0.3 to 7.3 signifying that the data are accurate. Table 1 shows the results of the tests. The standards were run in duplicate. None of the data from any of the 8 standard points needed to be excluded. Standard 1 and 8 were anchor points and could not have been included in the standard curve. The ULOQ was determined to be 6,400 ng/mL and the LLOQ was determined to be 4.2 ng/mL.

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Figure 2 is a graph of the standard curve using 8 PA spikes ranging from 4000 – 0.244 ng/mL.

Table 1: The results of the 8 concentrations of PA63 used to draw the standard curve for interpretation of results of Monkey ELISA tests

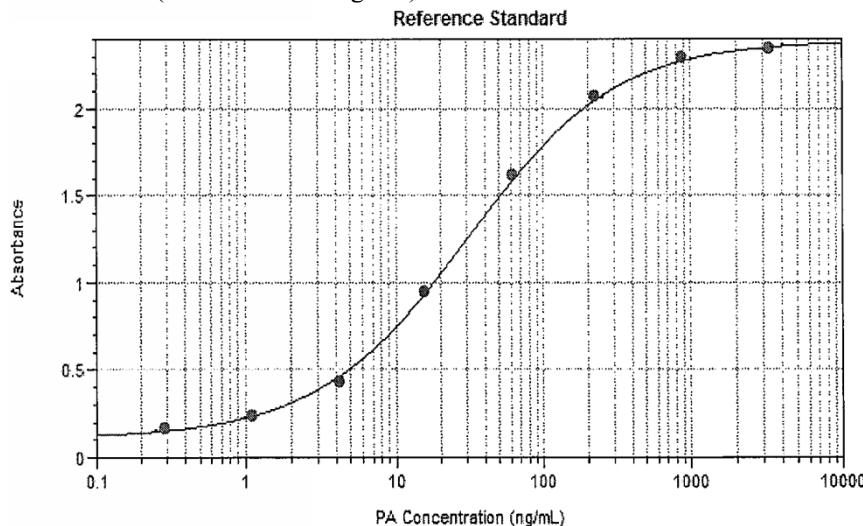
Sample	Concentration	Wells	Values	Mean Value	Std Dev	CV%	Result	Mean Result	% Recovery	Check 2
1	4000.000	A1	2.427	2.454	0.038	1.6	3374.785	3374.785	84.370	2.454
		A2	2.481							
2	1000.000	B1	2.359	2.371	0.017	0.7	1023.703	1208.433	120.843	2.371
		B2	2.383							
3	250.000	C1	1.984	2.071	0.150	7.3	134.489	212.835	85.134	2.071
		C2	2.177							
4	62.500	D1	1.673	1.704	0.043	2.5	64.364	69.163	110.661	1.704
		D2	1.734							
5	15.625	E1	0.963	0.993	0.041	4.2	14.978	15.966	102.183	0.993
		E2	1.022							
6	3.906	F1	0.419	0.433	0.020	4.6	3.183	3.389	80.768	0.433
		F2	0.447							
7	0.977	G1	0.226	0.225	0.001	0.3	0.937	0.932	95.417	0.225
		G2	0.225							
8	0.244	H1	0.165	0.168	0.004	2.2	0.401	0.422	172.867	
		H2	0.170							

Min Value = 0.168

Max value 2.454

Source: (b) (4) validation report

Figure 2: Example of Monkey PA reference Standard 8-Point Dilution Curve (4000 – 0.244 ng/mL)



Source: (b) (4) validation report

4.2.1.5. Accuracy

The criteria for the determination of accuracy were defined by the total error between the expected PA concentration and the test results. The percent total error (TE) accepted was $\leq 50\%$. The results were based on 306 data points. Table 2 shows the total error resulting from each nominal concentration used to draw the standard curve. One concentration (1,024 ng/mL) achieved the limit of 50% while the total error for the others samples ranged from 26% to 43%. Figure 3 shows the spread of test results relative to the actual concentrations of the standards. As stated previously, the resulting concentration 1,024 ng/mL is farthest removed from the expected concentration. The ULOQ – the highest concentration of PA that the assay can accurately quantitate, was determined to be 6,400 ng/mL

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and the LLOQ the lowest concentration of PA that can be measured by this assay was selected to be 4.2 ng/mL.

Table 3 shows the effect of variability based on operator, plates, and days on which the sample was tested. No difference resulted from the day on which samples were run, or the plate in which they were placed; the greatest variation was caused by the operator. At the concentration where the operator imprecision was highest the overall error was greatest, the opposite was also true. However, together these variables of operator, day, and plates met the < 50% acceptability criteria proposed by the applicant.

Accuracy also involved the amount of PA recovered as compared with the true concentration. The acceptability criteria stated by the applicant was that the amount of PA recovered must be $100 \pm 30\%$ of the nominal value. The percentage of PA recovered ranged from 80 – 120 ng/mL, which is acceptable. The bias tended to decrease with increasing concentrations of PA. Table 4 shows the recovery data at each level of concentration. The lowest amount of PA recovered was 80% from the concentration of 1,024 ng/mL. Not only were the low concentrations indicative of inaccuracy, but so were the concentrations that recovered more than 100% of PA.

Table 2: Total error between specific nominal concentrations of PA that were used to draw the standard curve

PA Conc. ng/mL	4.2	10.5	26.2	65.5	163.8	409.6	1,024	2,560	6,400
Pct Total Error	38%	32%	26%	21%	30%	36%	50%	43%	33%

Source: (b) (4) validation report

Table 3: Effect of variability by operator, plates, and days on the accuracy of the results

Conc. (ng/mL)	Predict. \log_{10} (Conc.) (ng/mL)	Predict. Log. Bias	Residual Variance	Non-Residual Variance (NRV) Breakdown				Sum of NRV	Total log Variation	Total log Error	Percent Total Error
				Operator	Day	Plate	Replicate				
4.20	0.693	0.070	0.009	0.006	0.000	0.000	0.006	0.012	0.021	0.161	38%
10.5	1.100	0.079	0.006	0.000	0.000	0.000	0.006	0.006	0.012	0.134	32%
26.2	1.410	-0.008	0.006	0.000	0.000	0.000	0.006	0.006	0.013	0.113	26%
65.5	1.781	-0.035	0.000	0.001	0.000	0.000	0.006	0.007	0.007	0.090	21%
164	2.161	-0.053	0.008	0.000	0.000	0.000	0.006	0.006	0.014	0.129	30%
410	2.623	0.010	0.014	0.003	0.000	0.000	0.006	0.009	0.023	0.152	36%

Source: (b) (4) validation report

Table 4: The percentage of PA recovered compared to nominal concentrations at various concentrations

PA Conc. ng/mL	4.2	10.5	26.2	65.5	163.8	409.6	1,024	2,560	6,400
Pct Recovery	117%	120%	98%	92%	88%	102%	80%	89%	82%

Pct = percentage Source: (b) (4) Validation report

Comments:

These results, although they fall within the applicant's acceptable limit, do not show a high degree of accuracy. The percentage total error (excluding 1,020 ng/mL) ranged from 21 ng/mL

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to 43 ng/mL. The operator effect was the most critical component in the determination of inaccuracy.

4.2.1.6. Limits of detection

The lower limit of detection (LLOD) is the lowest concentration of PA at which $\geq 95\%$ of the PA results obtained were detected from of the standard curve, but does not necessarily have to be accurately quantified. The upper limit of detection (ULOD) was the highest concentration of PA at which $\geq 95\%$ of results fell below the highest end of the standard curve. The LLOD was determined to be 0.87 ng/mL, which was <4.20 ng/mL, and the ULOD was calculated to be 24,000 ng/mL.

The limit of detection (LOD) is the lowest concentration of PA at which 95% of the lowest values can be accurately detected. The LOD was determined to be 4.2 ng/mL.

4.2.1.7. Linearity

The objective of testing this parameter was to determine if a diluted sample of PA63, dissolved in pooled naïve monkey serum, will obtain a proportionally accurate result. Three hundred and six values were used to compute the linearity. The maximum standard deviation was placed at <20% ; and the slope at 1 ± 0.064 . Figure 3 shows the linearity of the standard samples. The slope in the assay was 0.955 which was acceptable.

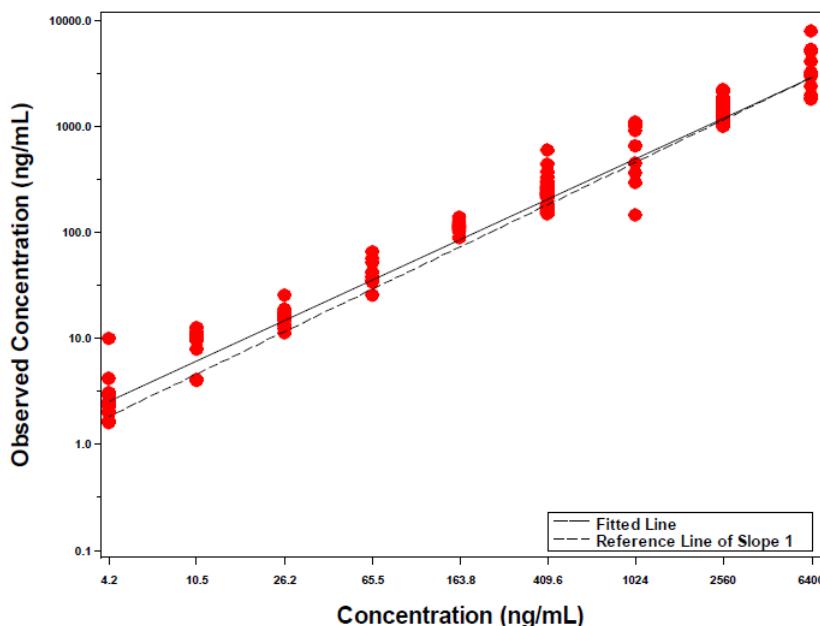
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Figure 3: Linear Relation between Predicted Concentration and True Concentration (Displayed on a Logarithmic Scale) Between 4.20 ng/mL to 6,400 ng/mL



Source: (b) (4) Validation report

4.2.1.8. Precision

Precision of the assay was determined by the results obtained when aliquots of the same sample were tested by the same or different operators, on the same or different runs, or on the same or different days. These different operations were classified as either intra-run or inter-run precision.

4.2.1.9. Repeatability tests:

Intra-run precision by one operator. This parameter was assessed when the results from aliquots of the same sample were tested within the same run, but not necessarily on the same plate. The acceptance criterion set by the applicant for agreement was a difference in values of < 30%. The concentrations tested were 10.5, 409.6 (410), 2,500, and 16,000 ng/mL. The results are shown in Table 5. The criterion was met in all but the highest concentration 16,000 ng/mL with a deviation of 49%. This means that reproducibility was inaccurate at the highest dilution and that results cannot be relied upon when such high concentrations of PA are present.

Table 5: Results of testing for Repeatability of PA from Monkey sera.

PAConc.ng/mL	10.5	409.6	2.560	16.000
Pct Rel Std Devn	19%	17%	27%	49%

Source: (b) (4) validation report

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Intermediate inter-run precision: This compares results of aliquots of the same specimen, but tested either on different days, different runs, or by different operators. The acceptable limit relative standard deviation (%RSD) for all concentrations (4.20 ng/mL to 6,400 ng/mL) must be ≤ 30%. The results of testing for intermediate precision are shown in Table 6. The table shows that the criteria were met only when the PA concentration was between 10.5 ng/mL to 164 ng/mL, and at the highest concentration 6,400 ng/mL. The results from 10.5 ng/mL to 164 ng/mL ranged from 19 to 28%. At the higher concentrations 410, 1,020, and 2,560 ng/mL, the values ranged between 36 and 41%. These three concentrations were also the concentrations where the operator variability was highest. The results suggest that, in this study, these values when repeated might not be accurate and that accuracy might depend, to a large extent, on the person who ran the test.

Table 6: Results of the analysis of Intermediate Precision showing the percentage relative standard deviation from each PA concentration

True Concentration (ng/mL)	Total Logarithmic (Base 10) Variance	%RSD (Observational Scale)
4.20	0.021	34%
10.5	0.012	25%
26.2	0.013	26%
65.5	0.007	19%
164	0.014	28%
410	0.023	36%
1,020	0.033	44%
2,560	0.030	41%
6,400	0.012	26%

Source: (b) (4) validation report

4.2.1.10. Specificity

Specificity for the assay was tested using five constituents which may influence the test results either by increasing, decreasing, or masking the PA. Three of the substances (EF, LF, antibiotic used for treatment) may be found in the blood of *Bacillus anthracis* infected monkeys, a fourth condition hemolysis, may be a consequence of blood collection technique. Plasma is sometimes used in place of serum for blood tests. The applicant also analyzed the effect of replacing serum with plasma. The substances tested were:

- Edema factor – one of the components of the tripartite toxin
- Lethal factor - also one of the components of the tripartite toxin
- Ciprofloxacin – from a class of drugs with which the non-human primates may be treated
- Hemolytic serum – resulting from a poor blood draw
- Plasma – resulting from unclotted blood which may be used as an alternative to serum

Specificity was tested using 142 samples in 12 plates, with blood obtained from 7 individual monkeys. The matrices were tested at concentrations of 6,400, 26.2 and 4.2 ng/mL. *Bacillus anthracis* PA63, LF and EF were obtained from (b) (4), serum and plasma were obtained from (b) (4), and ciprofloxacin from Aleken. The tests were run on 3 days by 3 operators. Pooled monkey serum from normal animals was also used as a matrix.

Analysis of results of the pooled serum against individual monkey samples was used to determine if, in future analyses, pooled serum can be used for comparison in tests, instead of serum from individual monkeys. If the results between the pooled serum and individual monkey

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sera differed by <30%, then pooled sera will be used in the future analyses. If $\geq 30\%$, then individual sera were to be used for comparing normal sera and sera with interferents. The results suggest that the difference did not fall within the acceptability zone. Therefore the serum samples were not pooled as a matrix for testing and individual monkey samples were used in the analyses.

Spikes of the interferents listed above at concentrations of 6,400, 26.2 and 4.20 ng/mL were placed in normal monkey serum matrices from each of 7 monkeys. The following combinations were:

1. Normal Naïve monkey serum + Ciprofloxacin 5 μ g/mL
2. Normal Naïve monkey serum + *Bacillus anthracis* EF and PA in 1:1 ratio
3. Normal Naïve monkey serum+ *Bacillus anthracis* LF and PA in 1:1 ratio
4. Normal Naïve monkey plasma
5. Hemolytic Naïve monkey serum
6. Normal Naïve monkey serum

The plate Layout is shown in Figure 4.

Figure 4 : Plate layout for Repeatability Precision and Specificity Testing

Layout	1	2	3	4	5	6	7	8	9	10	11	12
	All	HI	Low	A, B, and G 6400	A, B, and G 26.2	A, B, and G 4.2	C, D, and G 6400	C, D, and G 26.2	C, D, and G 4.2	E and F 6400	E and F 26.2	E and F 4.2
Dilution/P												
Repeatability												
1	10000.0	16000.0	409.6	A - 6400 no	A - 26.2 no	A - 4.2 no	C - 6400 no	C - 26.2 no	C - 4.2 no	E - 6400 no	E - 26.2 no	E - 4.2 no
2	40000.0	25600.0	10.5	A - 6400 Cl	A - 26.2 Cl	A - 4.2 Cl	C - 6400 Cl	C - 26.2 Cl	C - 4.2 Cl	E - 6400 Cl	E - 26.2 Cl	E - 4.2 Cl
3	16000.0	16000.0	409.6	A - 6400 lf	A - 26.2 lf	A - 4.2 lf	C - 6400 lf	C - 26.2 lf	C - 4.2 lf	E - 6400 lf	E - 26.2 lf	E - 4.2 lf
4	6400.0	2560.0	10.5	A - 6400 Ef	A - 26.2 Ef	A - 4.2 Ef	C - 6400 Ef	C - 26.2 Ef	C - 4.2 Ef	E - 6400 Ef	E - 26.2 Ef	E - 4.2 Ef
5	2560.0	16000.0	409.6	A - 6400 He	A - 26.2 He	A - 4.2 He	C - 6400 He	C - 26.2 He	C - 4.2 He	E - 6400 He	E - 26.2 He	E - 4.2 He
6	1024.0	2560.0	10.5	A - 6400 Pl	A - 26.2 Pl	A - 4.2 Pl	C - 6400 Pl	C - 26.2 Pl	C - 4.2 Pl	E - 6400 Pl	E - 26.2 Pl	E - 4.2 Pl
7	409.6	16000.0	409.6	B - 6400 no	B - 26.2 no	B - 4.2 no	D - 6400 no	D - 26.2 no	D - 4.2 no	F - 6400 no	F - 26.2 no	F - 4.2 no
8	163.8	2560.0	10.5	B - 6400 Cl	B - 26.2 Cl	B - 4.2 Cl	D - 6400 Cl	D - 26.2 Cl	D - 4.2 Cl	F - 6400 Cl	F - 26.2 Cl	F - 4.2 Cl
9	65.5	16000.0	409.6	B - 6400 lf	B - 26.2 lf	B - 4.2 lf	D - 6400 lf	D - 26.2 lf	D - 4.2 lf	F - 6400 lf	F - 26.2 lf	F - 4.2 lf
10	26.2	2560.0	10.5	B - 6400 Ef	B - 26.2 Ef	B - 4.2 Ef	D - 6400 Ef	D - 26.2 Ef	D - 4.2 Ef	F - 6400 Ef	F - 26.2 Ef	F - 4.2 Ef
11	10.5	16000.0	409.6	B - 6400 He	B - 26.2 He	B - 4.2 He	D - 6400 He	D - 26.2 He	D - 4.2 He	F - 6400 He	F - 26.2 He	F - 4.2 He
12	4.2	2560.0	10.5	B - 6400 Pl	B - 26.2 Pl	B - 4.2 Pl	D - 6400 Pl	D - 26.2 Pl	D - 4.2 Pl	F - 6400 Pl	F - 26.2 Pl	F - 4.2 Pl
13	1.7	16000.0	409.6	G - 6400 no	G - 26.2 no	G - 4.2 no	G - 6400 Ef	G - 26.2 Ef	G - 4.2 Ef	empty		
14	0.7	2560.0	10.5	G - 6400 Cl	G - 26.2 Cl	G - 4.2 Cl	G - 6400 He	G - 26.2 He	G - 4.2 He	empty		
15	0.3	16000.0	409.6	G - 6400 lf	G - 26.2 lf	G - 4.2 lf	G - 6400 Pl	G - 26.2 Pl	G - 4.2 Pl	empty		
16	0.1	2560.0	10.5	6400.0 po	26.2 po	4.2 po	6400.0 po	26.2 po	4.2 po	6400.0 po	26.2 po	4.2 po

Key: no = normal lf = Lethal Factor He = Hemolytic po = pool
 Cl = Cipro Ef = Edema Factor Pl = Plasma

(b) (4) Validation Protocol

The results showed that there was a significant difference with LF and EF at 6,400 ng/mL, where the values were lower than the controls by ratios of 0.41 and 0.81, respectively. The interferent levels of 4.2 and 26.2 ng/mL showed no significant difference from the original concentrations. In Table 7, the concentration of the LF and EF at a PA concentration of 6,400 ng/mL had estimates 0.391 and 0.199 and have significant levels of <0.0001 . In Table 8, the ratio of interferent to control of 0.41 and 0.63, again showed a reduction (<1) in concentration compared to the control. This indicated that at 6,400 ng/mL, LF and EF were likely to interfere with PA. Hemolysis was only marginally acceptable but again, the concentration was not stated. It is possible that the effect of hemolysis might be concentration dependent.

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Table 7: Pairwise Differences in the Logarithmic Domain to Compare Normal Serum Samples with Serum Samples having Potential Interferents separately within each Concentration of 4.2, 26.2, 6,400 ng/mL

Target PA Concentration	Interferent compared to Control	Estimates	Std. Error	DF	t-Value	Signif(unadj) ^{21,22}
4.2	Cipro	-0.053	0.118	86.1	-0.45	0.6533
	LF	-0.005	0.119	86.5	-0.04	0.9663
	EF	-0.134	0.119	86.8	-1.12	0.2655
	Hemolytic	-0.264	0.119	86.8	-2.21	0.0298
	Plasma	-0.010	0.118	86.3	-0.09	0.9293
26.2	Cipro	-0.007	0.064	80.4	-0.12	0.9079
	LF	-0.059	0.060	80.3	-0.97	0.3346
	EF	-0.058	0.062	80	-0.94	0.3486
	Hemolytic	-0.104	0.059	79.9	-1.76	0.0814
	Plasma	-0.046	0.059	79.9	-0.77	0.4414
6400	Cipro	-0.006	0.043	71	-0.14	0.8866
	LF	0.391	0.044	72	8.97	<0.0001 *
	EF	0.199	0.046	73.8	4.31	<0.0001 *
	Hemolytic	-0.101	0.045	71.5	-2.25	0.0277
	Plasma	-0.005	0.045	73	-0.12	0.9068

Source: (b) (4) Validation report

Table 8: Pairwise Ratio in the Observational Scale to Compare Normal Serum Samples with Serum Samples having Potential Interferents, Separately within each Concentration of 4.2, 26.2, 6,400 ng/mL

Target PA Concentration	Interferent compared to Control	Difference between log-means	RATIO (Interferent/Control)	95% Lower Conf Bnd ²³	95% Upper Conf Bnd ²⁴
4.2	Cipro	0.053	1.13	0.66	1.94
	LF	0.005	1.01	0.59	1.75
	EF	0.134	1.36	0.79	2.35
	Hemolytic	0.264	1.84	1.06	3.17
	Plasma	0.011	1.02	0.60	1.76
26.2	Cipro	0.007	1.02	0.76	1.36
	LF	0.059	1.14	0.87	1.51
	EF	0.058	1.14	0.86	1.52
	Hemolytic	0.104	1.27	0.97	1.66
	Plasma	0.046	1.11	0.85	1.46
6400	Cipro	0.006	1.01	0.83	1.23
	LF	-0.391	0.41	0.33	0.50 *
	EF	-0.199	0.63	0.51	0.78 *
	Hemolytic	0.101	1.26	1.03	1.55
	Plasma	0.005	1.01	0.82	1.25

(b) (4) validation Report

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Table 9: Summary of Assay Validation studies for AP201 Monkey serum assay for Protective Antigen by ELISA

Parameter	Validation Criteria	Test results of Parameter (ng/mL)	Validation Decision	Comment
Limits of Detection	ULOD \geq 16,000 ng/mL LLOD \leq 4.2 ng/mL LOD \geq 95% of samples have a PA result	23,988 (24,000) 0.87 4.2	Acceptable	None
Limits of Quantitation	ULOQ \geq 16,000 ng/mL LLOQ \leq 4.2 ng/mL %TE \leq 50% (all concentrations)	6,400 4.2	Acceptable	None
Accuracy	Absolute = %RE (bias) \leq 30%		Acceptable	
Recovery	100 \pm 30 %	80 – 120 %	Acceptable	
Intermediate Precision	%RSD \leq 30% For conc. Between (4.2 – 16,000)	RSD < 30 % up to 410 ng/mL 1020 – 6400 * $>$ 30	Acceptable to 410 ng/mL	Validated only up to 410 ng/mL
Repeatability	%RSD \leq 30%	10.5, 409.6, 2560 17 – 27% 16,000 = 49%*	Partially acceptable	Not repeatable at 16,000 ng/mL
Specificity for detection of PA63	Normal Pool vs individual specimens < 30% Ability to detect interferent – significance level 77-130%	Results were > 30%. All interferents passed except EF and LF at 6400 ng/mL recovered 81 % and 41% respectively	Acceptable	None
Stability PA63	Maximum 182 days frozen at \leq -70°C Freeze/Thaw = 5 cycles	NA	NA	None
Linearity of Standard curve	Range at LLOQ and ULOQ = \leq 30% Slope = 1 \pm 0.064	0.995	Acceptable	

RE= relative error; RSD= relative standard deviation

Comments:

- In terms of specificity, at lower concentrations of interferents (LF or EF) it appeared that there will be interference with PA. This is very important because the initial diagnosis of

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anthrax might depend on detection of minute levels of PA. In relationship to hemolysis, it appeared to have borderline interference with PA.

- *Table 9 summarizes the performance of the assay. The results in general, are not inaccurate enough to cause the assay to be discarded, but the operators must be aware of the deficiencies. The comments take into consideration the fact that this assay is not meant to be used primarily for diagnostic purposes.*
- *Operator variability played a leading role in the percentage of total errors. Additionally, values at the ULOQ may, at times, be inaccurate and even though they fit within the acceptable ULOQ limit, the samples should still be diluted.*
- *Recovery rate posed a problem in a specimen with PA at a concentration of 1,024 ng/mL. The recovery rate of this spike was 80% of the expected concentration, which was very low. But inaccuracy also is evident in values that are higher than the expected concentration such as 4.2 ng/mL and 10.5 ng/mL which were too high at 117% and 120 % respectively.*
- *Intra-run precision was out of range when 16,000 ng/mL of PA was diluted. At high levels intermediate precision was imprecise. The values above 410 ng/mL were out of range.*
- *In terms of interference by certain substances that may be present in the test samples, most of the possible interferents tested including hemolysis, EF, LF may have no significant effect on the PA concentration. However, LF and EF at 6,400 ng/mL may result in a reduction in the amount of PA in the sample.*
- *Cross-reactivity with factors produced by other microorganisms such as *Bacillus cereus* was not measured.*

4.2.2. Quantitation of Free PA in Monkeys

The assay designed to detect free PA from *Bacillus anthracis* in the serum of infected cynomolgus monkeys was performed at [REDACTED] (b) (4) [Appendix U for Study AP203; Study 1219-100005989; [REDACTED] (b) (4) Validation report VP-2012. 257 ([REDACTED] (b) (4) XVI-007-02)].

This assay meant to recapture both PA63 and PA83, utilized a 96 well plate coated with plate coating buffer (1X PBS pH7.4 containing 1.0 µg/mL ETI-204 (Elusys Therapeutics: Lot no: RS02), an anti-PA monoclonal IgG antibody, which was left to immobilize overnight. The coated wells were washed with BLOTTO, to remove any unbound antibody, washed again to remove coating and blotting mixture. After washing, the calibration standards in sera (CS 1-12) that comprised of recombinant PA63 (List Biological Laboratories) and quality control sera comprising of pooled rPA63 (Alpha diagnostics), as well as test sera from the cynomolgus monkeys, were added. The monkey sera, QC and standards were added to the ETI-204 coated wells. The plates were washed to remove unbound reagents. A 1:100 dilution of goat-anti-PA serum was added followed by a secondary antibody, horseradish peroxidase cyano anti-goat IgG antibody (Invitrogen), and the plates incubated at 37°± 2° C. The test and QC samples were either undiluted or diluted 1:10 in assay buffer. After incubation, the plates were washed to remove unbound PA. The reaction mixture was again re-incubated with goat-PA antiserum and then incubated with 3,3'5,5' tetramethylbenzidine, and the reaction was stopped by the addition of 2N sulfuric acid. The amount of PA that was captured was measured in a micro-titer plate reader at 450 nm (BioTek Elx800, SpectraMax Plus) or a similar instrument. Figure 1 is a depiction of the ELISA protocol. The concentrations of PA were calculated from a 4 parameter

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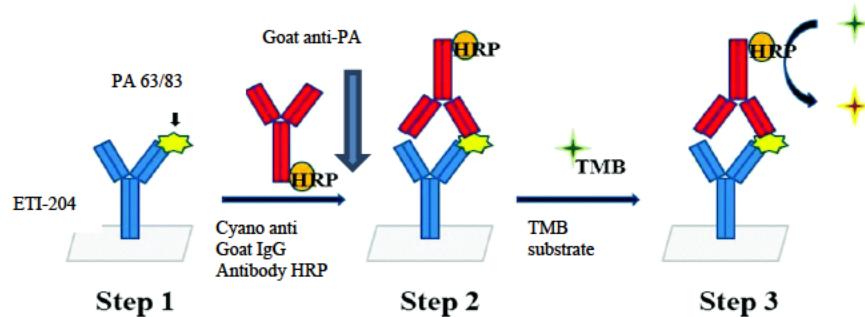
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logistic. Figure 2 shows the layout of samples, standards and QC, high = HQC, medium= MQC and low = LQC, on the micro-titer plate.

Figure 1: Flowchart of ELISA Protocol³



HRP = Horseradish peroxidase.
TMB= 3,3'5, 5'tetramethylbenzidine. Cyano = cynomolgus

Figure 2: Placement of Standards, Quality Control and test samples on a microtiter plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	CS11	CS12
B	TMB	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	CS11	CS12	4.88
C	HOC 28.1*	MQC 124*	LQC 28.1*	Blank <CS11	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8
D	HOC 28.1*	MQC 124*	LQC 28.1*	Blank <CS11	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8
E	TS9	TS10	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20
F	TS9	TS10	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20
G	TS21	TS22	TS23	TS24	TS25	TS26	TS27	TS28	HOC 24.3*	MQC 124*	LQC 28.1*	Blank <CS11
H	TS21	TS22	TS23	TS24	TS25	TS26	TS27	TS28	HOC 24.3*	MQC 124*	LQC 28.1*	Blank <CS11

* = These values represent target QC concentrations. Other QC concentration may be used as long as they are certified. The LQC must be <3X the LLOQ, and the HOC must be within 80% of the ULOQ.

Source: (b) (4) SOP

The applicant defined the acceptability criteria for each plate in terms of both the percent coefficient of variation (%CV) and percent relative error (% RE). The applicant's acceptance criteria are summarized below:

4.2.2.1. Acceptance Criteria

(1) Standard Curve:

- Calibration Standards (CS) within the validated range had replicates $\pm 20.0\%$ CV ($\pm 25.0\%$ CV at the LLOQ and ULOQ).
- Calibration Standards (CS) within the validated range were within $\pm 20.0\%$ RE of the nominal value ($\pm 25.0\%$ RE at the LLOQ and ULOQ).

(2) Quality Controls:

- QC replicates were $\pm 20.0\%$ CV.

³ Modified from U.Florida

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2. QC samples were within $\pm 20.0\%$ RE of the nominal value.
3. Each of six QCs (two at each level: HQC, MQC, and LQC) were evaluated for precision and accuracy. Four of six QCs demonstrated both a %CV of $\pm 20.0\%$ and a %RE of $\pm 20.0\%$. Also, there must be at least one passing QC at each level (HQC, MQC, and LQC).

(3) Sample:

1. The reportable value (RV) of the unknown test sample was taken from the average of the concentrations (from a single sample tested in replicate wells on one plate) interpolated from the passing reference standard curve. The average result was between (or be equal to) the LLOQ and ULOQ of the assay, and demonstrated an intra-dilution %CV of $\pm 20.0\%$.
2. If the sample demonstrated >20.0% CV, it was retested.
3. If the sample demonstrated a putative result <LLOQ (9.68ng/mL), then must not be retested, but reported as "<LLOQ."
4. If the putative result was >ULOQ (309 ng/mL), then it was diluted with pooled, naive cynomolgus monkey serum at a dilution appropriate for quantitation within the range of the assay, and re-tested. In cases where adjusted results whose unadjusted values fell between 9.68- 309 ng/mL, the value of the medium of the adjusted results should be reported.

4.2.2.2. Sensitivity

This included determination of the LLOQ and the ULOQ by the use of the standard curve (calibration curve). The spikes for the standard curve were prepared on each day of use from certified, frozen monkey serum. The frozen serum was thawed and diluted in a mixture of ELISA Wash buffer (Phosphate buffered saline and Tween 20) and Recovery buffer (Wash Buffer +3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate- CHAPS + EDTA) as shown in Table 1.

Table 1: Example of the preparation of stock serum for the preparation of the standard curve.

TUBE MATERIAL	TUBE LABEL	SOURCE LABEL	Source Conc. (ng/mL)	Trans. Vol. of Source (μ L)	Trans. Vol. of Diluent (μ L)	Initial Final Volume (μ L)	Remain. Volume (μ L)	Target Conc. (ng/mL)	Final Conc. (ng/mL)	%Difference from Target
Intermediate Stock	IS	Alpha	1000000	20	380	400	380	50000	50000	NA
Working Stock	WS	IS	50000	20	180	200	125	5000	5000	NA
Calibration Std 1	CS1	WS	5000	50	150	200	120	1250	1250	0.000
Calibration Std 2	CS2	WS	5000	25	175	200	120	625	625	0.000
Calibration Std 3 (ULOQ)	CS3	CS1	1250	50	150	200	150	313	313	0.000
Calibration Std 4	CS4	CS1	1250	30	130	160	110	234	234	0.160
Calibration Std 5	CS5	CS2	625	50	150	200	150	156	156	0.000
Calibration Std 6	CS6	CS2	625	30	130	160	160	117	117	0.160
Calibration Std 7	CS7	CS3	313	50	150	200	150	78.1	78.1	0.000
Calibration Std 8	CS8	CS4	234	50	150	200	200	58.5	58.6	0.160
Calibration Std 9	CS9	CS5	156	50	150	200	150	39.1	39.1	0.000
Calibration Std 10	CS10	CS7	78.1	50	150	200	150	19.5	19.5	0.000
Calibration Std 11 (LLOQ)	CS11	CS9	39.1	50	150	200	200	9.77	9.77	0.000
Calibration Std 12	CS12	CS10	19.5	50	150	200	200	4.88	4.88	0.000

*NOTE: The Intermediate Stock preparation in this example assumes that the PA63 source is Alpha Diagnostics and that the PA63 stock material is exactly 1.0 mg/mL. However, it is acceptable to utilize PA63 from an equivalent vendor, and PA63 of a different source concentration (i.e., which may get assigned during the bridging process) as long the final concentration of the Intermediate Stock (IS) is 50,000 ng/mL.

Source: (b) (4) validation report

The calibration curve was determined using concentrations of PA from 1,250 ng/mL in two-fold dilutions to 4.88 ng/mL. The applicant submitted 12 points that represented the concentrations

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of the standards from which the curve was drawn. The data for the calibration curve are shown below in Table 2. Figure 3 shows the standard curve.

Table 2: Data from twelve Standards that established the Calibration Curve for measuring Free Protective antigen in the serum of Monkeys

Free PA ELISA Calibration Curve Summary (PA63 Calibration Curve Concentrations and %RE)												
Level Concentration ¹ (ng/mL)	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	CS11	CS12
Run 1	1250	622	308	238	159	117	79.2	57.6	36.2	17.1	8.38	9.40
Run 2 (QC Fail)	1250	624	313	234	156	117	78.9	57.7	39.1	18.6	10.1	5.33
Run 3	1250	624	309	237	156	119	78.9	56.8	36.7	19.0	10.1	6.15
Run 4	1250	622	312	234	160	118	77.5	56.9	37.1	18.9	9.92	6.40
Run 5 (CS Edited)	Masked	Masked	315	230	161	114	79.0	59.3	38.3	19.3	9.68	5.18
Run 6	1240	636	315	224	160	117	78.7	59.4	39.0	19.2	9.35	4.33
Run 7	1250	631	311	230	158	118	79.7	59.3	37.9	18.9	9.57	5.25
Run 8	1250	635	301	237	158	122	73.6	59.1	37.0	18.3	10.2	5.55
Run 9	1250	624	312	236	154	119	77.9	58.2	39.1	19.3	9.70	5.26
Run 10	1230	650	305	226	158	118	81.2	59.1	40.1	18.9	9.25	3.97
Run 11	1250	624	309	238	157	118	79.1	56.6	37.6	19.5	10.1	5.45
Run 12	1270	597	330	239	150	115	80.5	56.8	37.3	19.3	9.68	6.92
Run 13	1260	605	323	242	156	114	80.6	55.9	35.3	18.7	10.1	6.90
Run 14	1260	613	323	235	151	117	80.9	57.5	37.5	19.4	9.69	5.80
Run 15	1260	614	319	235	157	115	80.7	57.9	35.9	19.5	9.62	6.13
Run 16	1240	634	307	239	151	116	81.3	60.2	39.2	20.4	8.79	3.80
Run 17 (QC Fail)	1250	622	317	236	152	113	80.0	61.4	39.2	19.8	9.10	4.30
Run 18 (QC Fail)	1260	614	321	233	155	113	79.0	60.7	37.8	19.2	9.48	5.65
Run 19 (QC Fail)	1250	624	312	237	154	115	80.6	60.0	36.6	19.2	9.47	5.76
Run 20	1250	633	310	230	156	119	79.9	57.5	39.6	19.1	9.46	4.64
Run 21	1250	627	313	230	159	118	79.0	57.7	38.7	19.0	9.49	5.56
Run 22	1250	626	315	228	161	114	79.9	58.5	39.0	19.4	9.35	5.07
Average (ng/mL)	1250	624	314	234	156	117	79.5	58.4	38.0	19.1	9.57	5.58
Standard Deviation (ng/mL)	8.3	11.5	6.56	4.57	3.11	2.35	1.34	1.44	1.33	0.619	0.447	1.20
%CV	0.7	1.8	2.1	1.9	2.0	2.0	1.7	2.5	3.5	3.2	4.7	21.4
%RE	0.0	-0.2	0.2	0.1	0.2	-0.3	1.7	-0.4	-2.8	-2.1	-2.0	14.4

¹ = PA concentration values, mean, and standard deviations are presented to three significant figures. The calculated %CV and %RE are presented to one decimal place. With the exception of PA concentration values over 1000 which were hand-typed in, the values were copy/pasted from the Softmax Pro file. The formatting in Excel has hidden decimals so no rounding errors have occurred. However, the reported PA concentrations in this spreadsheet may not match the Softmax Pro file exactly.

Appendix U This submission

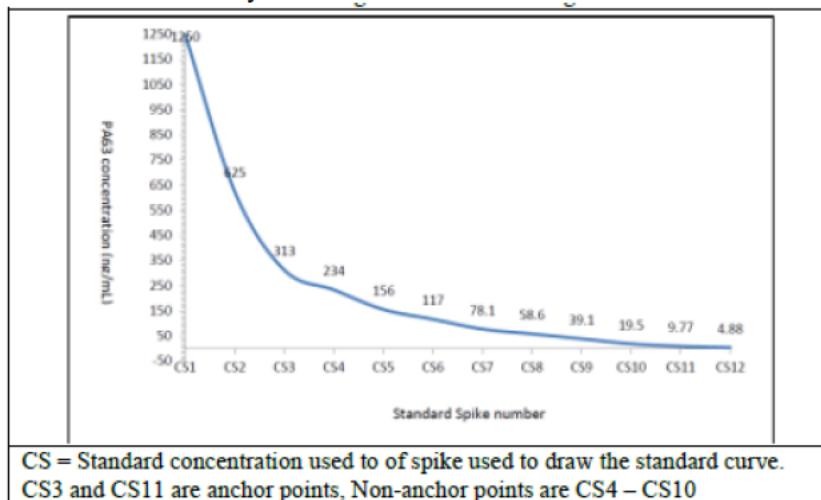
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Figure 3: Calibration Curve for measuring Free Protective antigen in the serum of Monkeys



4.2.2.3. Quality Control

The positive QC samples high, medium and low were prepared on the day of use from the same type of frozen monkey sample as the calibration standards. However, the stipulation was that the sample for the QC must not be taken from the same monkey sample used to draw the standard curve, if the QC and CS were run on the same plate. The samples were run in duplicate with two matrix blanks; each sample was diluted separately at 1:10 dilution. Each dilution of the QC was reconstituted in sufficient volume that it could be shared between all of the plates in the batch. The QC material was rPA63; the concentration of the lot used in this assay was 0.525 mg/mL. The applicant stated that it would be acceptable to use source material from other manufacturers, but that the concentration of each different lot of source material must be adjusted to 50,000 ng/mL. The preparation of the control samples is shown in Table 3. The QC sample was obtained from a different specimen than those used to draw the standard curve.

Table 3: Example of the preparation of the frozen Quality Control sample.

TUBE MATERIAL	TUBE MATERIAL	SOURCE LABEL	SOURCE CONC. (ng/mL)	Trans. Vol. of Source (μ L)	Trans. Vol. of Diluent (μ L)	FINAL VOLUME (μ L)	REMAINING VOLUME (μ L)	TARGET CONC. (ng/mL)	FINAL CONC. (ng/mL)	APPROX. #ALIQUOTS (200 μ L)**	VOL REMAINING (μ L)
Intermediate Stock*	IS-50K	List	525,000	100	950	1,050	170	50,000	50,000	NA	NA
Working Stock	WS-500	IS-50K	50,000	880	87,120	88,000	3,445	500	500	NA	NA
Upper Limit of Quantitation	ULOQ	WS-500	500	33,990	21,010	55,000	55,000	309	309	245	6000
High Quality Control	HQC	WS-500	500	32,500	32,500	65,000	65,000	250	250	295	6000
Mid Quality Control	MQC	WS-500	500	13,750	41,250	55,000	55,000	125	125	245	6000
Low Quality Control	LQC	WS-500	500	3,250	61,750	65,000	65,000	25	25	295	6000
Lower Limit of Quantitation	ULOQ	WS-500	500	1,065	53,935	55,000	55,000	9.68	9.68	245	6000
Matrix Blank**	-	-	-	-	55,000	55,000	55,000	<LOQ	<LOQ	245	6000

*List: List Biologicals, Inc. is a manufacturer of the rPA63 stock material. Lot No. 1748B1-040212 is assigned a source concentration of 0.525 mg/mL but other manufacturers, lots, etc. may be used as long as the concentrations are adjusted to create an IS-50K of 50,000 ng/mL.

**The actual number of aliquots may vary.

Source: This report

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4.2.2.4. Precision

The percent coefficient of variation (%CV) that measured precision was calculated (based on the formula: Standard deviation/mean X100), varied from 0.7% at the higher concentration to as much as 21.4% at the lowest level. The pattern in %CV showed that the high concentrations resulted in a low %CV and the low concentration a high %CV, this however, is a common outcome. The applicant set an acceptable ULOQ %CV limit of ≤ 25 at CS3 (Calibration Standard 3), the LLOQ %CV also ≤ 25 at CS11. For the non-anchor points CS4 to CS10 the %CV ranged from 3.5 to 1.7 ng/mL. The applicant defined acceptable limit was CV $\leq 20\%$. The results of the test in the non-anchor range of the curve CS4 (234 ng/mL) to CS10 (19.5 ng/mL) ranged from 3.5 to 1.7 ng/mL %CV, which is precise. The LLOQ was described by the applicant as the lowest amount of PA that could accurately be measured, and the ULOQ was the maximum amount of PA that could be accurately quantitated by this procedure.

Twenty-two runs were used to provide data for this curve, and each sample was run in duplicate. The %CV for CS12 was unacceptably high (21.4%); this eliminated CS12 from being used as the LLOQ. The applicant set the lower limit of quantitation to be CS11 at 9.77 ng/mL, and the upper limit to be CS3 at 313 ng/mL. This represented a range of 303 ng/mL between the lower and upper limits of quantitation. Figure 2 shows that CS1, CS2 fell outside of the slope of the other standards, and that CS3 fell on a section of the graph that was slightly curved. The applicant therefore could not validate the concentrations at this level. Therefore, the reportable range for this test was set from 9.68 to 309 ng/mL.

Comments:

- *A %CV of 20% suggests a wide range of variability of the results. It would have been preferable to have a %CV less than 10%.*
- *It appears from Figure 2 that the slope at the high concentration between CS3 and CS4 has shifted slightly upward. The ULOQ should be much closer to CS4. This is demonstrated in the instability of the higher concentrations of QC values, as shown in the section on quality control.*

4.2.2.5. Precision and accuracy of results of the quality control samples

The applicant stated that for testing of samples from animal efficacy study, each level of QC must be run in duplicate. The applicant further stated that at least 4/6 QC samples must be acceptable in both precision and accuracy (%CV and %RE), $\leq 20.0\%$ for CV and have a %RE ranging from (-20.0) to (+20.0). Additionally, there must be at least one passing value at each of the three levels. To be acceptable, the matrix blank must have an optical density that was less than that of the LLOQ.

The three quality control samples were high (HQC) - BM1902, medium (MQC) - BM1903 and low (LQC) - BM1907. The matrix blank was #1906. The working concentrations of the samples were 250, 125 and 25 ng/mL, respectively. Twenty-two runs of controls were tested in duplicate. The mean values obtained for the QC results were 284 ng/mL for the 250 ng/mL sample, 139 ng/mL for the 125 ng/mL sample and 25.6 ng/mL for the 25 ng/mL sample. All, except one of the high QC values, resulted in higher values than the nominal value of the respective control.

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The calculated standard deviation was 21.4. This indicated a wide difference in the test results. As stated above, only one of the results was lower than the expected value, and only 1/44 of the HQC values was equal to that of the nominal HQC. In 3/22 runs, the HQC results failed. For the LQC, 15/44 readings fell below the expected value of 25 ng/mL. The cumulative %CV for the high, medium and low levels of QC were 7.6, 6.9, and 7.5 respectively, which was acceptable. The %REs were 14.1, 11.9 and (-9.0), respectively. According to the applicant's criteria these values were acceptable. Tables 4 and 5 show the QC results.

Table 4: Test results of rPA63 concentration values for High, Medium and Low Quality Control samples in 22 runs.

Level Concentration ¹ (ng/mL)	BM1902		BM1903		BM1907		BM1906 ²	
	HQC-1 248	HQC-2 248	MQC-1 124	MQC-2 124	LQC-1 28.1	LQC-2 28.1	MB-1 OD	MB-2 OD
Run 1	<i>308</i>	293	<i>149</i>	141	23.8	25.4	0.126	0.151
Run 2 (QC Fail)	<i>305</i>	<i>301</i>	<i>153</i>	145	27.2	28.4	0.101	0.119
Run 3	256	288	148	136	26.4	25.6	0.080	0.079
Run 4	<i>304</i>	284	148	137	26.4	24.5	0.089	0.086
Run 5 (Curve Edited)	254	297	145	143	25.6	25.4	0.049	0.044
Run 6	286	272	142	138	26.1	26.4	0.046	0.045
Run 7	<i>301</i>	282	<i>141</i>	143	25.3	27.2	0.033	0.041
Run 8	277	280	141	138	24.6	24.9	0.032	0.030
Run 9	292	244	145	122	26.4	<i>21.6</i>	0.037	0.033
Run 10	289	250	143	125	24.7	22.9	0.051	0.037
Run 11	278	273	140	138	25.9	26.3	0.036	0.034
Run 12	255	261	121	123	23.7	23.9	0.035	0.030
Run 13	297	268	135	125	23.2	<i>23.1</i>	0.029	0.028
Run 14	266	268	127	132	24.3	<i>23.1</i>	0.034	0.041
Run 15	268	284	121	128	23.4	23.5	0.038	0.032
Run 16	253	266	125	129	25.7	25.8	0.039	0.035
Run 17 (QC Fail)	<i>310</i>	<i>307</i>	148	<i>149</i>	26.8	30.6	0.048	0.050
Run 18 (QC Fail)	<i>304</i>	<i>309</i>	145	145	27.8	27.4	0.047	0.046
Run 19 (QC Fail)	<i>343</i>	<i>307</i>	<i>149</i>	<i>149</i>	26.9	26.9	0.035	0.036
Run 20	289	274	<i>152</i>	137	27.9	25.5	0.023	0.022
Run 21	<i>301</i>	285	<i>150</i>	141	29.1	26.0	0.033	0.031
Run 22	<i>301</i>	245	148	124	28.0	<i>22.0</i>	0.036	0.028

¹ = PA concentration values, mean, and standard deviations are presented to three significant figures. The calculated %CV and %RE are presented to one decimal place. With the exception of PA concentration values over 1000 which were hand-typed in, the values were copy/pasted from the Softmax Pro file. The formatting in Excel has hidden decimals so no rounding errors have occurred. However, the reported PA concentrations in this spreadsheet may not match the Softmax Pro files exactly.

² = Matrix Blank (MB) are presented as raw optical density (OD) values rather than a concentration (ng/mL). ³ = Italicized values denote failure due to >20% I

Source: This report

Table 5: Summary of QC results

Level Concentration(ng/mL)	HQC (BM1902)	MQC (BM1903)	LQC (BM1907)	Negative (BM1906)
Nominal Concentration	248 ng/mL	124ng/mL	<i>28.1</i> ng/mL	OD
Cumulative Mean(ng/mL)	284	139	25.6	0.049
Cumulative Standard deviation ng/mL	21.4	9.63	1.91	0.029
Cumulative %CV	7.6	6.9	<i>7.5</i>	N/A
Cumulative %RE	14.3	11.9	-9.0	N/A

Comment

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The precision of the standard curve is demonstrated in the low %CV obtained for the controls. Only one of the %CV values, SC12, fell above 20 and 11/12 %CV were less than 5%. This is a high level of precision (Zady et al, 2014⁴). The precision of the QC testing is acceptable according to the applicant's criteria but the HQC was high; this might have been affected by the high value of the ULOQ in the calibration curve. The reagent blank served as the negative

⁴ Zady, Madelon F., Z-Stats / Basic Statistics www.westgard.com › Lessons › Z-Stats / Basic Statistics 2014.

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control. This is unacceptable. The negative control should comprise an analyte which gave a negative result.

Comment:

The accuracy of the QC results overall, are acceptable according to the applicants criteria but HQC values may, at times, not fall within acceptable limits. The applicant used a reagent blank for the negative control this is not appropriate – the sample used in this assay is a reagent blank. Another analyte should have been used as the negative control. The values obtained for the standard curve spikes showed a high degree of accuracy.

4.2.2.6. Background noise

The background noise was analyzed by the use of matrix blanks which must have an optical density (OD) less than that of the LLOQ (CS level 11). The matrix blank also served as the negative control. This was not appropriate.

4.2.2.7. Accuracy

The relative error that measured accuracy was calculated based on the formula:
(Mean-Nominal/Nominal X100)

The applicant stated that the acceptable value for both the ULOQ (CS3) and the LLOQ (CS11) may range between (-25%) to 25% and the CS3 through the CS11 - the non-anchor points, must have a %RE value between (-20%) to 20%. The Standard error was very low at all the concentrations (< 2%) except in CS9 and 12.

The accuracy (i.e., the closeness of the test values to the nominal value) of the data presented in the standard curve was appropriate; at higher concentrations of the standard from CS1 to 6 the value was less than 0.2. Five of six of the lowest standards had %RE values less than 5; CS12 had a relative error value of 14.4 and was not included in the standard curve. The accuracy parameter fell within the applicant's acceptable range.

4.2.2.8. Specificity

No requirements were stipulated to evaluate the specificity of the assay for detecting free PA63 other than the sera must come from healthy animals. The matrix described as a negative control was cynomolgus monkey serum from healthy animals. Toxins secreted by other *Bacillus* species which are implicated in infections of both humans and animals were not tested. *Bacillus cereus* is the only other confirmed major pathogen in this genus but *Bacillus licheniformis* and other species have been implicated in infections of man and animals; specimens to detect specificity for this assay could also have been taken from these bacteria. The effect of the compounds that were tested follow:

- ETI 204 – complete interference (94 – 100% inhibition). This is good because ETI-204 binds to PA.
- rEF- partial interference (18 - 43 % inhibition)
- rLF – partial interference (12 -38% inhibition)
- rPA83 - additive effect (48 – 266 % addition)
- Filtration – partial interference

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4.2.2.9. Linearity

The minimum assay dilution was 1:10. The applicant stated that the linearity was tested at two concentrations 40,000 ng/mL and 200,000 ng/mL. The data were not available for review. However, the validation report states that sample dilutions for both concentrations passed the acceptability criteria.

4.2.2.10. Stability

Prevention of the degradation of the target molecule is important. PA63 was dissolved in cynomolgus monkey serum and was found to be stable after freezing at -70°C for 182 days. The applicant stated that long term stability of this analyte is still ongoing. The integrity of PA63 could be affected by the number of freeze /thaw (F/T) cycles it undergoes. The tests recommended that the maximum number of F/T cycles a sample of PA63 could undergo without losing its potency, is five. PA63 rapidly loses its activity at room temperature and should not be held at RT longer than 15 minutes. Loss of activity could also occur at refrigerator temperature therefore the sample should be kept on ice while testing. HQC could not maintain its activity when held at room or refrigeration temperature for a long time. The unused portion of a sample used for testing, should be frozen again at -70°C, as soon as possible after use. The best way to preserve the integrity of the sample is to keep it frozen except when aliquots are being separated for immediate use.

Comments:

The applicant stated that the maximum number of F/T cycles a sample could withstand and still maintain its activity is 5 and that samples should not be kept at room temperature for longer than 15 minutes. However, data were not available for review.

4.2.2.11. Result reporting

Test readouts were converted using Softpro Max. The results that were below the LLOD were reported as less than the LLOQ – 9.68 ng/mL; those that exceeded the ULOD = 309 ng/mL, were appropriately diluted with pooled naïve cynomolgus monkey serum, retested, and recalculated. Table 6 is a summary of the results of evaluation of the test parameters of the assay for monkeys.

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Table 6: Summary of Assay Validation studies for AP203 for Free PA in Monkeys

Parameter	Validation Criteria	Test results of Parameter	Validation Decision	Comment
Standard Curve Precision	ULOD %CV ≤ 25 at CS3. LLOD %CV ≤ 25 at CS11. Non-anchor %CV ≤ 25 CS4-10 LOD	2.1% 4.7% 1.9 -3.5% 9.68	Acceptable	Sample results at the high end of the scale should possibly be diluted
Standard Curve	ULOQ (CS3)and the LLOQ (CS11) -25% to 25% No anchor points -20% to 20%	ULOQ = 309 ng/mL LLOQ = 9.68 ng/mL	Acceptable	Ideally the ULOQ should be lower than 309 ng/mL, but this stated value meets the applicant's criteria
Specificity for detection of PA63	Ability to test positive and negative control values and interferences correctly	For the positive HQC, MQC, LQC and negative Matrix samples ETI-204 - complete inhibition rLF, rEF – partial inhibition	Acceptable	Applicant should have used different test samples to determine specificity such as, antibody to another <i>Bacillus</i> species Negative control should be an actual analyte not just the absence of PA in the matrix
Stability PA63	Maximum 182 days frozen at $\leq -70^{\circ}\text{C}$	NA	NA	Complete results not available
QC Precision	$\leq 20.0\%$ for CV.	%CV: H=7.6 M=6.9 L=7.5	Acceptable	Negative control not appropriate
QC Accuracy	%RE ranging from -20.0 to 20.0	%RE: H=14.3 M=6.9 L=-9.0	Acceptable	Although the accuracy of results for the high control is acceptable it is much less accurate than the results in the medium and low controls
Linearity of Standard curve	Not stated for the applicant	Sample diluted from 40,000 & 200,000 ng/mL On standard curve passed	N/A	

CS = concentration standard. ULOQ = upper limit of concentration. LLOQ= Lower limit of concentration

Source: This report

Comments:

The results of testing to support the accuracy, precision, specificity of testing for free PA by ELISA in cynomolgus monkeys are adequate and meet the criteria set forth for acceptability by the applicant. However, some of the limitations are outlined below:

- The stability testing for test samples under various conditions has not yet been completed. Based on the information reviewed, the assay supports stability of the PA63 and PA83 until 182 days. Test samples should not be kept at room temperature for longer than 15 minutes.
- The ULOQ should have been set lower to facilitate a more linear standard curve. The QC values at the upper end of the scale are high, but they have met the applicant's criteria for acceptability.

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- Twenty-seven percent of the high level QC values were above 300 ng/mL whereas the nominal value is 248 ng/mL. Additionally, 37% of the QC values are above 2 standard deviations and only 1/44 of the HQC values are below the nominal value. This indicates a problem at the high end of the curve. This problem might be related to the instability of the ULOQ cut-off.

More specific samples should have been used to test for the specificity of this assay for detecting PA than matrix for negativity, and PA quality controls. Hemolysis is very important in a sample especially when it is drawn from a small animal.

The level of testing, as indicated by the %CV and %RE, was appropriate. Overall, the assay supported the LOD of 9.68 ng/mL and ULOD of 309 ng/mL.

4.2.3. ELISA to Quantify Free Protective Antigen in Monkey Serum at

(b) (4)

The assay was designed to detect the presence of circulating (free) PA in the serum of monkeys infected with *Bacillus anthracis* (SOP (b) (4) V-061; Appendix K for Study AP202; Study number 2826-100020847).

The solid phase sandwich ELISA utilized an anti-PA monoclonal IgG antibody coated 96 well micro-titer plate to which controls, standards and test samples were added. The mixture was incubated to allow the antigen and antibody to react, then washed to remove any unbound antibody and serum proteins. The bound antibodies were detected by the addition of horseradish peroxidase (HRP)-conjugated mouse anti-PA antibody. Color was produced by the two component 3,3', 5,5' – tetramethylbenzidine (TMB) reagent comprising TMB peroxidase substrate and peroxidase solution B. In the presence of hydrogen peroxide the bound HRP oxidized the chromogen TMB resulting in a blue solution. The reaction was terminated by the addition of sulfuric acid. The acidic environment caused by the addition of sulfuric acid changed the color of the reaction solution to yellow. The color formed was measured spectrophotometrically.

The monkey quality control samples were prepared by dissolution of known amount of purified stock PA63 in specific monkey serum. Three levels of QCs used were high, medium and low. The reference standards (PA) were also diluted in species specific serum.

The validation tests were carried out by three operators on three days using 12 different plate layouts. The validation schedule is shown in Table 1.

Table 1: Validation Test Schedule.

	Day 1	Day 2	Day 3
Test Operator 1	I	I	I
	I	IV	V
	II	VI	X
	III	XI	XII
Test Operator 2	I	I	I
	II	I	IV
	III	V	VI
	VII	VIII	IX
Test Operator 3	I	I	I
	II	VII	I
	III	VIII	IX
	X	XI	XII

(b) (4) validation report

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4.2.3.1. Acceptance Criteria

Calibration Standards:

1. The value of the standards must be within $\pm 20.0\%$ of the nominal concentration except at the LLOQ and ULOQ, which must be within $\pm 25.0\%$.
2. The precision between the calibration standard replicates must be $\leq 20.0\%$ except at the LLOQ and ULOQ, which must be $\leq 25.0\%$.
3. Unless the precision of the anchor points was $\leq 20.0\%$, they must be recalculated. Anchor points were the points at the highest and lowest standard / reference concentrations. A minimum of 6 standard values must meet these criteria.
4. Data points were not to be calculated from only one well. At least 6 points must be used in the calculation of the standard curve.

Quality Control samples:

1. Each QC sample must be run in triplicate and at least two-thirds of the QC sample should be within $\pm 20.0\%$ of the nominal concentration for each analyte.
2. The measured concentrations of at least one replicate at each QC concentration must be within $\pm 20.0\%$ of the nominal concentration for each analyte.
3. A minimum of 2/3 of the replicates of each QC sample must be $\leq 20.0\%$ of the nominal concentration.

Study Samples

Test results of duplicates of study samples should be $\leq 20.0\%$ of each other and should fall within the range of the assay. Figure 1 shows the layout of samples in the plates.

Figure 1: Plate Map Containing Diluted RS (e.g., 4000 ng/mL and 1:4 Dilution Series),
Test Samples, QC's, and NC

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS-1 4000 ng/mL	RS-1 4000 ng/mL	TS-1 neat	TS-2 neat	TS-3 neat	TS-4 neat	TS-5 neat	TS-6 neat	TS-7 neat	TS-8 neat	QC-mid Neat	QC-low Neat
B	RS-2 1000 ng/mL	RS-2 1000 ng/mL	TS-1	TS-2	TS-3	TS-4	TS-5	TS-6	TS-7	TS-8	QC-mid	QC-low
C	RS-3 250 ng/mL	RS-3 250 ng/mL	TS-1	TS-2	TS-3	TS-4	TS-5	TS-6	TS-7	TS-8	QC-mid	QC-low
D	RS-4 62.5 ng/mL	RS-4 62.5 ng/mL	TS-1	TS-2	TS-3	TS-4	TS-5	TS-6	TS-7	TS-8	QC-mid	QC-low
E	RS-5 15.63 ng/mL	RS-5 15.63 ng/mL	TS-9	TS-10	TS-11	TS-12	TS-13	TS-14	TS-15	TS-16	NC	QC-high
F	RS-6 3.91 ng/mL	RS-6 3.91 ng/mL	TS-9	TS-10	TS-11	TS-12	TS-13	TS-14	TS-15	TS-16	NC	QC-high
G	RS-7 0.98 ng/mL	RS-7 0.98 ng/mL	TS-9	TS-10	TS-11	TS-12	TS-13	TS-14	TS-15	TS-16	NC	QC-high
H	RS-8 0.24 ng/mL	RS-8 0.24 ng/mL	TS-9	TS-10	TS-11	TS-12	TS-13	TS-14	TS-15	TS-16	NC	QC-high

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4.2.3.2. Sensitivity

Ten concentrations were used to compute the standard curve. The concentrations ranged from 160 ng/mL to 2.50 ng/mL using two-fold dilutions. The data for the standard curve were obtained from 11 runs. The LLOQ and the ULOQ were determined from this standard curve (calibration curve). The spikes for the standard curve were prepared on each day of use from certified, frozen monkey serum. The frozen serum was thawed and diluted in a mixture of ELISA Wash buffer (Phosphate buffered saline and Tween 20) and Recovery buffer (Wash Buffer +3-[*(3-Cholamidopropyl) dimethylammonio*]-1-propanesulfonate- CHAPS + EDTA). Table 1 shows the results from which the standard curve was drawn. The %CV and %RE were very low. The %CV ranged from 2.3 to 4.5 and the % RE from 1.9 to 3.2 signifying that the data were accurate and reproducible. The data were accurate enough so that there was no necessity to exclude any of the points in order to adjust the curve. Standards (STD) 1 and 10 were anchor points and could not be included in the standard curve. The ULOQ was determined to be 80 ng/mL, the value of spike # 9, and the LLOQ was determined to be 5 ng/mL which is based on the value of spike 2. Table 2 shows the results of the testing of 10 spikes for the calibration curve. Figure 2 shows the calibration curve for this assay.

Table 2: Precision and Accuracy of PA63 Antigen in Calibration Standards in Cynomolgus Monkey Serum for Study AP202

Run No.	Mean Replicate Data (ng/mL)									
	STD1*	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10*
1	2.48	4.98	9.98	16.1	19.4	39.3	57.8	70.7	86.1	149
2	2.48	4.96	10.1	15.6	19.7	38.7	65.2	64.1	84.4	158
3	2.48	4.88	10.9	15.2	19.0	38.9	64.8	68.8	77.5	173
4	2.54	4.76	10.7	15.0	19.6	40.3	58.0	72.0	80.7	158
5	2.49	4.93	10.3	14.9	19.6	40.4	61.6	68.1	79.9	162
6	2.52	4.83	10.1	15.9	19.6	38.5	62.3	71.2	77.3	164
7	2.58	4.76	10.0	15.5	20.9	37.7	61.4	66.4	87.2	156
8	2.46	4.97	10.3	14.9	19.6	41.7	58.8	65.9	86.0	157
9	2.49	NA ^b	10.0	15.1	19.6	41.4	57.7	69.0	83.1	158
10	2.46	5.11	10.2	14.6	19.9	40.8	58.8	70.4	80.4	160
11	2.52	4.96	9.94	14.8	21.3	37.4	61.4	68.6	85.4	155
Inter-Run Mean	2.50	4.91	10.2	15.2	19.8	39.5	60.7	68.7	82.5	159
Inter-Run SD	0.0371	0.110	0.302	0.462	0.664	1.45	2.71	2.43	3.55	6.05
Inter-Run CV (%)	1.5	2.2	2.9	3.0	3.3	3.7	4.5	3.5	4.3	3.8
Inter-Run RE (%)	0.0	-1.7	2.3	1.5	-0.8	-1.1	1.2	-1.9	3.2	-0.6
Inter-Run n	11	10	11	11	11	11	11	11	11	11

*Anchor points.

^bNA. Standard was masked due to individual CV > 20.0%. The LLOQ was raised to 10 ng/mL in Run 9.

CV (%): Coefficient of Variati STD -Standard

RE (%): Relative Error.

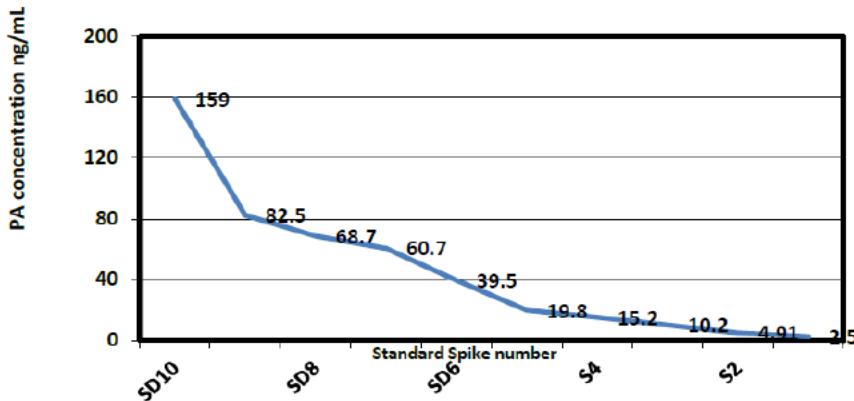
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Figure 2: Calibration Curve for measuring Circulating Protective Antigen in the serum of monkeys



PA concentrations in the following spikes: SD2 = 4.91 ng/mL, SD4 = 15.2 ng/mL, SD6 + 39.5 ng/mL, SD8 = 68.7 ng/mL, SD10 + 159 ng/mL ng/mL,

4.2.3.3. Quality Control

There were three positive QC samples high (HQC), medium (MQC) and low (LQC) with nominal values of 65.0 ng/mL, 40.0 ng/mL and 15 ng/mL, respectively. Three QC concentrations were prepared from 0.5 mg/mL of PA 63 stock (3 concentrations of 50,000 ng/mL, 5,000 ng/mL and 1,000 ng/mL). Table 3 shows the precision and accuracy of each level after 11 runs. The samples were run in duplicate resulting in 22 values. The intra-run precision was not calculated because there were only two samples. The inter-run precision values were 7.6, 8.2, and 8.6 % for the high, medium and low QC respectively, supporting reasonably high precision of the assay. The % RE ranged from 4.8 to 9.4 across the three levels. One value fell outside of the applicant's acceptable criterion, that was an intra-run LQC %RE of 22%.

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Table 3: Precision and Accuracy of PA63 Antigen in Quality Control Samples in Cynomolgus Monkey Serum for Study AP202

Run No.	Mean Replicate Data		
	HQC (65.0 ng/mL)	MQC (40.0 ng/mL)	LQC (15.0 ng/mL)
1	68.8 70.5	40.4 40.8	15.5 15.9
Intra-Run Mean	69.7	40.6	15.7
Intra-Run CV (%)	NA ^a	NA	NA
Intra-Run RE (%)	7.2	1.6	4.7
2	54.2 58.1	35.7 34.0	14.4 13.4
Intra-Run Mean	56.2	34.9	13.9
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	-13.6	-12.8	-7.3
3	66.4 74.0	50.3 ^b 44.6	19.4 ^b 17.4
Intra-Run Mean	70.2	47.5	18.4
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	8.0	18.6	22.5
4	75.0 69.8	45.2 40.9	17.0 15.7
Intra-Run Mean	72.4	43.1	16.3
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	11.4	7.7	8.8
5	65.1 65.9	43.0 38.9	17.6 15.6
Intra-Run Mean	65.5	40.9	16.6
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	0.8	2.3	10.7
6	63.8 74.1	45.3 43.4	17.6 16.8
Intra-Run Mean	68.9	44.3	17.2
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	6.0	10.8	14.7
7	63.4 68.9	42.3 42.5	16.5 17.2
Intra-Run Mean	66.2	42.4	16.8
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	1.8	6.0	12.2

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Table 3: Precision and Accuracy of PA63 Antigen in Quality Control Samples in Cynomolgus Monkey Serum for Study AP202 (continued)

Run No.	Mean Replicate Data		
	HQC (65.0 ng/mL)	MQC (40.0 ng/mL)	LQC (15.0 ng/mL)
8	68.6 69.9	44.2 40.1	16.5 14.8
Intra-Run Mean	69.3	42.1	15.7
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	6.6	5.4	4.6
9	NV ^c 68.7	44.9 42.1	18.2 ^b 14.5
Intra-Run Mean	68.7	43.5	16.3
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	5.6	8.8	8.9
10	70.3 75.0	46.5 44.5	18.3 ^b 16.7
Intra-Run Mean	72.7	45.5	17.5
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	11.8	13.8	16.5
11	68.5 70.8	42.9 41.5	15.8 16.3
Intra-Run Mean	69.6	42.2	16.1
Intra-Run CV (%)	NA	NA	NA
Intra-Run RE (%)	7.1	5.6	7.1
Inter-Run Mean	68.1	42.5	16.4
Inter-Run SD	5.20	3.50	1.42
Inter-Run CV (%)	7.6	8.2	8.6
Inter-Run RE (%)	4.8	6.2	9.4
Inter-Run n	21	22	22

CV (%): Coefficient of Variation.

RE (%): Relative Error.

This study

^aNot Applicable. CV not calculated for n≤ 2.

^bRE > 20.0% for the mean replicate result, included in statistical calculations.

^cNo reportable value. Value > ULOQ (80 ng/mL).

4.2.3.4. Stability

Data were not presented to justify the conditions under which PA maintained its integrity. However, the following criteria were stated by the applicant:

The coated plates will maintain integrity for 14 days. PA in test samples will maintain its potency for 4 hours at room temperature and at 4°C. The number of freeze/thaw cycles in which PA is expected to maintain its integrity is 5 cycles at -80°C, and can be stored at -80°C for 185 days. The applicant also stated that a mixture of protease inhibitors should be added to the sample to maintain the potency of PA if it is to be stored frozen for >3months. When the sample is stored at ≤ -60°C it is not necessary to add protease inhibitors.

Table 4 is a summary of the results of this study.

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Table 4: Summary of ELISA for PA63 in Monkey Serum by

(b) (4)

Parameter	Validation Criteria	Test results of Parameter	Validation Decision	Comment
Standard Curve	Accuracy RE% = -1.9 to 3.2%	Range 5.0 - 80 ng/mL	Passed	None
Precision	Precision% CV = 2.3 to 4.5%	LOQ 5ng/mL		
Standard Curve	Standards Replicates = \leq 20% LLOQ & ULOQ = \leq 25%	ULOQ = 80 ng/mL LLOQ = 5 ng/mL	Passed	None
Study Sample Precision	Replicates = \leq 20% and in quantitative range of standard curve			Not accepted if out of range
Stability PA63		5 F/T cycles at -80°C For 185 days		None
QC Precision	%CV = 7.6 to 8.6	Not calculated		No. runs = \leq 2
QC Accuracy	%RE = 4.8 to 9.4	Not calculated		None

Comments:

This study detects free PA 63 in cynomolgus monkeys. The applicant's acceptable criteria were overall very broad but the actual results showed a great deal of accuracy, in general, most of the % CV were less than 5. The ULOQ was calculated to be 80 ng/mL while the LLOQ was 5 ng/mL

4.2.4. Summary of Assay Validation studies for Free PA in Rabbits

The assay designed to quantitate free PA from *Bacillus anthracis* in the serum of NZW rabbits was performed at (b) (4) (SOP- (b) (4) X-180-04, (b) (4) XVI-006, Elusys Validation Report VP2012-256).

The method used was similar to that summarized above (for details see section 4.2.) using ETI-204 as the antibody to capture PA from the serum of rabbits infected with *Bacillus anthracis*, or spiked with rPA. Goat anti-PA antiserum was used for detection and the reporter is HRP-conjugated anti-gamma chain antibody. The assay is designed to quantify both PA63 and /or PA83. The assay was validated by the testing of three operators using 12 plate layouts. The initial range of concentrations used was 0.10 ng/mL to 100,000 ng/mL.

4.2.4.1. Acceptance Criteria

The sample acceptance criteria relate to the state of the entire microplate in which samples were tested. These incorporated the criteria used to evaluate the calibration standards, the quality control samples, and the reagent blanks. The absorbance of the blanks must be lower than that of

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the Lower Limit of Quantitation (LLOQ). In this assay the value of the LLOQ was represented by the calibration standard (CS) 11.

Calibration Standards

(1) Standard Curve:

1. CS within the validated range should have replicates $\pm 20.0\% \text{ CV}$ ($\pm 25.0\% \text{ CV}$ at the LLOQ and ULOQ). Calibration Standards within the validated range for accuracy were to be within $\pm 20.0\% \text{ RE}$ of the nominal value ($\pm 25.0\% \text{ RE}$ at the LLOQ and ULOQ). The accuracy of the standard curve points must have a %RE range from $\pm 20\%$ between CS4 to CS10. But at the LLOQ and the ULOQ the range could be $\pm 25\%$. The matrix blank of must be lower than the LLOQ.

(2) Quality Controls:

1. QC replicates had to be $\pm 20.0\% \text{ CV}$.
2. QC samples must be within $\pm 20.0\% \text{ RE}$ of the nominal value.
3. Each of six QCs samples (two at each level: HQC, MQC, and LQC) were to be evaluated for precision and accuracy. The HQC and MQC must fall within $\pm 30\%$ of their nominal value. The Low QC must fall within $\pm 50\%$ of its nominal value. The value of the negative control - naïve rabbit serum must have an optical density of ≤ 0.200 . Four of six QCs must demonstrate both a %CV of $\pm 20.0\%$ and a %RE of $\pm 20.0\%$. Also, there must be at least one passing QC at each level (HQC, MQC, and LQC) and at least two of the three QC samples must fall within the acceptable standard ranges.
4. QC was permitted to be censored one dilution at a time in order to have a percent coefficient of variation (%CV) of $\leq 30\%$.

(3) Sample:

1. If the sample demonstrated $>20.0\% \text{ CV}$, it must be retested.
2. If the sample demonstrated a putative result $<\text{LLOQ}$ (9.68 ng/mL), then no retesting was required, but it should be reported as " $<\text{LLOQ}$."

If the putative result was $>\text{ULOQ}$ (309 ng/mL), then it must be diluted with pooled, rabbit naive serum at a dilution appropriate for quantitation within the range of the assay, and re-tested. The results that were to be reported include the median of the adjusted results whose unadjusted results was between 9.68- 309 ng/mL.

Test samples

Stability of Samples

The stated conditions under which the samples must be kept in order to prevent degradation of the PA63 molecule were as follows:

Must be tested within 72 hours of collection and held at 2- 8 °C during this period. After 72 hours the samples must be stored at $\leq -70^{\circ}\text{C}$. For long term storage the samples must be held at $\leq -70^{\circ}\text{C}$ and tested with 3 months. The sample must be freeze/ thawed as little as possible. It is suggested that the number of (F/T) cycles be no more than 5.

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Sample Testing Criteria

If a samples was tested two independent times the %CV must be $\leq 35\%$. If greater than 35% the samples were to be repeated. If the quantity was insufficient and the %CV <35, then the median value of the test results must be reported.

Twelve plate layouts were used in the study; each layout was tested by at least two operators. Figure 1 shows the arrangement of the samples on one micro-titer plate.

Figure 1 Arrangement of Standards, Quality Controls and Test Specimens on a micro-titer preparation plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	CS1 1250	CS2 625	CS3 313	CS4 234	CS5 156	CS6 117	CS7 78.1	CS8 58.6	CS9 39.1	CS10 19.5	CS11 9.77	CS12 4.88
B	CS1 1250	CS2 625	CS3 313	CS4 234	CS5 156	CS6 117	CS7 78.1	CS8 58.6	CS9 39.1	CS10 19.5	CS11 9.77	CS12 4.88
C	HQC 250	MQC 125	LQC 28.1	Blank <CS11	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8
D	HQC 250	MQC 125	LQC 28.1	Blank <CS11	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8
E	TS9	TS10	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20
F	TS9	TS10	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20
G	TS21	TS22	TS23	TS24	TS25	TS26	TS27	TS28	HQC 250	MQC 125	LQC 28.1	Blank <CS11
H	TS21	TS22	TS23	TS24	TS25	TS26	TS27	TS28	HQC 250	MQC 125	LQC 28.1	Blank <CS11

(b) (4) Validation report

Comment:

Overall, the applicant's acceptable criteria are very wide and will not contribute to a high level of accuracy or precision.

4.2.4.2. Sensitivity

Twelve calibration standards were used to draw the standard curve. The standards (PA63) were prepared fresh on the day of use; they were reconstituted into naïve rabbit serum to obtain a final concentrations of 1,250 ng/mL, 625, 313, 234, 156, 117, 78.1, 58.5, 39.1, 19.5, 9.77, 4.88 ng/mL. Table 1 shows the reconstitution of the standards. Each standard curve was constructed by at least 6 points that were above zero. The calibration standards (CS) approximating the limits of quantitation were 313 ng/mL (CS3) and 9.77 ng/mL (CS11).The highest concentration of the assay was validated to be slightly less than the CS3 and so was estimated to be 309 ng/mL (ULOQ). The LLOQ was validated to be 9.68 ng/mL.

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Table 1 : Results of testing twelve standards to establish the standard curve and determination of LLOQ and ULOQ .

Reference Dilution		Volume (μ L) of CS	Recovery Buffer (μ L)*	Wash Buffer Diluent (μ L)**	Total Vol (μ L)	Final PA Conc (ng/mL)
Sample	PA63 (ng/mL)					
CS1	1250	20	40	140	200	250
CS2	625	20	40	140	200	125
CS3	313	20	40	140	200	62.6
CS4	234	20	40	140	200	46.8
CS5	156	20	40	140	200	31.2
CS6	117	20	40	140	200	23.4
CS7	78.1	20	40	140	200	15.6
CS8	58.5	20	40	140	200	11.7
CS9	39.1	20	40	140	200	7.82
CS10	19.5	20	40	140	200	3.90
CS11	9.77	20	40	140	200	1.95
CS12	4.88	20	40	140	200	0.98

*Recovery Buffer should be pre-warmed to 37°C

Source: (b) (4) validation report

4.2.4.3. **Quality Control**

Six QC samples, two at each level, were used in this assay: the High QC (HQC) with a concentration of 250 ng/mL, the medium QC (MQC) 125 ng/mL, and the low QC (LQC) 28.1 ng/mL. Each QC sample was diluted 1:5 fold for three successive times.

Other data

Accuracy (linearity and absolute accuracy): The inter-assay accuracy was 1.4 -13.6 % RE.
 Precision, repeatability and intermediate precision: The inter-assay precision was 7.8 -13.1 %CV.

Table 2 is a summary of the assay.

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Table 2: Summary of assay performance of Free PA in Rabbits

Parameter	Validation Criteria	Test results of Parameter (ng/mL)	Validation Decision	Comment
Limits of Quantitation	ULOQ & LLOQ %CV = ± 25% , %RE = ± 25%	ULOQ = 309 LLOQ = 9.68	Acceptable	None
Accuracy Inter assay& intra assay	Inter and Intra assay accuracy %CV & %RE = ± 20	HQC = 248 MQC = 124 LQC = 27.8	Acceptable	None
Intermediate Precision	HQC, MQC, LQC %CV, %RE = ±25			
Specificity for detection of PA63	Ability to detect PA in the presence of other components No acceptance criteria stipulated	% inhibition of PA ETI-201 – 100% rLF (45 -85%) rEF (21- 54%) ----- % increase rPA83 (30-183 %)	Acceptable	None
Stability PA63 Freeze/Thaw	Tested 3 times - 2/3 samples must meet % CV and RE = ≤ 20	Freeze/thaw cycles maximum = 5 % CV and %RE = ≤ 20	Acceptable	Acceptable
Linearity of Standard curve	2/3 of dilution samples must meet 156, 78.1, 39.1 ng/mL must meet ≤ 20 % CV and RE	3/3 each of: 156, 78.1, 39.1 ng/mL met criteria	Acceptable	None

4.2.5. ELISA for Detection of *Bacillus anthracis* Protective Antigen in Rabbits The assay designed to quantitate bound and free PA from *Bacillus anthracis* circulating in the serum of NZW rabbits was performed at [REDACTED] (b) (4) (SOP-[REDACTED] (b) (4) X-180-03, [REDACTED] (b) (4) XVI-006, [REDACTED] (b) (4) Validation # VP2008-201, Elusys Validation Report VP2012-256).

ETI-204 was the antibody to capture PA from the serum or plasma of rabbits infected with *Bacillus anthracis* or spiked with rPA. The assay was validated by the testing of three operators using 12 plate layouts, each plate was tested by at least two operators. The initial range of concentrations used was 0.10 ng/mL to 100,000 ng/mL.

4.2.5.1. Acceptance Criteria

The primary acceptance criteria relate to the state of the entire microplate in which samples were tested. These incorporated the criteria used to evaluate the calibration standards, the quality control samples, and the reagent blanks. The absorbance of the blanks must be lower than that of

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the Lower Limit of Quantitation (LLOQ). In this assay the value of the LLOQ was represented by the calibration standard (CS) 11.

Calibration Standards

(1) Standard Curve: CS within the validated range should have replicates $\pm 20.0\% \text{ CV}$ ($\pm 25.0\% \text{ CV}$ at the LLOQ and ULOQ). Calibration Standards within the validated range were to be within $\pm 20.0\% \text{ RE}$ of the nominal value ($\pm 25.0\% \text{ RE}$ at the LLOQ and ULOQ).

The accuracy of the standard curve points must have a %RE range from -20% to +20%. But at the LLOQ and the ULOQ the range could be between -25% to +25%

(2) Quality Controls:

QC replicates had to be $\pm 20.0\% \text{ CV}$. QC samples must be within $\pm 20.0\% \text{ RE}$ of the nominal value. Each of six QCs samples (two at each level: HQC, MQC, and LQC) was to be evaluated for precision and accuracy. The HQC and MQC must fall within $\pm 30\% \text{ of their nominal value}$. The Low QC must fall within $\pm 50\% \text{ of its nominal value}$. The value of the negative control naïve rabbit serum must have an optical density of ≤ 0.200 . Four of six QCs must demonstrate both a %CV of $\pm 20.0\%$ and a %RE of $\pm 20.0\%$. Also, there must be at least one passing QC at each level (HQC, MQC, and LQC) and at least two of the three QC samples must fall within the acceptable value ranges described above. QC was permitted to be censored one dilution at a time in order to have a percent coefficient of variation (%CV) of $\leq 30\%$.

(3) Sample

The reportable value (RV) of the unknown test sample must be taken from the average of the concentrations (from a single sample tested in replicate wells on one plate) interpolated from the passing reference standard curve. The average result must be between (or be equal to) the LLOQ and/or ULOQ of the assay and demonstrated an intra-dilution %CV of $\pm 20.0\%$. If the sample demonstrated $>20.0\% \text{ CV}$, it must be retested. If the sample demonstrated a putative result $<\text{LLOQ}$, then no retesting was required, but it should be reported as " $<\text{LLOQ}$." If the putative result was $>\text{ULOQ}$, then it must be diluted with pooled, naive serum at a dilution appropriate for quantitation within the range of the assay, and re-tested. The results that were to be reported include the median of the adjusted results whose unadjusted results was between the LLOQ and ULOQ.

Test samples

Stability of Samples

The stated conditions under which the samples must be kept in order to prevent degradation of the PA63 molecule were as follows:

Must be tested within 72 hours of collection and held at 2- 8 °C during this period. After 72 hours the samples must be stored at $\leq -60^{\circ}\text{C}$. For long term storage the samples must be held at $\leq -60^{\circ}\text{C}$ and tested with 3 months. The sample must be freeze/ thawed as little as possible.

Sample Testing Criteria

If a sample was tested two independent times the %CV must be $\leq 35\%$. If the %CV is greater than 35% the sample was to be repeated. If the quantity was insufficient and the %CV <35 , then the median value of the test results was taken.

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Twelve plate layouts were used in the study; each layout was tested by three operators. Figure 1 shows the arrangement of the samples on one micro-titer plate. The samples are diluted in individual columns down the plate. The two reference standards were each diluted 7 times. Each QC and test sample was diluted 1:5-fold for three successive times. The relative standard deviation (RSD) between equal concentrations of the same substance must be <20. The standard curve calibrators ranged from 4000 ng/mL diluted four-fold to 0.244 ng/mL.

Figure 1 Arrangement of Standards, Quality Controls and Test Specimens on a micro-titer preparation plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS-1 4,000 ng/mL	RS-1 4,000 ng/mL	VTS-1 neat	VTS-2 neat	VTS-3 neat	VTS-4 neat	VTS-5 neat	VTS-6 neat	VTS-7 neat	VTS-8 neat	QC-Mid neat	QC-Low neat
B	RS-2 1,000 ng/mL	RS-2 1,000 ng/mL	VTS-2 1:5	VTS-3 1:5	VTS-4 1:5	VTS-5 1:5	VTS-6 1:5	VTS-7 1:5	VTS-8 1:5	VTS-9 1:5	QC-Mid 1:5	QC-Low 1:5
C	RS-3 250 ng/mL	RS-3 250 ng/mL	VTS-1 1:25	VTS-2 1:25	VTS-3 1:25	VTS-4 1:25	VTS-5 1:25	VTS-6 1:25	VTS-7 1:25	VTS-8 1:25	QC-Mid 1:25	QC-Low 1:25
D	RS-4 62.5 ng/mL	RS-4 62.5 ng/mL	VTS-1 1:125	VTS-2 1:125	VTS-3 1:125	VTS-4 1:125	VTS-5 1:125	VTS-6 1:125	VTS-7 1:125	VTS-8 1:125	QC-Mid 1:125	QC-Low 1:125
E	RS-5 15.6 ng/mL	RS-5 15.6 ng/mL	VTS-9 neat	VTS-10 neat	VTS-11 neat	VTS-12 neat	VTS-13 neat	VTS-14 neat	VTS-15 neat	VTS-16 neat	NC	QC-High neat
F	RS-6 3.9 ng/mL	RS-6 3.9 ng/mL	VTS-9 1:5	VTS-10 1:5	VTS-11 1:5	VTS-12 1:5	VTS-13 1:5	VTS-14 1:5	VTS-15 1:5	VTS-16 1:5	NC	QC-High 1:5
G	RS-7 0.98 ng/mL	RS-7 0.98 ng/mL	VTS-9 1:25	VTS-10 1:25	VTS-11 1:25	VTS-12 1:25	VTS-13 1:25	VTS-14 1:25	VTS-15 1:25	VTS-16 1:25	NC	QC-High 1:25
H	RS-8 0.24 ng/mL	RS-8 0.24 ng/mL	VTS-9 1:125	VTS-10 1:125	VTS-11 1:125	VTS-12 1:125	VTS-13 1:125	VTS-14 1:125	VTS-15 1:125	VTS-16 1:125	NC	QC-High 1:125

(b) (4) Validation report

Comment:

Overall, the applicant's acceptable criteria are very wide and will not contribute to a high level of accuracy or precision.

3.2.5.2. Sensitivity

Eight calibration standards were used to draw the standard curve. The standard PA63 concentrations (10.5, 26.2, 65.5, 164, 410, 1020, 2,560, 6,400 ng/mL) were prepared fresh on the day of use with reconstitution into naïve rabbit serum. The acceptance criterion was $\leq 50\%$ of the nominal value. The LLOQ was determined to be 10.5 ng/mL and ULOQ was 6,400 ng/mL. Table 1 shows the results for concentration points in the calibration curve.

Table 1: Results of testing eight standards to establish the standard curve and determination of LLOQ and ULOQ .

Conc. (ng/mL)	Predict. \log_{10} (Conc.) (ng/mL)	Predict. Log. Bias	Residual Variance	Non-Residual Variance (NRV) Breakdown				Sum of NRV	Total \log_{10} Variation	Total \log_{10} Error	Percent Total Error
				Operator	Day	Plate	Replicate				
10.5	0.973	-0.048	0.002	0.001	0.000	0.001	0.001	0.003	0.004	0.082	19%
26.2	1.404	-0.014	0.004	0.002	0.000	0.001	0.001	0.004	0.008	0.091	21%
65.5	1.799	-0.017	0.008	0.001	0.006	0.001	0.001	0.009	0.017	0.130	31%
164	2.181	-0.034	0.003	0.000	0.002	0.001	0.001	0.004	0.008	0.094	22%
410	2.517	-0.095	0.002	0.005	0.007	0.001	0.001	0.014	0.016	0.158	38%
1,020	2.941	-0.070	0.001	0.014	0.007	0.001	0.001	0.024	0.024	0.171	41%
2,560	3.340	-0.068	0.006	0.010	0.006	0.001	0.001	0.018	0.024	0.169	40%
6,400	3.757	-0.050	0.014	0.014	0.000	0.001	0.001	0.016	0.030	0.180	43%

Source: (b) (4) validation report

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4.2.5.3. Quality Control

Six QC samples, two at each level, were used in this assay: the High QC (HQC) with a concentration of 1000 ng/mL, the medium QC (MQC) 100 ng/mL, and the low QC (LQC 10 ng/mL. Each QC sample was diluted five-fold for three successive times.

4.2.5.4. Accuracy

The criterion for the determination of accuracy was defined by the total error between the expected PA concentration and the concentration after testing. The percent total error (TE) accepted was $\leq 50\%$. The results were based on 264 reportable pieces of data. Initially, concentrations ranging from 0.1 to 100,000 ng/mL were tested. The total logarithmic error range was high at the highest and lowest concentrations when the values included 0.1 to 100,000 ng/mL, and included variables such as day to day, operator and residual variables at each concentration. The data were therefore restricted to the range normally used in the assays of 4.2 to 16,000 ng/mL, the variances were separated and the number reduced from 49 to 35. The results of the corrected values are shown in Table 2. Table 2 shows that the results at the highest and lowest concentrations of PA fell outside of the acceptance limit of $\leq 50\%$ TE. Figure 2 is a comparison of the two sets of values –

A - represents concentration range 0.1 to 100,000 ng/mL and

B - concentration range 4.2 to 16,000 ng/mL.

Table 3 shows the results of testing between 10.5 ng/mL and 6,400 ng/mL; all of the values were below 50%. The LLOQ and ULOQ were therefore computed to be 10.5 and 6400 ng/mL respectively.

Table 2: The Corrected concentration range restricted to 4.2 to 16,000 ng/mL by percent total error

Concentration	Predicted \log_{10} (Concentration)	Predicted Logarithmic Bias ⁸	Residual Variance	Sum of Non-Residual Variance	Total Logarithmic Variation	Total Logarithmic Error ¹⁰	Percent Total Error ¹¹
4.2	0.569	-0.054	0.055	0.005	0.060	0.250	62.676
10.5	0.975	-0.046	0.003	0.005	0.008	0.100	23.266
26.2	1.404	-0.015	0.004	0.007	0.011	0.105	24.524
65.5	1.801	-0.015	0.011	0.008	0.019	0.137	32.332
163.8	2.172	-0.042	0.005	0.005	0.010	0.110	25.663
409.6	2.512	-0.100	0.004	0.014	0.017	0.166	39.639
1024.0	2.946	-0.064	0.000	0.024	0.024	0.169	40.427
2560.0	3.336	-0.073	0.007	0.021	0.027	0.180	43.252
6400.0	3.755	-0.051	0.014	0.019	0.033	0.188	45.416
16000.0	4.222	0.018	0.037	0.026	0.062	0.250	62.755

(b) (4) Validation report

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Figure 2: Comparison of total logarithmic errors of concentration ranges 0.1 to 100,000 ng/mL and 4.2 to 16,000 ng/mL

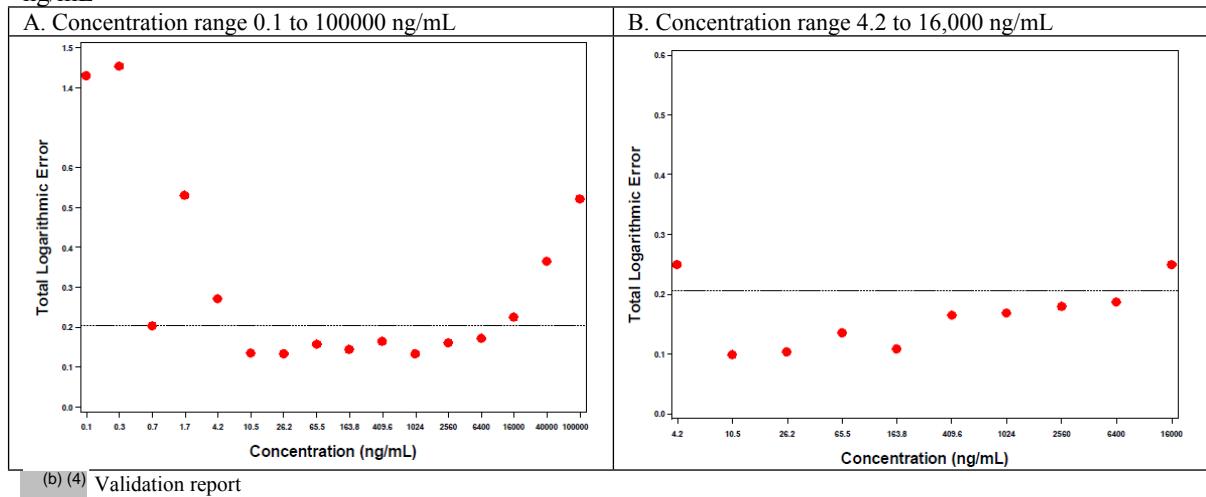


Table 3: Percent total error and other variances at concentrations between 10.5 ng/mL and 6,400 ng/mL of PA in the assay for New Zealand White Rabbits

Concentration	Predicted \log_{10} (Concentration)	Predicted Logarithmic Bias ¹³	Residual Variance	Sum of Non-Residual Variance	Total Logarithmic Variation	Total Logarithmic Error ¹⁴	Percent Total Error ¹⁵
10.5	0.973	-0.048	0.002	0.003	0.004	0.082	19.131
26.2	1.404	-0.014	0.004	0.004	0.008	0.091	21.178
65.5	1.799	-0.017	0.008	0.009	0.017	0.130	30.719
163.8	2.181	-0.034	0.003	0.004	0.008	0.094	21.857
409.6	2.517	-0.095	0.002	0.014	0.016	0.158	37.546
1024.0	2.941	-0.070	0.001	0.024	0.024	0.171	40.880
2560.0	3.340	-0.068	0.006	0.018	0.024	0.169	40.326
6400.0	3.757	-0.050	0.014	0.016	0.030	0.180	43.404

(b) (4) Validation report

The accuracy of the assay with regard to the amount of PA recovered is shown in Table 4. The acceptability criteria stated by the applicant was $100 \pm 30\%$. The percentage of PA recovered ranged from 80 – 97%. The amount of PA recovered was relatively stable throughout the concentration range. The lowest amount of PA recovered was 80% in the spiked specimen concentration of 410 ng/mL. The recovery results met the acceptability criteria.

Table 4: The percentage of PA recovered compared to nominal concentrations at various levels

PA conc. ng/mL	10.5	26.2	65.5	164	410	1,020	2,560	6,400
Percent. recovery	90	97	96	93	80	85	85	89

(b) (4) Validation report

4.2.5.4. Limits of Detection

The LLOD is the lowest true concentration at which $\geq 95\%$ of the concentration of PA can be detected. The LLOD was determined to be 1.30 ng/mL. The upper (ULOD) limits of detection defined as the concentrations where at least 95% of the PA results, below the upper end of the standard curve, can be determined. The ULOD was determined to be 26,297 ng/mL.

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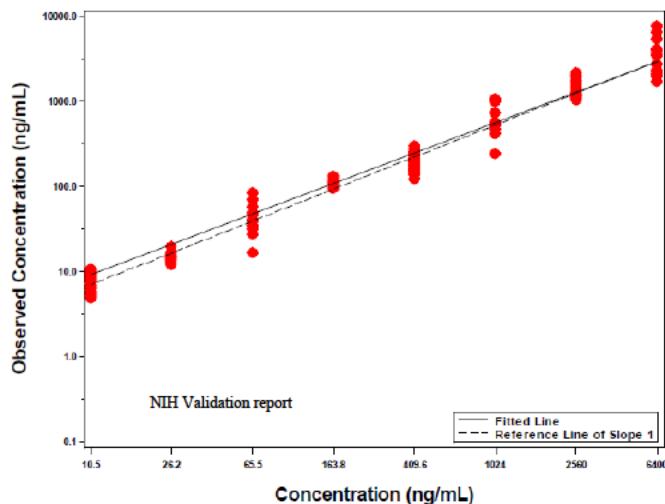
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4.2.5.5. Linearity

The results for linearity were collected from all values 10.5 ng/mL to 6,400 ng/mL.

The slope was 0.98 which met the acceptability criterion of within 1 ± 0.0645 . The line is shown in Figure 3.

Figure 3: Linear relationship between nominal and predicted relationship in rabbit serum PA between values 10.5 ng/mL and 6,400 ng/mL



4.2.5.6. Precision

Repeatability tests: This parameter was assessed using 8 samples that comprised 10.5, 410, 2560 and 16,000 ng/mL concentrations; each concentration was tested in three separate plates, run by separate operators. The acceptance criterion set by the applicant for agreement was < 30% relative standard deviation. The results are shown in Table 5. The criterion was met in all but the highest concentration 16,000 ng/mL which had a deviation of 39%. This meant that reproducibility was inaccurate at the highest dilution and that results cannot be relied upon even when such high concentrations are duplicated. At high concentrations the sample should be diluted.

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Table 5: Results of tests run for repeatability on four concentrations of PA spiked Rabbit serum.

Model	Variance Component	Concentration (ng/mL)	Pooled Variance Estimate	Standard Deviation	Percent Relative Standard Deviation
Separate Residual Variance	Operator	10.5	0.003	N/A	
		410	0.010		
		2,560	0.025		
		16,000	0.035		
	Residual	10.5	0.002	0.045	10%
		410	0.003	0.052	12%
		2,560	0.008	0.089	21%
		16,000	0.026	0.162	39%

(b) (4) Validation study

Intermediate inter-run precision: This parameter compares results of aliquots of the same specimen but tested on either different days, different runs, or by different operators. Acceptable limit (%RSD) was

≤ 30%. The results of testing for intermediate precision are shown in Table 6. The table shows that the criterion was met only from PA concentration 10.5 ng/mL to 410 ng/mL. The RSD results for 10.5 ng/mL to 410 ng/mL concentrations ranged from 16 to 30%. At the higher concentrations 1,020, 2560 ng/mL and 6,400 ng/mL, the values ranged between 37 and 42% which indicated that, in this study, these values when repeated were not accurate and are therefore not validated. These were also the concentrations where the operator variability was highest.

Table 6: Intermediate Precision analysis based on Percentage Relative Standard Deviation versus Concentration within the Analytical Range

True Concentration (ng/mL)	%RSD (Observational Scale)
10.5	16%
26.2	21%
65.5	30%
164	20%
410	30%
1,020	37%
2,560	37%
6,400	42%

Source Modified from NIH report

4.2.5.7. Specificity

As in the monkey studies, specificity was measured at three concentrations of PA: 6,400, 26.2 and 4.20 ng/mL. The physiochemically interference substances and matrices tested were : ciprofloxacin, LF, EF, hemolytic serum and plasma. The first part of the specificity testing was used to determine if the results obtained from pooled sera were different from those obtained from individual rabbit serum for use as matrices in testing interferents. The effect of pooled sera over individual sera was not significant therefore the normal serum samples were combined for testing of the interferents. A second part of specificity testing related to the effect of interferents in the test specimens. Normal serum samples were compared with samples that contained interferents. Table 7 shows the results of comparison between the control sera and those with

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interferents. Only two of the interferents made significant difference and those were LF and EF at 6,400 ng/mL. Table 8 is a summary of the results of the assay.

Table 7: Pairwise Differences in the Logarithmic Domain to Compare Normal Serum Samples with Serum Samples Having Potential Interferents, Separately within each Concentration. Concentrations 4.2, 26.2, 6,400 ng/mL

Target PA Concentration	Interferent compared to Control	Estimates	Std. Error	DF	t-Value	Signif(unadj) ^{21,22}
4.2	Cipro	-0.053	0.118	86.1	-0.45	0.6533
	LF	-0.005	0.119	86.5	-0.04	0.9663
	EF	-0.134	0.119	86.8	-1.12	0.2655
	Hemolytic	-0.264	0.119	86.8	-2.21	0.0298
	Plasma	-0.010	0.118	86.3	-0.09	0.9293
26.2	Cipro	-0.007	0.064	80.4	-0.12	0.9079
	LF	-0.059	0.060	80.3	-0.97	0.3346
	EF	-0.058	0.062	80	-0.94	0.3486
	Hemolytic	-0.104	0.059	79.9	-1.76	0.0814
	Plasma	-0.046	0.059	79.9	-0.77	0.4414
6400	Cipro	-0.006	0.043	71	-0.14	0.8866
	LF	0.391	0.044	72	8.97	<0.0001 *
	EF	0.199	0.046	73.8	4.31	<0.0001 *
	Hemolytic	-0.101	0.045	71.5	-2.25	0.0277
	Plasma	-0.005	0.045	73	-0.12	0.9068

(b) (4) validation report

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Table 8 : Summary of assay performance for Total PA in Rabbits

Parameter	Validation Criteria	Test results of Parameter (ng/mL)	Validation Decision	Comment
Limits of Detection	ULOD \geq 16,000 ng/mL LLOD \leq 4.2 ng/mL LOD \geq 95% of samples have a PA result	26,297 1.30 4.2	Acceptable	None
Limits of Quantitation	ULOQ \geq 16,000 ng/mL LLOQ \leq 4.2 ng/mL %TE \leq 50% (from LLOQ all Conc. to ULOQ) %RSD \leq 30% (LLOQ to ULOQ)	410 10.5	Acceptable	None
Accuracy	Absolute = %RE (bias) \leq 30% at each concentration	80 to 97 %	Acceptable	
Recovery	100 \pm 30 % at each concentration	80 – 97%	Acceptable Acceptable	
Intermediate Precision	%RSD \leq 30% For each concentration	RSD \leq 30 % up to 410 ng/mL 1020 – 6400 * 37-42%	Acceptable to 410 ng/mL	Validated only up to 410 ng/mL
Repeatability	%RSD \leq 30%	10.5, 410, 2560 10 - 21% 16,000 = 39%*	Partially acceptable	Not repeatable at 16,000 ng/mL
Specificity for detection of PA63	Normal Pool vs individual specimens < 30% Acceptance criteria = 77-130% Ability to detect interferent – significance level ($p \leq 0.15$) Ciprofloxacin, EF, LF, Hemolytic serum	Results were not significant Specific for plasma or interferents if Ratios 77% to 130%	Individual samples and pooled samples combined Interferents -Not significant <u>except</u> At 6,400 ng/mL LF (41%) EF 63%) significant	Acceptable except at 6,400 ng/mL.
Stability PA63	Maximum 182 days frozen at \leq -70°C	NA	NA	Complete results not available
Linearity of Standard curve	Range at LLOQ and ULOQ = \leq 30% Slope = 1 ± 0.0645	Regression slope 0.98	Acceptable	

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Comments:

- *The results of testing to demonstrate the accuracy, precision, specificity of testing for free PA in rabbits using this ELISA assay are adequate, because the results met the criteria set forth for acceptability by the applicant. The assay showed acceptable linearity but only between 10.5 and 410 ng/mL.*
- *Specificity tests show that PA will not be affected by most of the interferents that are expected to be found in specimens of infected animals. This includes a fluoroquinolone with which the infected animal is likely to be treated. It is also important to know whether there would be a difference in results between serum and plasma. The other components of the tri-partite toxin LF and EF do not affect PA except at high levels of PA when it reduces the recovery of PA. One of the major problems in this assay as with other assays in this study is that the level of hemolysis was not clearly defined.*
- *Sensitivity was demonstrated in the minute quantity of PA that could be detected. This is important because it means that the assay can detect PA activity very early in the infection, when it is most critical to administer therapy. ULOQ tended to pose some problems with inaccuracy as it did with the non-human primates. Therefore it might be wise to repeat the samples, with dilutions, when results fall at the upper limits of quantitation. The LLOD was determined to be 4.2 ng/mL while the LLOD was 10.5 ng/mL*
- *The applicant states that this assay, and its companion in monkeys, are meant to track toxemia.*

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4.3. Enzyme linked immunosorbent assay for quantitating anti-PA IgG antibodies

4.3.1. Anti-PA monoclonal Antibody in Monkeys

SOP ^{(b) (4)} X-101-12. Validation Report VP2008-221(G607605).

This was an indirect ELISA designed to measure the specific IgG antibody to the PA in the serum of cynomolgus monkeys infected with, or exposed to, *Bacillus anthracis*. The active response of the body to a foreign antigen is the formation of antibodies. The antibodies found in the blood are located in the serum when blood clots. It is this serum that is tested in the indirect ELISA. Antibodies bind with varying avidity, to the specific antigen which stimulated their production.

The principle of the assay is different from that described in section 4.2. Briefly, the antigen recombinant PA is immobilized in the well. Sera from infected animals are added which, if specific for PA, will bind to the immobilized antigen. A secondary antibody of the same class as anti- PA IgG is linked to a labeled reporter molecule. The secondary antibody (goat anti-non-human primate IgG Horseradish Peroxidase conjugate (Fitzgerald), binds to the primary antibody in the sample and therefore must be compatible with the primary antibody. Anti-PA antibody that is not bound to rPA in the plate is washed out while the bound PA-anti-PA – enzyme-conjugated anti-IgG complex remains. The intensity of the reporter molecule in the plate is read by a read-out instrument such as a spectrophotometer. The applicant states that in the future, this assay will be used to detect anti-PA IgG antibodies in the sera of patients using an anti-human IgG conjugated antibody.

In this assay, a 96 well microtiter plate was coated with 1 µg/mL rPA in 1X phosphate buffered saline pH7.4 and stored until use. The unbound rPA in the microtiter plates were washed, when ready to use. One hundred microliter of 2X diluted standard samples, test samples, and 1X diluted quality controls were added to specific wells in the plate, incubated, and washed. The conjugate was added to the washed plate and re-incubated. After incubation and washing, species specific, horseradish peroxidase conjugated anti-gamma chain antibody, goat anti-NHP IgG or goat anti-rabbit IgG Horseradish peroxidase which were the secondary antibodies (dependent on the specific serum being tested) were added. This combination was incubated, and stop solution added. The tests were read on a 96 well microplate reader (Bio Tek Elx800 or a similar spectrophotometer at a wavelength of 405 nm). The endpoint of the assay was reported in µg/mL of anti-PA IgG. The assay was performed at ^{(b) (4)} to evaluate the following:

- Sensitivity
 - Limit of Detection
- Accuracy
 - Repeatability
 - Dilutional Linearity
- Precision
- Specificity
 - Matrix effect

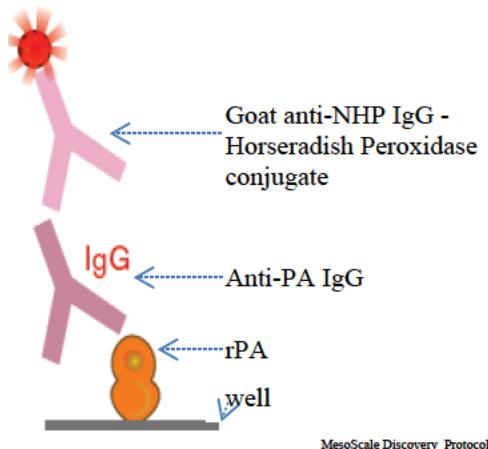
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Figure 1: Diagram of Indirect ELISA for detection of Anti-PA IgG



4.3.1.1. Acceptance Criteria

The following definitions are important in the evaluation ELISA for Windows (EFW) Censors:

1. CenA = Censor optical density (ODs) below user selected value. The selected value is typically the average OD of the negative control, but the average OD of the reagent control may also be used depending on the study.
2. CenB = Censor ODs outside the range of the reference standard.
3. CenC = Censor ODs where the within-dilution is CV > 20%.
4. CenE = Censor ODs that are within 5% of the reference standard maximum OD (S1).
5. CenF = Censor both replicates in a dilution series if one is censored.
6. N/C (Non-Calculable) = Censor values that fall outside the range of the reference standard asymptotes.

Plate Acceptability

The entire plate must meet all of the acceptability standards described for the parameters relating to standards, quality controls, and test results as indicated in other parameters described below.

Plate acceptability is called the primary acceptance, and affects all samples on the plate. If the plate acceptability fails, all samples on the plate will fail.

Calibration Standards

The stock concentration of 159.916 µg/mL was diluted two times starting by diluting the standard 1:900. Standard 1 (S1) = 0.178 µg/mL subsequent two-fold dilutions resulted in S7 = 0.0028 µg/mL, the lowest undiluted concentration of anti-PA IgG. Since test samples were tested at a starting dilution of 1:50 the lowest concentration that would result in an OD > S7 would be 0.140 µg/mL – the lowest concentration of anti-PA IgG in a sample that would give an acceptable OD i.e., greater than that of S7. Figure 2 shows 3 calibration samples diluted 7 times vertically down the plate. The reference standard 7-point dilution series, must demonstrate a minimum goodness-of-fit by having a regression coefficient (R^2) of ≥ 0.9800 .

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Quality Control

Duplicate samples of high, low and negative controls were added to each plate. Figure 2 shows the layout of each plate.

- The QC values must meet all of the criteria listed below.
- The High (HQC) and Low quality control (LQC) results must have a %CV of <20.
- Both the HQC and LQC must have an optical density that falls within the range of the reference standard and must be at least 5% below the value of the maximum standard. In Figure 3 the OD range was 1.61 (S1) to 0.17 (S7) and the maximum OD was 1.61.
- HQC and LQC must fall within a pre-determined range for the concentration. The HQC must have an OD approximately between S2 and S3 and the LQC approximately between S5 and S6 on the standard curve.
- The Negative QC must have a mean OD of <0.2.

Figure 2: Layout of the ELISA Plate for IgG testing

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-HIGH
B	S2	S2	S2	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-HIGH
C	S3	S3	S3	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-LOW
D	S4	S4	S4	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-LOW
E	S5	S5	S5	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-QUAL
F	S6	S6	S6	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	QC-QUAL
G	S7	S7	S7	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	NC
H	S8	S8	S8	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	NC

Key:

S1-S8 = Reference Standard Serum, Dilutions 1-8
TS# = Test Samples 1-4
QC-HIGH = High Concentration Serum Control
QC-LOW = Low Concentration Serum Control
QC-QUAL = Candidate Concentration Serum Control under Qualification (not included in this validation)
NC = Negative Serum Control

Note: Columns 1 - 3 are reference standard samples diluted serially 2-fold down the plate, and Columns 4-11 are test samples diluted serially 2-fold down the plate.

(b) (4) validation report

Test Samples:

Achievement of the secondary acceptance criteria of the assay was dependent upon the extent to which the individual sample acceptance criteria were met. The test samples were first diluted 1:50 and were run in duplicate. The amount of anti-PA IgG for a test sample was calculated as the average of the acceptable dilution from the validation test sample, read from the standard curve. Figure 3 is an example of the standard curve. If a test sample on a specific plate does not meet the secondary QC requirements, it can be repeated and its result does not affect the other

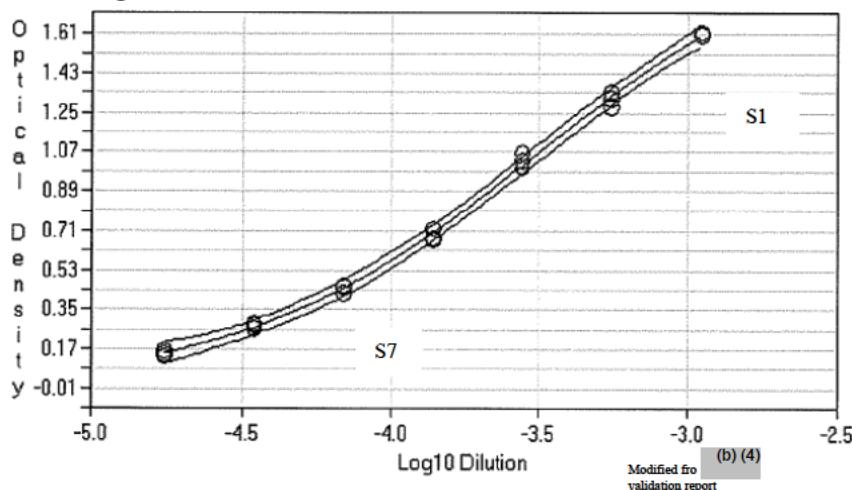
samples on that plate. Acceptable OD of samples must range between S1 and S7.

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Figure 3: Reference Standard Curve



- Acceptable OD values: OD values of a test sample must fall between those of the minimum and maximum standards of the curve S1 to S7 and at least 5% below the OD of the maximum standard, and have a within-dilution of $\leq 20\%$.
- Within-dilution %CV: If the %CV of a pair of dilutions is $\geq 20\%$ the mean of the pair cannot be calculated unless the standard deviation (SD) is \leq the current LLOQ of the assay. This situation ($SD \leq LLOQ$) will occur only if the sample is a test that has a low titer.
- Intra-assay %CV: Intra-assay should be $<20\%$. If this is $\geq 20\%$ and there is an outlier, that outlier pair must be censored. The maximum number of outliers pairs censored per run should be no greater than 3. Only one pair can be censored at a time.

4.3.1.2. Sensitivity

Limit of Detection

The lower limit of detection (LLOD) and the upper limit of detection (ULOD) were first calculated, then the result was multiplied by the dilution factor. LOD is the lowest concentration of the analyte (anti-PA IgG) that can reliably and consistently be detected in a sample using this method. It refers to the amount of analyte that is present in the original concentration of the sample.

Seventy-seven plates were used to calculate the LOD. Four of the samples did not meet acceptance criteria and had to be discarded. The LOD was calculated from the 73 plates that passed the criteria. The limit of detection for this assay was 1.6 µg/mL.

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4.3.1.3. Linearity

Dilutional Linearity

This parameter was performed by spiking naïve negative monkey serum or plasma, with monkey serum that was positive for anti-PA IgG. Four spiked specimens, BM1269, BM 1280, BM1289, BM1290, were initially chosen to determine dilutional linearity. Two of the four specimens were acceptable, BM1269 and BM1280. Results for BM1290 indicated that two dilutions (1:16 and 1:32), fell outside of the acceptability range. In specimen BM1281, one sample (1:96 dilution) tested lower than was initially expected; these results made the relevant curves unacceptable. Table 1 shows the two spiked samples and dilutions that were used to determine the dilutional linearity.

The concentrations of all of the dilutions fell within the acceptance limit, except dilutions from validation test samples (VTS)15 and 16 of BM1280, which passed the criteria but had medians less than the LOD. Figure 4 shows the 4 curves drawn from the results of testing the 4 original samples.

The slope of -1.01 was acceptable and the linearity was validated to range from 4 µg/mL, the lowest concentration in Bin 2, to 2048 µg/mL. When corrected for bias, the working range of the assay was determined to be 5.42 µg/mL to 2360 µg/mL. The LLOQ was therefore 5.42 µg/mL.

The ULOQ was set at 256 µg /mL.

Table 1: Two Spiked Specimens used to Calculate the Dilutional Linearity
For the Monkey ELISA test for the Detection of Anti-PA IgG

VTS	Specimen	Dilution Factor	Bin	Total Number of Tests	Number of Successful Tests	Geometric Mean	R ₂
1	BM1269	1	10	7	5	3040	26.1%
2		5	8	10	6	495	30.3%
3		20	6	10	6	121	6.15%
4		80	4	10	7	27.0	15.3%
5		160	3	10	7	12.8	12.3%
6		320	2	8	6	7.23	6.17%
7		640	1	8	6	3.82	8.24%
8		1280	0	7	5	2.44	6.55%
9	BM1280	1	10	5	5	1450	18.0%
10		4	8	5	5	351	28.7%
11		16	6	5	5	84.4	10.6%
12		64	4	5	5	21.8	28.2%
13		128	3	7	4	10.3	7.28%
14		256	2	7	4	5.44	4.52%
15		512	1	5	NA ²		
16		1024	0	6	NA ²		

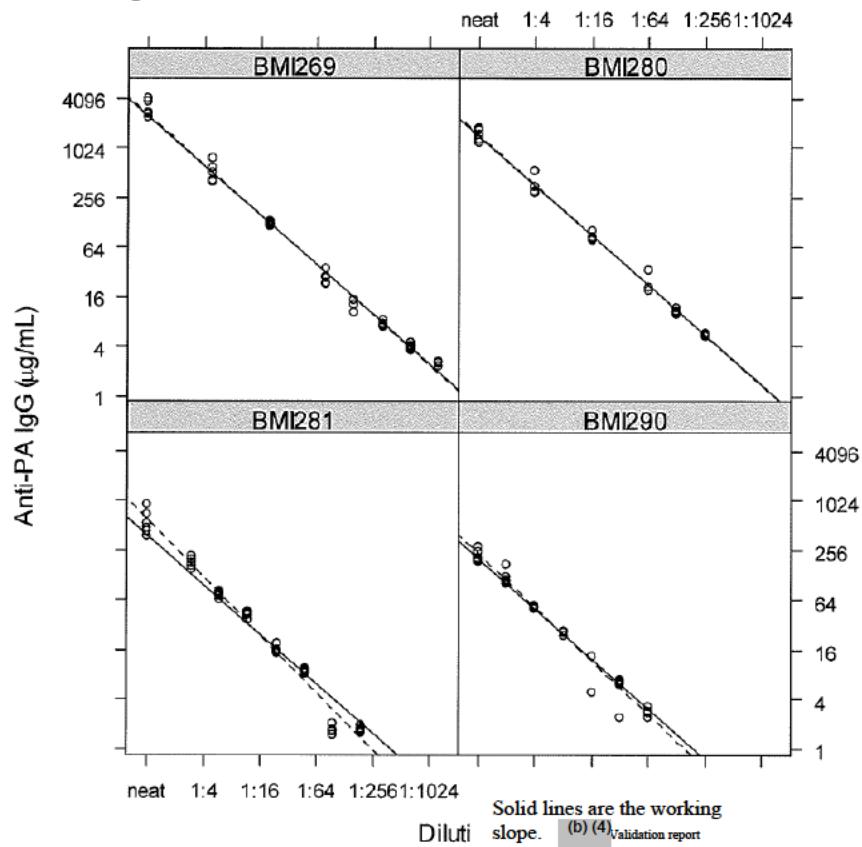
VTS 15 16 passed the primary acceptance criteria had medians less than the estimated LOD and were excluded from the dilutional linearity analysis. R₂ = regression analysis (b) (4) VP2008-221

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Figure 4: Dilutional Linearity Plot for the Four Specimens Tested



4.3.1.4. Precision

The assessment of precision was made from 52 specimens. Of these, the median activity of 4 was below that of the LOD and so these specimens were excluded from the calculations. The relative variability (R_2), of the plate repeatability was 18.4 %. The R_2 of the intermediate precision was 21.1%. The required acceptability results were less than the stipulated 30% and 40%, respectively. Therefore the assay passed precision.

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Table 2: Composite results of the 52 Validation test samples used for Precision of the ELISA used for Monkey testing

High Level	Low Level	Variance	Percentage of Total	Standard Deviation	R ₂
Repeatability	Residual	0.0389	51.2%	0.197	14.7%
	Plate	0.0206	27.1%	0.144	10.5%
		0.0595	78.4%	0.244	18.4%
Intermediate Precision	Date	0.00270	3.55%	0.0519	3.67%
	Technician	0.0137	18.1%	0.117	8.46%
		0.0760	100%	0.276	21.1%

(b) (4)_{VP2008-221}

4.3.1.5. Specificity

Normal serum from BM1293 and BM 1280 were spiked to provide matrices that measured the effect of hemolysis and ciprofloxacin on the specificity of the assay, to detect anti-PA IgG. Plasma was used instead of serum. Specimen BM1269 was spiked 1:5 and 1:80 and specimen BM1280 was spiked 1:4 and 1:64 with diluents that were hemolytic, or had ciprofloxacin. Table 3 is a summary of the data used to analyze specificity. Table 4 shows the effect of the hemolysis, ciprofloxacin and plasma on the detection of the activity of anti-PA IgG antibody. When the amount of the matrix was 10% the activity of the anti-PA IgG was high, but when the amount of diluent was high the activity was low -0.0714 and 0.0290 for hemolysis and plasma, respectively. The result for ciprofloxacin it was -0.172, indicating that the activity was suppressed to a greater extent by ciprofloxacin than by the other two matrices. Tables 3 and 4 show these results.

Table 3: Data used to Analyze Specificity of Assay for three Matrices in Tests for anti-PA IgG

VTS	Specimen	Dilution Factor	Diluent	Bin	Number of Tests	Number of Successful Tests	Geometric Mean	R ₂
02	BMI269	5	BMI293	8	10	6	495	30.3%
33	BMI269	5	Hemolytic	8	7	6	417	48.5%
34	BMI269	5	Cipro	8	7	6	423	31.8%
35	BMI269	5	Plasma	8	5	5	378	40.9%
04	BMI269	80	BMI293	4	10	7	27.0	15.3%
36	BMI269	80	Hemolytic	4	9	6	18.8	3.98%
37	BMI269	80	Cipro	4	10	6	17.4	9.90%
38	BMI269	80	Plasma	4	10	6	31.1	12.2%
10	BMI280	4	BMI293	8	5	5	351	28.7%
39	BMI280	4	Hemolytic	8	9	6	433	48.1%
40	BMI280	4	Cipro	8	5	5	357	16.0%
41	BMI280	4	Plasma	8	5	5	353	24.2%
12	BMI280	64	BMI293	4	5	5	21.8	28.2%
42	BMI280	64	Hemolytic	4	5	5	20.9	7.99%

(b) (4) validation Report

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Table 4: The effect of hemolysis, ciprofloxacin and plasma on the activity of anti-PA IgG

		Hemolytic	Cipro	Plasma
intercept and slope to spike estimated from data	10%	-0.306	-0.408	-0.213
	90%	-0.0714	-0.172	0.0290
adjusted mean estimated from data assuming slope ≡ 1	10%	-0.295	-0.419	-0.223
	90%	-0.0489	-0.173	0.0267

(b) (4) validation Report

Table 5 is a summary of the acceptance criteria and the results that were obtained for each criterion. The assay passed all parameters, except for the testing of samples in which ciprofloxacin was included.

Table 5: Summary of Monkey Anti-PA IgG assay

Parameter	Validation Criteria	Test results of Parameter ($\mu\text{g/mL}$)	Validation Decision	Comment
Limits of Detection	LOD \geq 95% of samples have an Anti-PA IgG Result . No greater than 5 $\mu\text{g/mL}$	1.6	Acceptable	None
Limits of Quantitation	ULOQ LLOQ Variance no greater than 0.236 %TE \leq 50% (from LLOQ all Conc. to ULOQ)	256 5.42 TE acceptable in Bins 2 and 5-7	Acceptable	None
Precision			Acceptable	None
Intermediate Precision	R2 = < 40%	Intermediate precision = 21.1%		
Repeatability	R2 = <30%	Repeatability R2 = 18.4%		
Specificity for detection anti-PA IgG	Reference Standard <50% when diluted in other matrices	< 25% failure of samples		Hemolysis & plasma = Pass Ciprofloxacin = Fail
Accuracy - Linearity of Standard curve	Within ± 0.322 on the log scale (80 to 125%)	2/4 met criteria Range 5.42 to 1280	Acceptable	

Comment:

This assay is validated for the testing of monkey serum samples for the presence of anti-PA IgG.

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It cannot be used for early identification because the antibody is formed approximately 7-10 days after infection. The assay can be used for follow-up. The secondary antibody will have to be changed to a species specific antibody conjugate e.g., goat anti-rabbit IgG horseradish peroxidase conjugate, if antibody IgG is to be detected in rabbit serum or goat anti-human IgG horseradish peroxidase conjugate, if antibody IgG is to be detected in human serum. Cross-reactivity with antibodies against other organisms, even within the same genus, was not examined.

4.3.2. Anti-PA monoclonal Antibody in Rabbits

SOP ^{(b)(4)}X-101-12. Validation Report VP2004-108 , Appendix -B, 2004-108

- This assay is the same as is described for the monkey anti-PA IgG except that goat anti-rabbit IgG horseradish peroxidase conjugate was used as the secondary antibody.
- The tests were performed at ^{(b)(4)}. This report reviews the validation of the following parameters:
- Sensitivity
 - Limits of Quantitation
- Limit of Detection
- Accuracy
 - Dilutional Linearity
- Precision
 - Repeatability
 - Intermediate precision
- Specificity
 - Matrix effect

4.3.2.1. Acceptance Criteria

Repeatability:

- Plates High1, Mid1 and Low1 - % Relative Standard Deviation (RSD) must be $\leq 20\%$
- Plates Low2 and Low3 - %RSD must be $\leq 30\%$
$$\%RSD = (\text{Plate Standard Deviation} \div \text{Plate Mean}) \times 100$$

Intermediate Precision:

- Plates High, Mid1 and Low1 - must be $\leq 20\%$
- Plates Low2 and Low3 - %RSD must be $\leq 30\%$

Dilutional Linearity:

Diluted sample must fall within the standard curve. Dilutional Linearity will be assessed by

- Percent Relative Error (%RE) = $\leq 30\%$; and Percent Recovery (%Rec) = $100\% \pm 30\%$.
- But as many as 11 of 126 points may be excluded from this calculation
 $\%RSD$ at each dilution level is as follows:
- R^2 from linear regression model Log_{10} nominal vs Log_{10} observed
- Within-dilution %CV must be $<20\%$. If %CV $\geq 20\%$ then the dilution pair must be censored
- Within-assay %CV must be $<20\%$.

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Specificity:

The secondary antibody for this assay was goat anti-rabbit IgG-horseradish peroxidase conjugate (Biomedical Aurora, Ohio). All results must have %RE = $\leq 30\%$ and %Rec $100\% \pm 30\%$ from the nominal concentration.

Sensitivity:

Results must have a %RE of $\leq 30\%$ RE, %Rec = $100\% \pm 30\%$, RSD + $\leq 40\%$

Calibration Standard

The concentration of anti-PA IgG in the reference Standard BMI001 was $79.89 \mu\text{g/mL}$. The starting dilution was 1:100. The sample was then diluted 1:50 for testing. Each plate used in this study comprised two positive and one negative control sample.

4.3.2.2. Sensitivity

In order to establish a range for the assay, the concentration of samples spiked to $1 \mu\text{g/mL}$ was used to establish the minimum LOQ.

In addition to the $1 \mu\text{g/mL}$ spiked samples, 8 samples from rabbits, with approximately $1 \mu\text{g/mL}$ rabbit anti-PA IgG were diluted 1:50, and tested by 3 operators. The results of tests with $1 \mu\text{g/mL}$ were validated. It was found that all samples with a concentration of $\geq 1 \mu\text{g/mL}$ were always positive. Therefore, the applicant decided that $1 \mu\text{g/mL}$ would be an appropriate LOD. Analysis of results from the 8 rabbit samples using this LOD, showed that one positive sample registered as negative and that another fell above the upper limit of acceptance. Sixteen additional tests were performed to confirm the LOD and LOQ. The tests were run by 3 operators. The results confirmed that the LOD should be $1 \mu\text{g/mL}$. The applicant retrospectively assessed previous results of samples with a concentration of IgG $< 3.0 \mu\text{g/mL}$. It was found that 74.47 % of the times the samples tested resulted in a %RSD of $\leq 40\%$. This retrospective analysis also indicated that an acceptable level ($< 40\%$ RSD) of rabbit anti-PA IgG was achieved at concentrations $< 5 \mu\text{g/mL}$. This concentration, $5 \mu\text{g/mL}$, was selected as the limit of quantitation LOQ.

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4.3.2.3. Accuracy

Dilutional Linearity

Dilutional linearity assesses whether the results of a dilution of a sample are representative of the undiluted sample. Dilutional linearity is important for accurately determining the value of a sample that has a high concentration of an analyte, in this case, rabbit anti-PA IgG. For the test of this parameter, the applicant spiked a positive sample (BM1002) concentration of 470.2 $\mu\text{g}/\text{mL}$, into a negative sample (BM1012) to prepare concentrations of IgG of 80, 40, 20, 10, 5, 2.5 and 1.25 $\mu\text{g}/\text{mL}$. The 7 samples were tested in duplicate by 3 operators on 3 days. These original concentrations were adjusted because the trend of results indicated that the original concentrations were inaccurately prepared. The test concentrations were adjusted to 71.77, 35.89, 17.94, 8.97, 4.49, 2.24, and 1.21 $\mu\text{g}/\text{mL}$. Acceptability was calculated as follows:

Accuracy was calculated by utilizing the mean bias (%RE) and the percent recovery (%Rec).

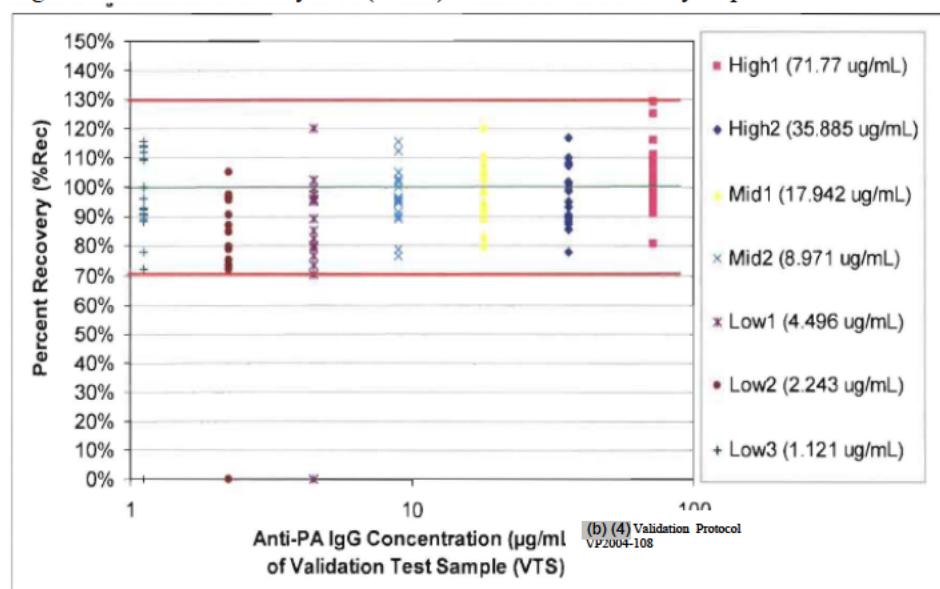
Percent relative error (bias) was calculated by using the following categories of concentrations:

%RE = [(observed-nominal)/nominal] X100. The acceptance criterion, $\leq 30\%$

Percent recovery of IgG, was calculated as follows: %Recovery = (observed/nominal) x 100.

Acceptance criterion was defined as $100\% \pm 30\%$ but up to 11 of the 126 results may be excluded. Figure 1 and Table 1 show the results. All test concentrations fell within the prescribed acceptable limits for dilutional linearity.

Figure 1: Percent Recovery Plot (%Rec) of Dilutional Linearity Reportable Values



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Table 1: Summary of Dilutional Linearity Percent Recovery (%Rec) Data

Dilutional Linearity Percent Recovery Summary			Result	Pass/Fail
71.77 µg/mL	%RE		29.18%	Pass
	Min	%Rec	80.74%	Pass
	Max		129.18%	Pass
35.89 µg/mL	%RE		21.83%	Pass
	Min	%Rec	78.17%	Pass
	Max		116.76%	Pass
17.94 µg/mL	%RE		20.74%	Pass
	Min	%Rec	80.39%	Pass
	Max		120.74%	Pass
8.97 µg/mL	%RE		23.46%	Pass
	Min	%Rec	76.54%	Pass
	Max		115.46%	Pass
4.49 µg/mL	%RE		29.54%	Pass
	Min	%Rec	70.46%	Pass
	Max		119.91%	Pass
2.24 µg/mL	%RE		27.78%	Pass
	Min	%Rec	72.22%	Pass
	Max		105.26%	Pass
1.12 µg/mL	%RE		27.74%	Pass
	Min	%Rec	72.26%	Pass
	Max		115.61%	Pass

(b) (4) Validation Protocol VP2004-108

Percent Relative Standard Deviation (%RSD) of the dilutional linearity was calculated as follows:

% RSD = Standard deviation of the Concentration/ Mean concentration of the concentration)
 X100.

The acceptance criteria were $\leq 20\%$ of each concentration, except the lowest concentration which is $\leq 30\%$.

Percent relative standard deviation is shown in Figure 2. All concentrations fell below 20% of the nominal concentration. This demonstrated accuracy in dilution of the original sample.

When the sources of variability were investigated, it was found that the persons who conducted the tests performed well. The technician effect did not adversely affect the results. The errors were greatest at the high concentrations. Figure 2 shows the results. Table 2 shows the effect of technician and day of test performance, on the results, for 6/7 occasions the technician performance was of a high standard.

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Figure 2 : Percent Relative Standard Deviation (%RSD) of Dilutional Linearity

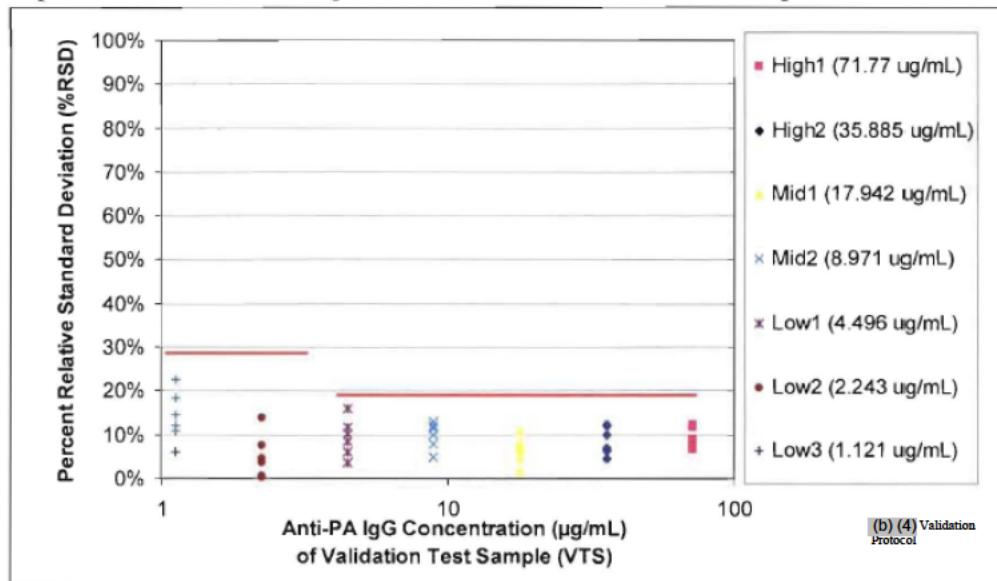


Table 2: Sources of Variability, Total Variability, and Relative Standard Deviation for the Dilutional Linearity Data.

Anti-PA Concentration Level	Sources of Variability			Total Variability	%RSD
	Technician Effect SD	Day Effect Nested within Technician Effect SD	Error Component SD		
High 1	0.000 ¹	4.064	7.849	8.839	11.946
High 2	0.000 ¹	2.687	2.619	3.752	10.707
Mid 1	0.000 ¹	1.142	1.605	1.970	11.292
Mid 2	0.000 ¹	0.264	0.932	0.969	11.194
Low 1	0.000 ¹	0.359	0.485	0.604	15.054
Low 2	0.000 ¹	0.000 ¹	0.280	0.280	14.197
Low 3	0.017	0.065	0.130	0.147	13.365

¹Model estimated a negative variance for the effect; thus, the SD was set to zero.

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Accuracy was also determined by checking if bias was present. The researcher added 6 rabbit anti-PA IgG positive serum samples to one negative sample in order to obtain a concentration of the antibody of 40 and 80 µg/mL. Eighteen samples, numbered 25 to 42 were tested by 3 operators. A mean was calculated for the results of each concentration. Acceptable limits were that each sample result must have an error rate of $\leq 30\%$ of the nominal value, and a repeatability rate of $100\% \pm 30\%$. Table 3 shows the results. The error rate ranged from 0 to 33 %. Only one sample failed - test sample # 25 which has a concentration of 1 µg/mL and a %RE of 33%. The error rate range of the other samples was 0 % to 19%.

For repeatability, sample 25 failed again. An investigation showed that the sample was prepared incorrectly. After the investigation #25 was replaced by 3 new specimens, diluted to 1 µg/mL. This substitution enabled the test to pass validation.

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Table 3: The Error and recovery rates of 18 rabbit anti-PA spiked samples to test for sample bias.

VTS ID	Mean Conc. of Observed VTS ($\mu\text{g/mL}$)	Nominal Conc. ($\mu\text{g/mL}$)	%RE	%Rec	Pass/Fail
VTS #25	1.33	1.00	33%	133%	Fail*
VTS #26	0.98	0.90	8%	108%	Pass
VTS #27	1.14	1.00	14%	114%	Pass
VTS #28	0.96	1.00	4%	98%	Pass
VTS #29	1.00	1.00	0%	100%	Pass
VTS #30	0.85	1.00	15%	85%	Pass
VTS #31	42.58	40.00	6%	106%	Pass
VTS #32	39.90	35.89	11%	111%	Pass
VTS #33	41.29	40.00	3%	103%	Pass
VTS #34	43.62	40.00	9%	109%	Pass
VTS #35	40.96	40.00	2%	102%	Pass
VTS #36	38.20	40.00	4%	96%	Pass
VTS #37	87.16	80.00	9%	109%	Pass
VTS #38	85.30	71.78	19%	119%	Pass
VTS #39	89.39	80.00	12%	112%	Pass
VTS #40	88.57	80.00	11%	111%	Pass
VTS #41	80.13	80.00	0%	100%	Pass
VTS #42	73.16	80.00	9%	91%	Pass

4.3.2.4. Precision

Repeatability

Repeatability evaluated the similarity of results obtained from specimens within a run. This was measured by 3 operators, who prepared the samples independently using 5 concentrations of anti-PA IgG, for testing on 3 days. The five concentrations were High 1, Mid 1, and 3 levels of Low namely, 1, 2 and 3 in concentrations of 80, 20, 5, 2.5 and 1.25 $\mu\text{g/mL}$, respectively. Each of the concentrations was assayed 36 times. Table 4 shows the results. All concentrations met the acceptance criteria.

Table 4: Results of Repeatability of Five Concentrations of Rabbit Samples

	80 $\mu\text{g/mL}$ (Plate 1)	20 $\mu\text{g/mL}$ (Plate 2)	5 $\mu\text{g/mL}$ (Plate 3)	2.5 $\mu\text{g/mL}$ (Plate 4)	1.25 $\mu\text{g/mL}$ (Plate 5)
Acceptance Criteria	$\leq 20\% \text{ RSD}$			$\leq 30\% \text{ RSD}$	
Max Plate Repeatability (%RSD)	9.73%	10.39%	12.26%	12.16%	21.47%
Validation Acceptance of Repeatability at 1.25 $\mu\text{g/mL}$	PASS	PASS	PASS	PASS	PASS

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Intermediate precision

Intermediate precision, evaluated the results obtained from specimens between runs, in order to assess the variability across the plates, operators and times the specimens were tested. The results of the five specimens 80, 20, 5, 2.5, 1.25 µg/mL ran 18 times by 3 operators on 3 days using 2 separate preparations, were compared. The inter-plate mean and inter-plate standard deviation were calculated and the inter-plate percentage relative standard deviation was calculated as follows (%RSD):

$$\text{Inter-plate \%RSD} = (\text{Inter-plate standard deviation}/\text{Inter-plate mean}) \times 100$$

All of the acceptance criteria were met. Table 5 shows the results.

Table 5: Results of Intermediate Precision of Five Concentrations of Rabbit Samples

	80 µg/mL	20 µg/mL	5 µg/mL	2.5 µg/mL	1.25 µg/mL
Acceptance Criteria	≤ 20% RSD			≤ 30% RSD	
Inter-Plate %RSD	11.73%	10.53%	15.21%	15.29%	28.43%
Validation Acceptance of Repeatability at 1.25 µg/mL	PASS	PASS	PASS	PASS	PASS

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The applicant also assessed some random effects using the same concentrations as those used in intermediate precision. The random effects were variability by day, analyst, error and overall variability. Table 6 shows the results. There was no difference in analyst results. Although the relative standard deviation percentage met the acceptance criteria, they were generally very high. Low level 3 fell just within the level of acceptance criteria, and only at Low level 3 did the technician have any adverse effect.

Table 6: Variability between Analysts, Day, Error and Overall Variability in testing five concentrations for anti-PA IgG in Rabbits

Anti-PA Concentration Level	Sources of Variability			Total Variability	%RSD
	Technician Effect SD	Day Effect Nested in Technician Effect SD	Error Component SD		
High 1	0.000	5.077	7.704	9.227	12.470
Mid 1	0.000 ¹	1.142	1.605	1.970	11.292
Low 1	0.000 ¹	0.000 ¹	0.728	0.728	18.421
Low 2	0.000 ¹	0.000 ¹	0.352	0.352	18.209
Low 3	0.105	0.081	0.270	0.301	29.017

¹Model estimated a negative variance for the effect; thus, the SD was set to zero.

(b) (4) validation Report
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4.3.2.5. Specificity

Specificity was tested in 3 ways (i) Two PA positive sera - one from recombinant Protective Antigen (rPA) vaccinated, and the other from Anthrax Vaccine Adsorbed (AVA) - vaccinated rabbit. These provided high and low concentrations of 6 different anti-PA IgG sera (ii) These 6 different anti-PA IgG sera were spiked into one negative sample to create high, medium and low concentrations (iii) Interference from substances that might normally be found in the sample of an infected individual e.g., LF or EF, or that might be hemolytic or lipemic, or contain medication used to treat the infection e.g., ciprofloxacin. The applicant therefore used the following matrices, normal naïve rabbit serum, rabbit sera with ciprofloxacin or levofloxacin, hemolytic rabbit serum, lipemic rabbit serum and serum that were obtained from rabbits that were vaccinated using rPA or AVA. Table 7 shows the results of the interferents. The test was able to identify rabbit anti-PA IgG in the presence of the interferents that were tested.

Table 8 is a summary of the results of the assay.

Table 7: Results of interferents used in the test for Specificity

Specificity Matrix	Validation Test Sample ID	Nominal Conc. ($\mu\text{g/mL}$)	Observed Conc. ($\mu\text{g/mL}$)	%RE ($\leq 30\%$)	Average %RE ($\leq 30\% \text{ RE}$)	%Rec ($100 \pm 30\%$)	Pass/Fail
Normal Naïve #1	VTS #1	71.770	68.783	4.16%	8.57%	95.84%	Pass
	VTS #7	1.794	2.039	13.68%		113.68%	Pass
	VTS #13	71.770	72.233	0.64%		100.64%	Pass
	VTS #19	1.794	1.510	15.81%		84.19%	Pass
Normal Naïve #2	VTS #2	71.770	69.797	2.75%	7.52%	97.25%	Pass
	VTS #8	1.794	1.731	3.49%		96.51%	Pass
	VTS #14	71.770	69.541	3.11%		96.89%	Pass
	VTS #20	1.794	1.422	20.72%		79.28%	Pass
Ciprofloxacin Naïve	VTS #3	71.770	68.213	4.96%	2.17%	95.04%	Pass
	VTS #9	1.794	1.791	0.15%		99.85%	Pass
	VTS #15	71.770	70.578	1.66%		98.34%	Pass
	VTS #21	1.794	1.759	1.93%		98.07%	Pass
Levofloxacin	VTS #4	71.770	67.505	5.94%	5.01%	94.06%	Pass
Naïve	VTS #10	1.794	1.760	1.91%		98.09%	Pass
	VTS #16	71.770	72.040	0.38%		100.38%	Pass
	VTS #22	1.794	1.582	11.80%		88.20%	Pass
Hemolytic Naïve	VTS #5	71.770	77.679	8.23%	3.26%	108.23%	Pass
	VTS #11	1.794	1.779	0.84%		99.16%	Pass
	VTS #17	71.770	72.140	0.52%		100.52%	Pass
	VTS #23	1.794	1.732	3.46%		96.54%	Pass
Lipemic Naïve	VTS #6	71.770	80.422	12.05%	16.07%	112.05%	Pass
	VTS #12	1.794	1.485	17.22%		82.78%	Pass
	VTS #18	71.770	61.182	14.75%		85.25%	Pass
	VTS #24	1.794	1.514	15.61%		84.39%	Pass

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Table 8: Summary of Rabbit Anti-PA IgG assay results

Parameter	Validation Criteria	Test results of Parameter ($\mu\text{g/mL}$)	Validation Decision	Comment
Limits of Detection LOD (1 $\mu\text{g/mL}$)	LOD RE \leq 30% Rec 100% \pm 30% RSD \leq 40%	RE 15.17% Rec 84.83 – 113.70% RSD 12.95% The LOD = 1 $\mu\text{g/mL}$	Acceptable	None
Limits of Quantitation LOQ (5 $\mu\text{g/mL}$)	LOQ RSD \leq 40%	Real World 5.504 -10.78 $\mu\text{g/mL}$ LOQ = 5 $\mu\text{g/mL}$	Acceptable	None
Precision	RSD \leq 20% High, Mid, Low1&2 RSD \leq 30% Low 3	RSD High 1(71.77 $\mu\text{g/mL}$) = 11.95% High 2 (35.89 $\mu\text{g/mL}$) = 10.71 % Mid 1 (17.94 $\mu\text{g/mL}$) = 11.29 % Mid 2 (8.97 $\mu\text{g/mL}$) = 11.19 % Low 1 (4.49 $\mu\text{g/mL}$) = 18.42 % Low 2 (2.94 $\mu\text{g/mL}$) = 18.2 % Low 3 (1.12 $\mu\text{g/mL}$) = 29.01 % ----- RSD (i) 11.29 -18.42 % (ii) 18.21 -29.02 %	Acceptable	None
Intermediate Precision	RSD (i) \leq 20 % for 80,20,5 $\mu\text{g/mL}$ (ii) \leq 30 for 2.5 and 0.25 $\mu\text{g/mL}$			
Repeatability	RSD (i) \leq 20 % for 80, 20, 5 $\mu\text{g/mL}$ (ii) \leq 30 for 2.5 and 1.25 $\mu\text{g/mL}$	RSD (i) 9.73% - 12.26% (ii) 12.16 -21.47%		
Specificity for detection anti-PA IgG	%RE = \leq 30% and %Rec 100% \pm 30% from the nominal concentration.	Ave RE% Hemolysis = 3.26% Ciprofloxacin2.17% Levofloxacin =5.01 % Lipemia = 16.07% Range Rec (range) = 79 % - 112 %	Acceptable	None
Accuracy - Linearity of Standard curve	RE \leq 30% Rec 100% \pm 30% RSD \leq 20 % for 80,40,20,10 $\mu\text{g/mL}$ \leq 30 for 5, 2.5 and 1.25 $\mu\text{g/mL}$ R2>0.95	RE -29.54% Rec range 70.46%-129.18% RSD 1.81-15.99% RSD 0.30 – 22.58% R2 slope 0.9902	Acceptable	

Comments

All parameters tested passed the acceptable validation criteria for the quantitation of anti-PA IgG in rabbits.

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4.4. High throughput Toxin Neutralization Assay (htp TNA)

(b) (4) X-143-06 . VP2006-152

Toxin Neutralizing Assay (TNA) was used for measurement of the neutralizing anti-lethal toxin using serum samples from rabbits, monkeys and humans. One of the protective mechanisms of the immune system against the *Bacillus anthracis* toxin, including the PA, is the production of an anti-toxin which is a neutralizing antibody. Neutralizing antibody is also formed in response to vaccination, recombinant protective antigen and Anthrax Vaccine Adsorbed (AVA). In the absence of the neutralizing antibody, PA forms a complex with LF to form lethal toxin, and enters the cell causing cell death, by down-regulating the mitogen-activated protein (MAP) kinase pathway. The *Bacillus anthracis* lethal toxin that comprises the protective antigen and lethal toxin is cytolytic, it causes apoptosis, in some macrophage cells, and this activity is the basis of the neutralizing antibody assay. Specifically, the assay looks at the ability of serum from animals that have been infected with *Bacillus anthracis*, or vaccinated with anthrax vaccine, to neutralize the cytolytic effect of the lethal toxin.

As stated above, ETI- 204 is a monoclonal antibody directed against PA which it binds, and competitively prevents the binding of the LF and EF, critical components for the establishment of anthrax disease. This action prevents the endocytosis of these toxins into susceptible host cells. Cell based toxin neutralization assay was performed to detect neutralizing antibodies to lethal toxin of *Bacillus anthracis* in human, monkey and rabbit sera. As noted earlier, it is unethical to test humans with *Bacillus anthracis* (Animal Rule). Therefore, human efficacy is based on a comparison between the TNA titer that protects animals, and the TNA titer obtained when humans are vaccinated against anthrax.

A mouse cell line, J774.A 1, (BEI Resources of ATCC), that comprises macrophage/monocyte - like cells, are susceptible to the lethal toxin of *Bacillus anthracis*. The assay is performed using a monolayer of J774.A 1 cells incubated with a specific quantity of lethal toxin (LeTx), prepared by mixing equal concentration of rPA and rLF (BEI Resources of ATCC), in the presence or absence of ETI-204, and rabbit, monkey, or human sera or plasma containing anti-PA antibodies. The cells are then washed and tested for mitochondrial activity, which is a reflection of viability, metabolism, and proliferation of the cells. The activity is measured colorimetrically using an indicator, (MTT) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma Chemical Company), a water soluble tetrazolium salt. The activity is indicated by the enzyme mitochondrial reductase present in living cells which reduces the yellow colored MTT to a purple formazan which can be read at a wavelength between 500 to 600 nm (Figure 1). Dead cells, in this case those that are killed by the lethal toxin, will not reduce MTT and so the assay medium will remain yellow. The variation in color will be proportional to the amount of MTT reduced, which in turn, is proportional to the number of live cells.

The endpoint of the assay in this study, was primarily the Effective Dilution 50 (ED50), and secondarily the Neutralization Factor 50 (NF50), the dilution of the sample that reduces 50% of the toxic factor of the amount of purified antibody neutralizing 50% of the cyto-toxicity in the cell. All testing was done at (b) (4) laboratories.

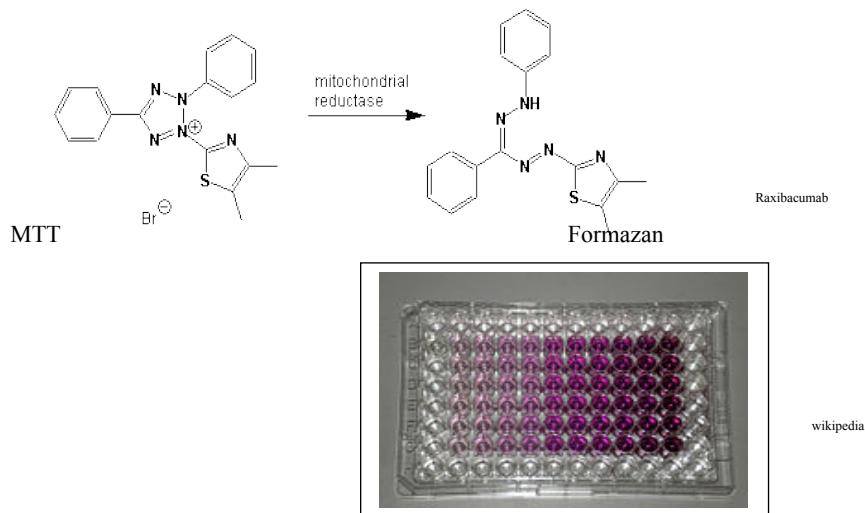
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Figure 1. Mechanism of the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide



J774.A 1 cells, obtained from the American Type Culture Collection and harvested from passages 4 to 11, were incubated in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with fresh glutamine, 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ and 85% humidity. The flat bottomed microtiter plates were seeded with 100µL of approximately 4 X10⁵ J774.A.1 cells/mL. The confluence of cells at the time of testing must be approximately 40 to 70 % of the original inoculum (Cell Plate).

Recombinant PA (rPA) and recombinant LF (rLF) stock were prepared in growth medium to a concentration of 0.1 mg/mL. Lethal toxin (LeTx) was prepared by combining appropriate concentrations of rPA and rLF stock in growth medium.

Diluted test sera, controls and standards were added to wells and serially diluted. Lethal toxin was added to some wells and growth media to other wells including some of those with LeTx. All plates were placed on a plate shaker for 15 seconds at 620 rpm then incubated at 37°C for 30 minutes (Neutralization step).

The intoxication step occurred by the addition of diluted sera or controls to wells in the same rows that corresponded to rows in the cell plate and incubated at approximately 37°C in CO₂ for 4 hours.

After incubation, MTT (viability indicator) was added to each cell-LeTx mixture and the plates re-incubated at 37°C in CO₂ for an additional two hours. This was followed by the addition of a stop buffer, then the assay re-incubated overnight at 37°C in CO₂. Solubilization buffer was added to the wells causing the cells to lyse, release, and dissolve the cellular granules. After 16 – 20 hours of incubation at 37°C for 16 to 20 hours, the plates were read on a microplate reader at a wavelength of A₅₇₀ - A₆₉₀. Figure 2 shows the general layout of the plates and figure 3 shows

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the parameters for which the plates were used. The optical densities were read and exported to a SAS program.

Figure 2 : Plate format used for testing

	1	2	3	4	5	6	7	8	9	10	11	12**
A	RS1-1	TS1-1	TS2-1	TS3-1	TS4-1	RS2-1	TSS-1	TS6-1	TS7-1	TS8-1	TS9-1	QC-1
B	RS1-2	TS1-2	TS2-2	TS3-2	TS4-2	RS2-2	TSS-2	TS6-2	TS7-2	TS8-2	TS9-2	QC-2
C	RS1-3	TS1-3	TS2-3	TS3-3	TS4-3	RS2-3	TSS-3	TS6-3	TS7-3	TS8-3	TS9-3	QC-3
D	RS1-4	TS1-4	TS2-4	TS3-4	TS4-4	RS2-4	TSS-4	TS6-4	TS7-4	TS8-4	TS9-4	QC-4
E	RS1-5	TS1-5	TS2-5	TS3-5	TS4-5	RS2-5	TSS-5	TS6-5	TS7-5	TS8-5	TS9-5	QC-5
F	RS1-6	TS1-6	TS2-6	TS3-6	TS4-6	RS2-6	TSS-6	TS6-6	TS7-6	TS8-6	TS9-6	QC-6
G	RS1-7	TS1-7	TS2-7	TS3-7	TS4-7	RS2-7	TSS-7	TS6-7	TS7-7	TS8-7	TS9-7	QC-7
H	NC	TS1*	TS2*	TS3*	TS4*	NC	TS5*	TS6*	TS7*	TS8*	TS9*	QC*

RS = Reference Serum sample

QC = Quality Control sample

TS = Test sample

NC = Negative Control sample

*Serum Control Wells; No Lethal Toxin is added to these cells.

**For plates 11 and 12, this column contained TS10 in lieu of a QC sample.

(b) (4) VP2006-138

Figure 3: Utility of plates and schedule of testing.

TNA Day	Repeatability	Intermediate Precision	Dilutional Linearity	LOD/LOQ	Specificity	Test Operators	Lab
Run 1	Plate 1	Plates 2-3		Plates 8-10	Plates 11-12	A and B	(b) (4)
Run 2			Plates 4-7	Plates 8-10	Plate 11	A and C	
Run 3		Plates 2-3		Plates 8-10	Plates 11-12	A and D	
Run 4			Plates 4-7	Plates 8-10	Plate 12	B and C	
Run 5		Plates 2-3	Plates 4-7	Plate 8		B and D	
Run 6		Plates 2-3	Plates 4-7		Plates 11-12	C and D	
Run 7			Plates 13 - 16			A and B C and D	

(b) (4) VP2006-138

Comment:

The placement of samples in the microtiter plates is important since the environment is likely to affect the samples around the periphery to a greater extent than it will affect the samples in the internal wells. This is particularly important in this TNA assay, because of the long incubation periods. Additionally, in this assay, reagents from wells have to be transferred to matching wells in a corresponding plate therefore placing is critical for accuracy.

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Acceptance Criteria

In this study the ED₅₀ is defined as the reciprocal of the dilution of the sample that neutralizes 50% of the toxic effect of PA. Validation was done in 16 runs on 7 consecutive days. Statistical models were used to assist in the computation of data.

Repeatability:

- The difference between the highest positive value column and the lowest negative value column must be <50% in the 95% quantile of the percent relative maximum fold of bias (PRMFB_{0.95}).
- The total error must be < 50%
- Percent relative total precision (PRTP) versus ED₅₀ for pooled samples, combined pooled + real world samples, and specificity samples must be <50%

Intermediate Precision:

- RSD for ED₅₀ & NF₅₀ - must be <0.5
- Precision profile standard deviation (PRSD) takes into account repeatability and intermediate precision versus ED₅₀ must be <50%

Dilutional Linearity 7 days, 4 operators.

- 75% of slope must lie between 0.67 and 1.50
- LLOQ bias must not be >50%
- ED₅₀ has an %RSD not greater than 50%
- LLOQ ED₅₀ must be <100

Specificity:

- Comparison between normal serum diluent and hemolyzed serum or normal plasma diluent: 95% confidence interval of ED₅₀ must lie between 0.67 to 1.50
- No more than 50% change in performance between normal and test matrix.

Sensitivity:

ED₅₀ LOD must be less than 50

Calibration Standard/controls:

- Reference Standard (RS) – optical density (OD) values must fall between 0.10 to 2.8. Must generate a dilution dependent OD response when LT is added.
- Lowest dilution of reference serum must be ≥ 0.85
- Highest dilution of RS must be within 0.25 of the mean OD of the negative control. %CV of NC must be $\leq 20\%$
- Coefficient of Variation for each dilution must be $\leq 20\%$. It is allowed that one dilution could be $> 20\%$ but $\leq 30\%$
- Difference between the first and last dilution must be OD >0.55
- Negative Control = OD must be ≤ 0.45

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Samples:

- Validation Test Samples (VTS) were prepared by spiking samples positive for anti-LT activity into pooled normal naïve NHP serum or plasma. ‘Real world’ samples were those from NHP that were immunized against *Bacillus anthracis*, and found to have an appropriate level of neutralizing antibodies.
- Special matrices were used in order to simulate the type of matrices that may be encountered in testing animal specimens. These were in addition to normal naïve serum, the following sera were obtained - levofloxacin naïve, hemolytic naïve, hyper-lipemic naïve serum, and plasma obtained by the addition of EDTA.
- In general, the positive samples were added to pools of negative naïve samples.
- The test sample must have an R^2 value ≥ 0.89

4.4.1. Validation of the (b) (4) High Throughput Toxin Neutralization Assay in Monkeys

Monkey VP2006-152, SOP were (b) (4) X-143-06

This study was performed subsequent to the rabbit TNA study, and the protocol is the same as that for the rabbit study. The monkey review is placed before that of the rabbit to allow for consistency in placement in the overall review.

The validation tests were performed at (b) (4)

(b) (4). The documents reviewed were SOP were (b) (4) X-143-06 and VP2006-152

Monkey VP2006-152 constitutes a partial evaluation of the activity in Non Human Primates and assesses the validation of intermediate precision, reproducibility, dilutional linearity, specificity, limit of quantitation and detection.

Samples

Positive monkey samples were added to pooled normal naïve samples from Rhesus and Cynomolgus monkeys. Two hundred and fifty-one samples were used in this validation exercise.

Comparison of results between rooms was based on predicted ED_{50} and NF_{50} values originating from the laboratories (b) (4). In general the ED_{50} values were higher in room (b) (4) by 50%, but the NF_{50} values tended to be only 9% higher in (b) (4)

Initially, it was planned to compare two reportable results from each of 226 validation test samples from each laboratory. However, only 79 of these were usable because either results of some tests were zero, or sample results were missing. Therefore, the results of the useable samples were averaged and then compared.

The analysis examined means, trends and outliers. Figures 1A and 1B show plots of the results. In figure 1A the ED_{50} values tended to be greater than 1 whereas in Figure 1B, the values were spread more evenly around 1. This indicated that the general trend in the patterns of results in the

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two rooms were similarly distributed because they were both centered around 1. There was one very low outlier which came from Room [REDACTED] (b) (4).

Figure1A: Ratio of Geometric Mean ED50s ((b) (4)) vs. Geometric Mean ED50 Across Rooms

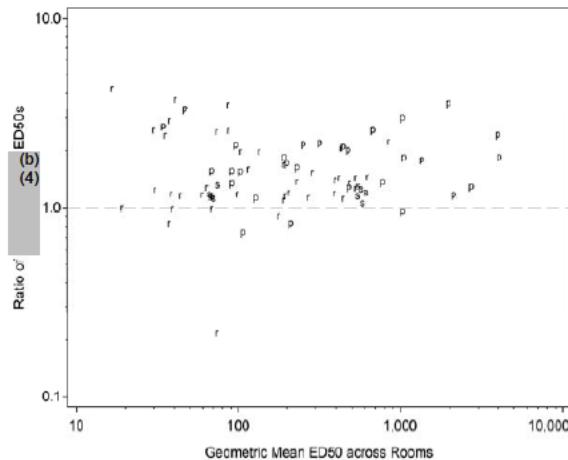
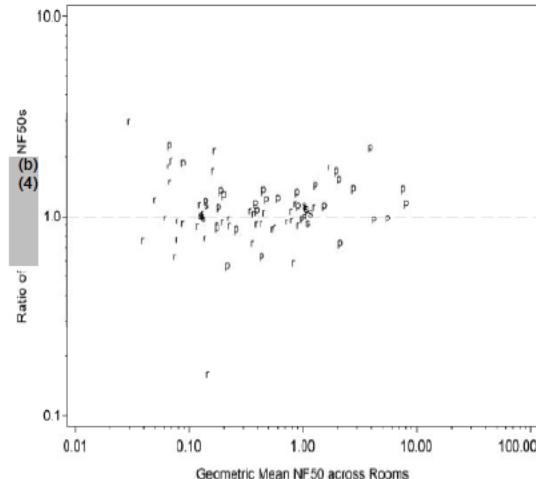


Figure1B: Ratio of Geometric Mean NF50s ((b) (4)) vs. Geometric Mean NF50 Across Rooms



(b) (4) VP2006-152

The results of ED_{50} and NF_{50} were used to make a comparison of the level of work done within the separate laboratories. The relative standard deviation was used to determine the 'within' laboratory (intra-laboratory) variability of ED_{50} and NF_{50} . The intra-laboratory standard deviations for ED_{50} and NF_{50} were 0.17 and 0.16, respectively. Acceptance for 'between' laboratory (inter-laboratory) variability was set at a range of 0.67 to 1.5. The inter-laboratory comparison of ED_{50} failed but the NF_{50} passed.

4.4.1.3. Sensitivity

The limits of detection (LOD) for ED_{50} and NF_{50} were determined by using only samples with a starting dilution of 1:50, and used the naïve monkey serum as the diluent. Only values above zero were included in the calculations, no allowance was made for non-zero values. Because of the omission of zero values, the LOD would be greater than it would be if zeros had been included. Using logistic regression analysis with at least 95% probability, the LOD for ED_{50} was 36.6 and that for NF_{50} was 0.074. Table 1 shows the estimated limit of detection and the 95% confidence interval. Figures 2 and 3 depict the LODs.

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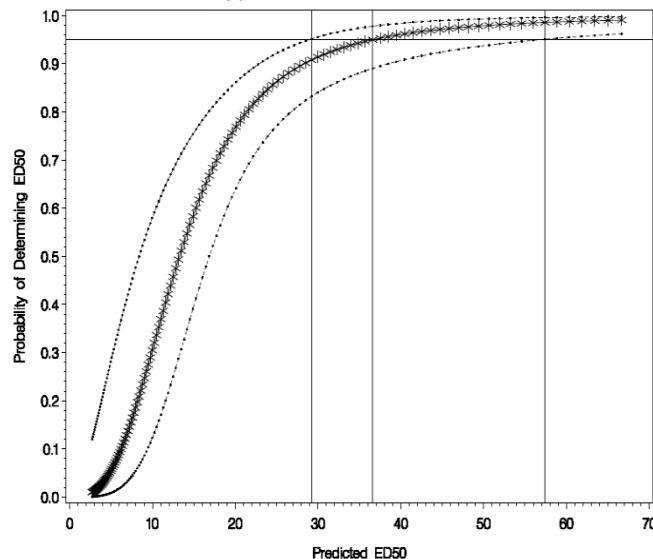
Table 1: Estimated Limits of Detection (LOD) and Lower and Upper 95% Confidence Interval Bounds by ED₅₀ and NF₅₀

Endpoint	Estimated LOD	Lower 95% Confidence Bound	Upper 95% Confidence Bound
ED ₅₀	36.6	28.9	56.9
NF ₅₀	0.074	0.058	0.117

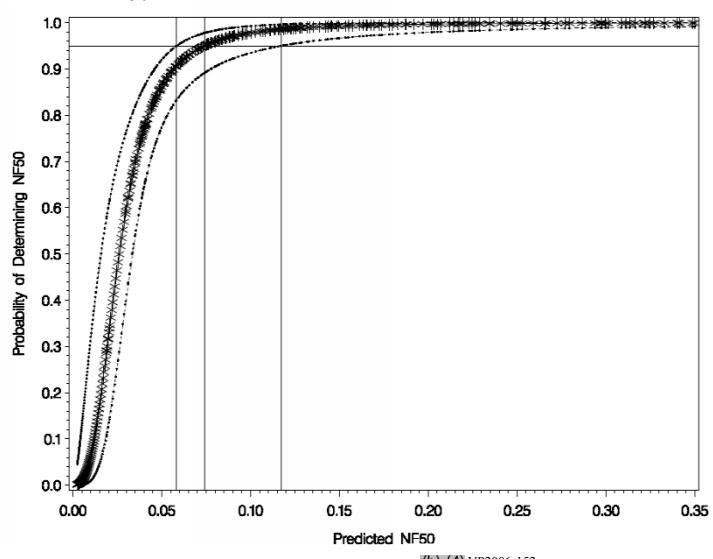
(b) (4) VP-2006-152

Figure 2: Estimated Limits of Detection ED₅₀ and NF₅₀ in Monkey Toxin Neutralization Assays

Estimated LOD and Lower and Upper 95% Confidence Interval Bounds - ED₅₀



Estimated LOD and Lower and Upper 95% Confidence Interval Bounds. NF₅₀



(b) (4) VP2006-152

4.4.1.4. Accuracy

Dilutional Linearity: to determine dilutional linearity spiked and unspiked samples were tested. Four positive samples were used to spike negative, naive serum. The unspiked samples were tested at a starting ED₅₀ and NF₅₀ dilution from 1:50 up to a final 1:1600. For the spiked level, the first sample was undiluted with subsequent two-fold dilutions to 1:64. The acceptance criterion for ED₅₀ was that the ratios must fall between 0.67 and 1.50. There were no performance criteria for NF₅₀. Tables 2, 3 and 4 show the ED₅₀ and NF₅₀ values. For ED₅₀, both the starting and up to 1:800 dilution, and spiked level dilutions up to 1:32 were acceptable. For NF₅₀ the starting dilution was acceptable but the spiked level dilution was not. The applicant stated that real measurements were available up to the starting dilution of 1:400, and that starting dilutions 1:800 and 1:1,600 were extrapolated from the previous dilutions up to, and including 1:400. The dilutional linearity for starting samples is acceptable up to 1:400.

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The LLOQ was defined as the point at which the values forming a constant width begin to expand. The requirement for validation is that the ED₅₀ LLOQ is <100. The NF₅₀ must be < 0.152. The ED₅₀ LLOQ was estimated to be in the range from 55 to 70. The LLOQ was reported as 55. The acceptability criterion for NF₅₀ was stipulated to be between 0.105 and 0.135, and was reported to be 0.105.

Table 2: Relative Accuracy via Dilutional Linearity at the Upper Starting Dilution and the Upper Spike Level

Endpoint	Factor	Value	Relative Accuracy
ED ₅₀	Upper Starting Dilution (a)	50	1.00
		100	1.09
		200	1.19
		400 ¹	1.30
		800	1.41
		1600	1.54
NF ₅₀	Upper Starting Dilution (b)	50	1.00
		100	1.09
		200	1.19
		400 ¹	1.30
		800	1.42
		1600	1.55
ED ₅₀	Spike Level (c)	1	1.00
		2	1.09
		4	1.18
		8	1.28
		16	1.39
		32	1.50
		64 ²	1.63
		128	1.77
		256	1.92
		512	2.08
		1	1.00
		2	1.04
NF ₅₀	Spike Level (d)	4	1.09
		8	1.14
		16	1.19
		32	1.24
		64 ²	1.29
		128	1.35
		256	1.41
		512	1.47

(a) Log_eED₅₀ log starting dilution effect regression slope = 0.1246.

(b) Log_eNF₅₀ log starting dilution effect regression slope = 0.1265.

(c) Log_eED₅₀ log spike level effect regression slope = 0.1177.

(d) Log_eNF₅₀ log spike level effect regression slope = 0.0617.

¹ Extrapolation beyond starting dilution 400 is considered extrapolation beyond the range of the data.

² Extrapolation beyond spike dilution 64 is considered extrapolation beyond the range of the data.

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Table 3: Section of table showing Means and Standard Deviations of Seven-Point Moving Averages Within Bins of Absolute Values of Residuals from Analysis of Variance Fit to Log ED₅₀s

Bin No.	No. of Moving Averages within Bin	Mean of Moving Averages Within Bin	S. D. of Moving Averages Within Bin	Range of Bin, Log Scale		Range of Bin, ED ₅₀ Scale	
				Minimum (≥)	Maximum (≤)	Minimum (≥)	Maximum (≤)
1	5	0.28	0.031	2.75	3.00	16	20
2	16	0.28	0.095	3.00	3.25	20	26
3	25	0.31	0.141	3.25	3.50	26	33
4	16	0.22	0.021	3.50	3.75	33	43
5	24	0.28	0.090	3.75	4.00	43	55
6	32	0.18	0.095	4.00	4.25	55	70
7	25	0.19	0.051	4.25	4.50	70	90

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Table 4: Section of table showing Means and Standard Deviations of Seven-Point Moving Averages Within Bins of Absolute Values of Residuals from Analysis of Variance Fit to Log NF₅₀s

Bin No.	No. of Moving Averages within Bin	Mean of Moving Averages Within Bin	S. D. of Moving Averages Within Bin	Range of Bin, Log Scale		Range of Bin, NF ₅₀ Scale	
				Minimum (≥)	Maximum (≤)	Minimum (≥)	Maximum (≤)
1	3	0.27	0.008	-3.50	-3.25	0.030	0.039
2	24	0.37	0.112	-3.25	-3.00	0.039	0.050
3	16	0.20	0.057	-3.00	-2.75	0.050	0.064
4	24	0.21	0.078	-2.75	-2.50	0.064	0.082
5	20	0.27	0.054	-2.50	-2.25	0.082	0.105
6	34	0.16	0.084	-2.25	-2.00	0.105	0.135
7	19	0.21	0.058	-2.00	-1.75	0.135	0.174

4.4.1.5. Precision

The assessment of intermediate precision was based on the repeat values of the relative standard deviation in each room. The data used were pooled from both rooms.

Two hundred and twenty-six samples were used to determine the intermediate precision. The standard used to define precision was ED₅₀. The acceptance criterion for ED₅₀ was recommended to be a relative standard deviation (RSD) of <0.5. The resulting ED₅₀ and NF₅₀ RSD within the two rooms were 0.24 and 0.19, respectively. This was acceptable.

4.4.1.6. Specificity

Positive neutralizing antibody sera from nine cynomolgus monkeys were spiked into three normal naïve samples to assess the specificity of the assay. The negative matrices were normal naïve serum, severely hemolyzed naïve serum, and normal naïve plasma. There were 30 normal naïve samples (10 rhesus and 20 cynomolgus monkey samples). After mixing with the negative matrices, the original values of each positive serum were confirmed. The acceptance criterion for ED₅₀ values stated that they must range between 0.67 and 1.5 at a 95% CI.

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The test found that the values obtained in the specificity parameter were much lower than that for the original positive values. Therefore the criterion failed; this means, that hemolysis and plasma would affect the TNA results.

Table 5 shows these results. The significance level for ED₅₀ ranged from 0.017 to 0.050, and for NF₅₀ 0.008 to 0.020 respectively, for hemolyzed serum and plasma, respectively. Table 6 is a summary of the results of the Toxin Neutralization assay.

Table 5: Summary of Specificity results using spiked positive monkey serum

Endpoint	Diluent Compared to NHP Normal Naïve Serum	Ratio (Diluent/ NHP Normal Naïve Serum)	Significance Level	95% Lower Confidence Bound	95% Upper Confidence Bound
ED ₅₀	Hemolyzed naïve serum	0.76	0.017	0.61	0.95
NF ₅₀	Hemolyzed naïve serum	0.91	0.008	0.84	0.97
ED ₅₀	Naïve plasma	0.77	0.050	0.60	1.00
NF ₅₀	Naïve plasma	0.92	0.020	0.85	0.99

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Table 6 : Summary of Validation Parameters of Monkey Toxin Neutralization Assay

Parameter	Validation Criteria	Test results of Parameter	Validation Decision	Comment
Limits of Detection	LOD < 50	LOD = 36.6	Pass	None
Limits of Quantitation	LLOQ = <100	LLOQ = 55	Pass	None
Precision			Pass	None
Intermediate Precision	RSD-ED50 & NF50 = 0.5	RSD ED50 = 0.24 NF50 = 0.19		
Repeatability	75% of slope must lie between 0.67 and 1.50	100% of starting dilution slope with mean of 1.14		
Specificity	95% confidence interval of ED50 between normal and hemolyzed serum & plasma must range 0.67 to 1.50	Hemolyzed serum : normal serum = 0.76 (p = 0.017) Plasma : normal serum = 0.77 (p = 0.05)	Pass	None
Accuracy (Relative)	Upper starting dilution (usd) of 1:50 & 1.0 = 0.67 to 1.50	Usd 1:800 relative accuracy = 1.41	Pass	For starting dilutions above 1:400 the results were extrapolated 1:400 = 1:32
Dilutional linearity of assay range	Upper spike Level (usl) of 1 = 0.67 to 1.50	Usl = 32 relative Accuracy = 1:50		Spike levels above 64 were extrapolated

Comment

Statistical models were widely used to assist in the computation of the results in this assay. The primary endpoint for this assay was ED₅₀ and the secondary was NF₅₀. The NF₅₀ value was calculated based on the value of ED₅₀ (NF₅₀ = ED_{50 test}/ED_{50 std}). The value that was used in all parameters of this validation study was ED₅₀. NF₅₀ was used to a lesser extent and was used only in conjunction with ED₅₀. The assay was validated.

This assay cannot be used as one of the primary assays for diagnosis, because neutralizing antibodies will be detected only subsequent to the production of the PA toxin.

Even though the level of hemolysis has been more descriptive in the TNA than in other assays, in this assay the hemolysis was described as severe, the need for more accurate and measurable amounts of hemolysis still exists.

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4.4.2. Validation of the [REDACTED]^{(b) (4)} High Throughput Toxin Neutralization Assay in Rabbits

VP2006-138

4.4.2.1. Plates

The set up and placement of samples in this assay are important to test accuracy. The criteria for acceptability stated that 75% of the plates must pass the acceptance criteria, and that for the plates that are acceptable at least 75% must pass the test sample acceptance criteria. Table 1 shows the results of acceptability of plates and samples by 4 operators conducting TNA tests. A total of 154 plates were used in this assay, of these, 116 passed the acceptance criteria (75.3%). This falls just within the limit of plate acceptability. The sample acceptable rate was 99.6%.

Comment:

Overall, the plate parameter passed the acceptance criteria. However, approximately one-quarter of the plates failed. This failure rate is very high.

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Table 1: Validation Run History and Summary of Sample and Plate Failure Rates

run	analyst	try	mmdd	passed sample counts per plate layout												failures			passes									
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	r	p	s	r	p	s			
(b) (4)	1	1	0918	9	9	9	.	.	.	9	9	8	10	10	1	1	8	73					
	1	2	1018	-	-	-	.	.	.	-	-	1	-	-		1	1	1					
1	1	1	0918	9	9	9	.	.	.	9	8	9	10	10	1	1	8	73					
	1	2	1025	-	-	-	.	.	.	-	1	-	-	-		1	1	1					
2	1	1	0919	.	.	0	0	0	0	0	0	0	0	0	†								
	2	2	1004	.	.	9	8	9	9	*8	9	9	10	1	1	8	71					
2	3	1	1023	.	.	-	1	-	-	-	-	-	-	-		1	1	1					
	2	1	0919	.	.	9	9	9	9	9	9	9	10	1	8	73						
2	2	2	1011	.	.	‡	-	-	-	-	-	-	-	-		‡							
3	1	1	0920	0	0	0	.	.	.	0	0	0	0	0	†								
	3	2	1004	9	9	9	.	.	.	*8	9	9	10	10		1	8	73					
3	1	1	0920	0	0	0	.	.	.	0	0	0	0	0	†								
	3	2	1004	9	9	9	.	.	.	*8	9	9	10	10		1	8	73					
4	1	1	0925	.	.	0	0	0	0	0	0	0	0	0	.	0	.	.	.	1	8							
	4	2	1009	.	.	0	-	0	0	0	0	0	0	0	1	7							
4	3	1	1018	.	.	9	9	9	9	*8	9	9	.	10		1	8	72					
	4	1	0925	.	.	9	0	0	0	0	0	0	0	0	.	0	.	.	.	1	7	1	9					
4	2	2	1010	.	.	-	9	9	9	*8	9	9	.	10	1	7	63						
5	1	1	0926	9	0	9	9	9	9	9	9	1	1	7	63					
	5	2	1017	-	9	-	-	-	-	-	-	-	-	-		1		9					
5	1	1	0926	9	0	0	0	0	0	0	9	6	1	2	18					
	5	2	1010	-	9	9	9	9	8	0	-	1	1	1	5	44				
5	3	1	1017	-	-	-	-	-	-	9	-		1	9						
	5	3	1023	-	-	-	-	-	-	1	-		1	1						
6	1	1	0927	.	9	9	9	9	9	9	0	.	.	.	10	10	.	.	.	1	1	7	65					
	6	2	1030	.	-	-	-	-	-	9	.	-	-	-		1	1	9					
6	1	1	0927	.	9	9	9	9	9	9	.	.	.	10	10	.	.	.		1	1	8	74					
	7	1	1002	0	0	0	9	1	3	1	9						
7	2	1016	9	9	*8	-			1	3	26						
	7	1	1003	9	9	9	9			1	4	36						
7	1	1002	0	0	0	0	1	4	1	4	35						
	7	2	1016	9	9	*8	9			1	4	36						
7	1	1003	9	9	9	9			1	4	36						
sample failure rate:				$4/(4+1017) = 0.4\%$												total samples for all runs:			4	1017								
plate failure rate:				$38/(38+116) = 24.7\%$												total plates for all runs:			38	116								
plate failure rate for passed runs:				$9/(9+106) = 7.8\%$												total plates for passed runs:			9	106								
run failure rate:				$5/(5+17) = 22.7\%$												total runs:			5	17								

* depleted sample

. layout not included in the study design

- already passed plate and not rerun

† deviation from SOP

‡ inadvertently rerun

(b) (4) VP2006-138

Comparison of the performance in the two laboratories was the first step in the evaluation process. This enabled the researcher to look at the similarities in the results of the two laboratories.

The results that originated from each room were similar in variability in ED₅₀ and NF₅₀. As with the monkey assay, test results for ED₅₀ in both rooms tended to trend in the vicinity of 1 but NF₅₀ appeared to be more evenly distributed around 1. The scatterplot for this parameter in the rabbit study is similar to 1A and 1B for the ED₅₀ and NF₅₀ plots in the monkey assay. The plots show a tighter grouping of NF₅₀ than the results of ED₅₀ did.

The applicant stated that in studies with rabbits and humans, the acceptance criteria for descriptive analyses indicated that 75% of the ratios should fall between 0.67 and 1.50. Table 2 shows the ranges in which the ED₅₀ and NF₅₀ fall in the two rooms. ED₅₀ did not meet the acceptance criteria but the plots suggest that the values obtained in the two laboratories were similar.

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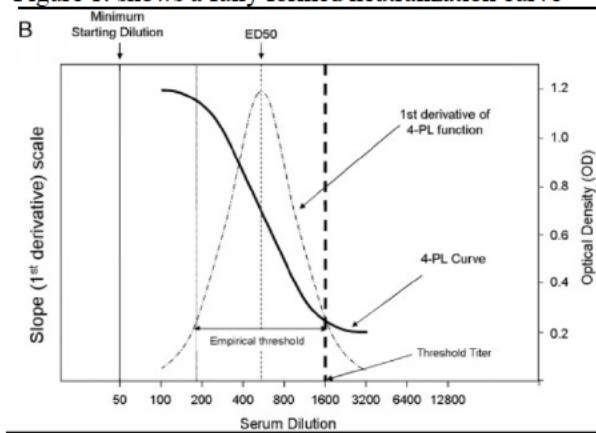
Table 2: Ranges of the results of ED₅₀ and NF₅₀ as compared in the two rooms where tests were performed

Numbers and Percentages of Ratios Falling into Range Categories			
Endpoint	Range of JM3-71/JM3-47 Ratios	No. of VTS Samples	Percent of VTS Samples
ED ₅₀	<0.67	1	1.3
	0.67-1.5	43	54.4
	>1.5	35	44.3
NF ₅₀	<0.67	5	6.3
	0.67-1.5	63	79.8
	>1.5	11	13.9

(b) (4) VP2006-138

Distinctive curves, peculiar to the analysis of data from TNA assay, were used to describe neutralization activity of samples. Samples that were positive were described as reactive, and samples with no neutralizing activity were described as non-reactive. Positive samples form a curve in which the beginning and end of the curve fall within the threshold titer, and usually assume a sigmoidal shape (Figures 1, 2A). The threshold titer is the highest serum dilution that corresponds to the acceptable range of dilutional linearity, represented by first dilution of a sample. A partially formed curve has one point in the lower end in the threshold limit, while the upper end of the curve is located outside the upper range (Figure 2B and 2C). A negative curve is a flat line (Figure 2D). Figure 2 shows a normal curve of neutralizing activity with both start and end points within the threshold titer.

Figure 1: shows a fully formed neutralization curve⁵



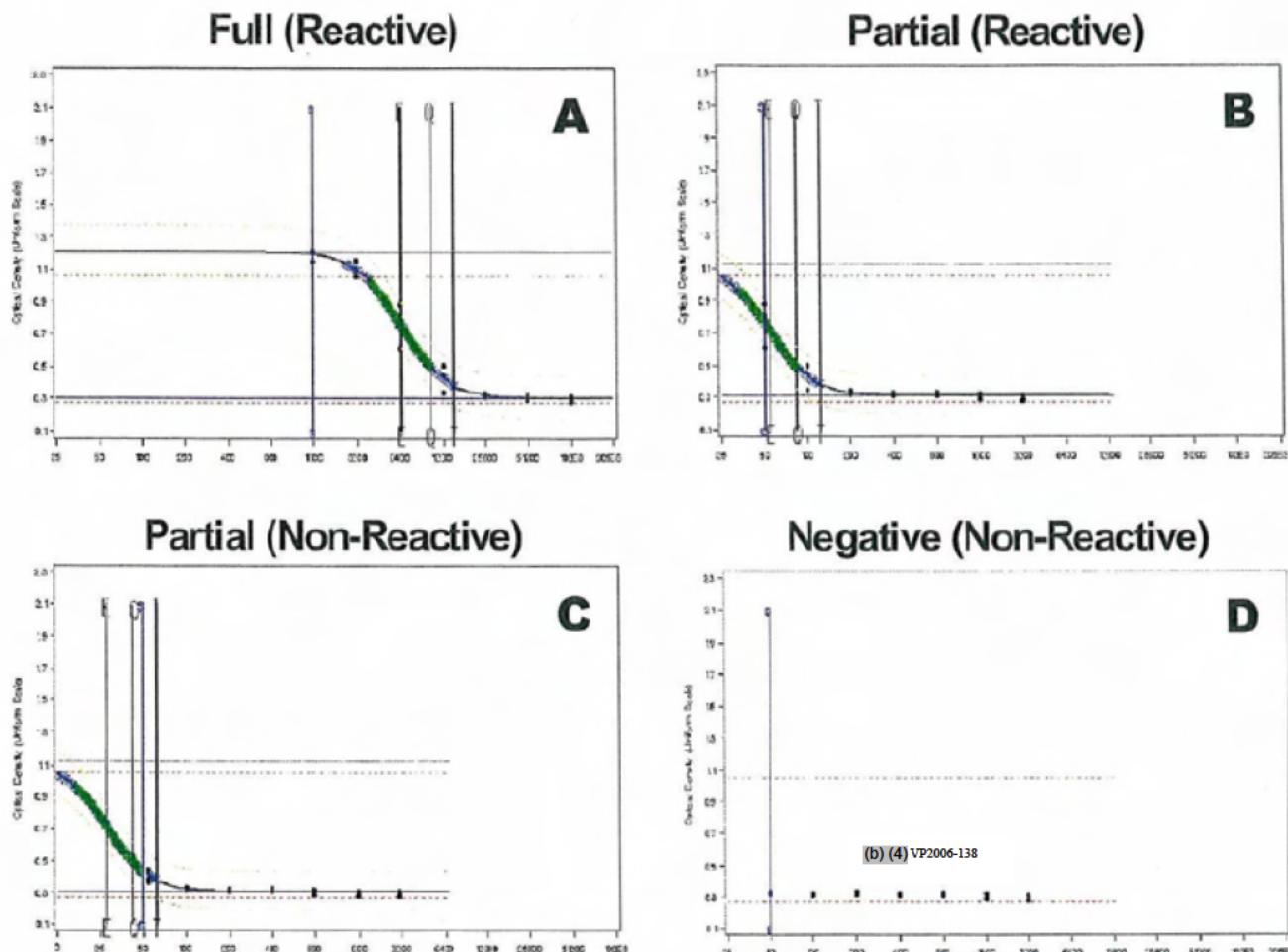
⁵ Li et al., Standardized, mathematical model-based and validated in vitro analysis of anthrax lethal toxin neutralization, Journal of Immunological Methods 333

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Figure 2: Curve Patterns and their Associated Reactivity Thresholds



4.4.2.2. Sensitivity

The LOD is the lowest ED₅₀ for which an assay has a 95% chance to detect a non-zero value. The acceptable criterion was that the LOD must be less than 50. Pooled sera, specimens from infected animals, and specimens with low ED₅₀ were tested in different plate layouts to determine the LOD. Regression analysis of the results was applied in order to determine the LOD. Figure 3 shows the curve that established the Limit of Detection. The LOD was determined to be 23.

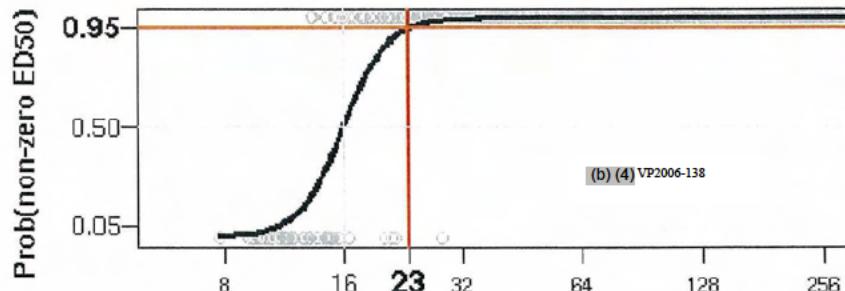
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Figure 3: Curve that demonstrates the Limit of Detection



4.4.2.3. Accuracy

Dilutional linearity

Six positive sera were used to test for accuracy. All were from animals that had been vaccinated with rPA or AVA. The source of vaccination and the PA titers are shown in Table 3. The preparation of spiked sera is shown in Table 3; it shows the dilution of the positive sera that was added to negative naïve sera, the expected ED_{50} , and the starting dilution.

The parameter also tested the range over which the assay had dilutional linearity. Different ED_{50} concentrations, each made separately from different positive serum lots, were spiked in the same lot of negative naïve serum and tested in 12 plates. Three plates tested samples from infected animals.

Table 3: Characteristics of the positive sera used for specificity and accuracy

Positive Serum ID	Unique Serum Lot Number	Vaccine Type	Mean ED_{50} Titer ¹
A	(b) (4) 01	rPA	1090
B	02	rPA	3667
C	09	rPA	5268
D	019	rPA	255
E	020	AVA	985
F	026	rPA	99

¹Geometric Mean ED_{50} titer determined during assay development.

(b) (4) VP2006-138

Dilutional linearity was tested using samples which were first diluted 1:50. It was found that for specimens tested only once, 80% of the samples initially diluted 1:50 were accurate up to a 16-fold dilution. Linearity for this dilution, 1:50, began at 39 and continued to the level of 624 (39x16). The range of the slope in which dilutional linearity existed was 39 to 624. For samples with higher dilutions 1:100 to 1:600, it was found that dilutional linearity would be maintained up to 5-fold. The best ranges for possible dilutions are found in Table 4.

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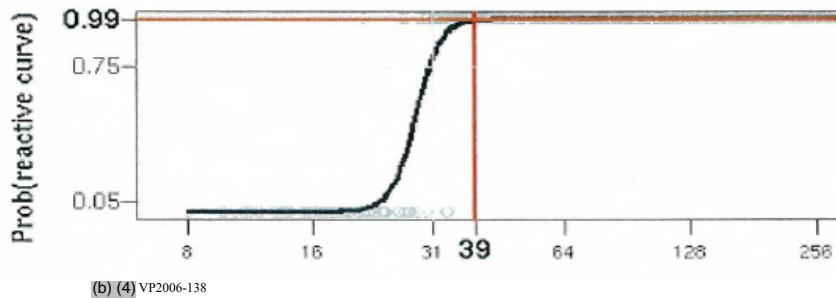
Table 4: Range of Dilutional Linearity for Samples with Specific Preliminary Dilutions

preliminary dilution	analytic range		fold	sample range		PRTE _{0.90}
	lower	upper		lower	upper	
1:50	39	624	16	39	624	49.7
1:100	200	1000	5	400	2000	38.0
1:200	200	1000	5	800	4000	42.3
1:300	200	1000	5	1200	6000	45.0
1:400	200	1000	5	1600	8000	47.0
1:600	200	1000	5	2400	12000	49.9
1:1200	200	1000	5	4800	24000	55.2
NR 1:50	39	624	16	39	624	57.9

(b) (4) VP2006-138

The LLOQ was determined using pooled, infected animal samples, and specimens used to determine ED₅₀ values. Analysis using the data obtained from the dilutional linearity determined that the LLOQ for specimens tested only one time should be 39 as in the lower range of the 1:50 dilution, and the ULOQ was 12,000 as in the highest value in 1:600 dilution. For samples tested twice however, the LLOQ was 35 and the ULOQ 24,000. The 1:1200 dilution was not included in the calculation, even though the total error fell within the acceptable limit. Figure 4 shows the LLOQ curve.

Figure 4: Curve depicting Lower Limit of Quantitation



(b) (4) VP2006-138

4.4.2.4. Precision

The researchers tested precision at three levels.

Repeatability: The ability to obtain the same result on a sample that is run more than once is a measure of repeatability. Within run precision assessed the values of the same sample within the same run. The purpose of testing repeatability was to ensure that there would be no discrepancy. The specimen used was diluted eight-fold 1:2, 1:16 and 1:128 and tested according to 3 plate outlays. In one outlay, each sample was used in the same plate 3 times in 3 different locations. In other outlay, each sample appeared once per plate in specific locations. A two-stage model was used to measure this parameter. The first stage was adjusted for effect due to sample variance, day, and operator. The second stage looked at the maximum difference between the columns in which the sample was placed. In the plate where three samples from the same

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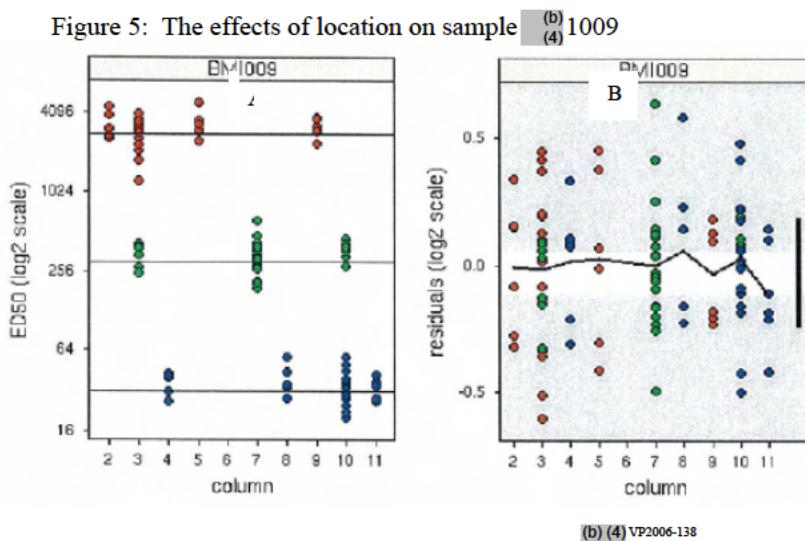
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specimens were placed in three different locations, the percent relative standard deviation (PRSD) was 20% . The validation criterion outlined above states that PRSD must be <50%. The percent relative maximum fold bias (PRMFB_{0.95}) which defined the difference between the highest positive value column and the lowest negative value column must also be <50%. Using the values in columns 8 and 11 the PRMFB_{0.95} was 33.7 %. Figure 5 shows the results. The different colors represent different dilutions of BM1009 samples (red= 1:2, green = 1:16, blue =1:128). The lines in Figure A represent the mean ED₅₀

Intermediate Precision: Assessed the precision between days, operators and equipment.

Intermediate precision was assessed as part of the determination of the total error for dilutional linearity. The total relative error was set up to be < 50% for all initial dilutions. It follows that the intermediate precision would also be <50% the acceptance criterion. The intermediate precision was calculated to be either <27 or <28 depending on the layout of the plate in which the sample was tested.

Reproducibility: For this study the applicant stated that the difference between equipment was representative of activity between laboratories. Therefore reproducibility was tested as intermediate precision. That was acceptable.



4.4.2.5. Specificity

This was the parameter used to assess whether the assay is affected by elements found in the serum samples that were tested by TNA for infection by *Bacillus anthracis*. Work performed prior to this validation study identified the lack of non-specific activity in the normal naïve rabbit serum. The matrices used in the former assay assessed the level of anti-LT activity. The matrices used in this validation assay were levofloxacin, hemolytic rabbit serum, lipemic serum, plasma resulting from the addition of the anti-coagulant EDTA. Each of the four matrices was spiked into two pooled positive sera (rabbits vaccinated with either AVA or rPA) and a normal negative serum. The assay compared the effect of operator, laboratory, day and plate. Figure 6 shows the shift from normal of each of the matrices to the spiked assay. Although the spiked

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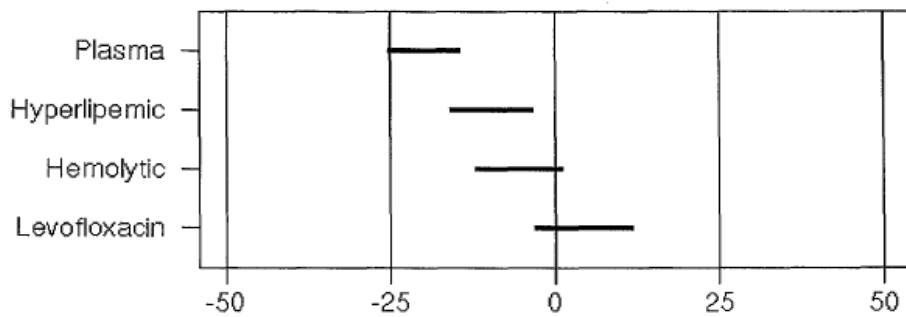
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samples tended to shift to the left, giving a lower value than normal. All spiked valued fell within $\pm 25\%$ of normal.

Acceptance criteria stipulated that there should be $< 50\%$ change. Therefore the assay specificity passed validation. This indicates that antibodies found in the positive sera were not masked significantly by the matrices tested e.g. if lipemic sera in infected rabbits were tested, the presence of the neutralizing antibodies would still be detected.

Figure 6: Specificity Results of tests for samples that are Lipemic, Hemolytic and contain metabolized Levofloxacin and Plasma



(b) (4) VP2006-138

Table 4 is a summary of the validation parameters for the TNA in rabbit serum.

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Table 4 : Summary of Validation of Rabbit Toxin Neutralization Assay

Parameter	Validation Criteria	Test results of Parameter	Validation Decision	Comment
Limits of Detection	LOD \leq 50	LOD = 23	Pass	None
Limits of Quantitation	ED ₅₀ Bias <50% LLOQ = <100	LLOQ = 39 ULOQ = 24,000	Pass	None
Precision	Pooled samples & pooled + real world - Total precision versus ED ₅₀ = <50%	PRTE overall = 33.7% Pooled samples = \leq 27%	Pass	None
Intermediate Precision				
Repeatability	Total error of Intermediate Precision + Dilutional linearity + Repeatability = \leq 50%	Pooled + real world = 28%		
Specificity	All matrices \leq 50% change Lipemia, hemolysis, levofloxacin, plasma	All matrices <30% change	Pass	None
Accuracy	Output shall be the sample range = LLOQ – ULOQ. LLOQ ED ₅₀ = <100 ED ₅₀ range for each initial dilutional level	Sample tested at 1:50 = 16-fold Sample diluted above 1:50 = 5-fold Lowest ED ₅₀ with reactive value = 39. LLOQ of 39 = <100 ULOQ = 24,000	Pass	Suggested usable range for each initial dilution when sample is tested one time 1:50 = 39 - 624 1:100 = 400 - 2000 1:200 = 800 - 4000 1:300 = 1200 - 6000 1:400 = 1600 - 8000 1:600 = 2400 - 12000 1:1200 = 4800 - 24000
Dilutional linearity of assay range				

PRTE = percent relative total error

Comment:

All of the parameters tested in this study were validated. The specificity of this assay using matrices that would probably be found in testing rabbit serum or plasma was accurate, because none of the matrices caused a change greater than 30 in the amount of anti-LT activity. One of the important characteristics of this assay is that the series of dilutions were outlined and the accuracy depended on the initial dilution of the sample. The accuracy tended to decrease with increasing initial factors of dilutions. When the sample was tested more than once the error rate decreased, this is normally expected. The purpose of this assay in real life, would be to determine the antibody response to infection or vaccination.

The assay described three types of curves full/reactive, partial reactive and non-reactive. The results between the two laboratories varied but not enough to cause the parameter to fail the acceptability criteria.

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4.5. Analytical Method for the quantification of ETI-204 by MesoScale Discovery (MSD) Electrochemiluminescence (ECL) Assay

4.5.1. Quantitation of ETI-204 in Rabbit Serum

Study No.TNJS11-010

The principle of the ECL assay is described in section 4.1. In the Tandem ECL assay the plates are coated with Streptavidin to which Biotinylated rPA63 is added. When ETI-204 conjugated to routhentlated protein A/G binds to the rPA63, light proportionate to the amount of binding, is emitted and measured. This assay utilizes a sandwich format. The purpose of the study is to quantitate ETI-204 using MesoScale Discovery (MSD) technology. The assay was run at
[REDACTED] (b) (4).

4.5.1.1. Acceptance Criteria

The acceptance criteria were outlined as follows:

Standards

- Back calculation of each non-anchor standard should have a %CV = < 20%
- On the standard curve each non-anchor point should be \pm 20% of the nominal value except at the ULOQ and LLOQ where the result must be \pm 25%
- At least 6 points are required to draw the standard curve

Sample

- Run in duplicate

Quality Control

- Run in duplicate (high, medium, low)
- At least four out of six must meet the criteria, and at least one must be acceptable for each level

Matrix effect

- At least 80% of animals must recover at least \pm 25% of the nominal value of the drug

Linearity

- Sample must fall within \pm 20% of the nominal value and have a % CV of \leq 20

Inter-assay and intra-assay accuracy and precision

- Accuracy: The acceptance level for the inter-and intra-assay must have \pm 20% Bias, except for the anchor points where a bias of \pm 25% is acceptable
- Precision: \leq 20%CV

(i) Plate acceptable criteria must meet the criteria for

- Standard curve
- Quality Control

(ii) Criteria related to the experiments

- Testing of parameters

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The following parameters will be tested:

Sensitivity

- Standard curve

Accuracy

- Linearity

Precision

- Inter- and intra-assay precision

Selectivity

- Matrix effect
- Protective antigen interference

The study will be run by at least 2 analysts.

4.5.1.2. **Sensitivity**

The sensitivity of the assay was determined as the value where the signal was twice that of the matrix blank (2:1). To test sensitivity, the LQC sample of 150 ng/mL was diluted two-fold to 0.5859 ng/mL. Table 1 shows that for Sensitivity sample 3, the signal to noise ratio(S/N) is 2.3, Sensitivity 4 was 1:8 and Sensitivity 2 was 3:9. Therefore, the sensitivity of the assay was selected as the value of Sensitivity 3 equal to 18.75 ng/mL.

Table 1: The Sensitivity level of this Tandem ECL assay

Sample ID	ETI-204 (ng/mL)	Replicate 1	Replicate 2	Mean Instrument Response	S/N
Sensitivity 1	75.0	2829	2731	2780	6.6
Sensitivity 2	37.50	1630	1625	1628	3.9
Sensitivity 3	18.75	978	989	984	2.3
Sensitivity 4	9.375	746	781	764	1.8
Sensitivity 5	4.6875	585	579	582	1.4
Sensitivity 6	2.3436	503	544	524	1.2
Sensitivity 7	1.1719	440	458	449	1.1
Sensitivity 8	0.5859	427	421	424	1.0
Blank	0	435	410	423	N/A

(b) (4)

All concentrations are expressed as ng/mL.

The samples used for the standard curve were prepared in pooled rabbit serum. Ten concentrations valued at 8,000, 6,000, 4,000, 2,000, 1,000, 500, 250, 100, 50, 25 ng/mL were tested, the anchor points were 8,000 and 25 ng/mL. A set of standards was run on each plate. The data for the standard curve were generated by three analysts on two days. Table 2 shows the results of the standard curve. The %CV at the anchor points were 13 and 14.8 respectively, for the 25 ng/mL and 8,000 ng/mL. The %CV of the non-standard points ranged from 1.8 to 8.2 %. These results are acceptable, the standard curve generated by these data is acceptable.

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Table 2: Results from eight runs of the Standard Curve

Nominal Conc. (ng/mL)	Incubation Times	STD 10 ^A	STD 9	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1 ^A
Run 20	Long	25.0	50.0	100	250	500	1000	2000	4000	6000	8000
Run 21	Long	25.2	49.3	102	245	506	997	2050	3960	5760	8320
Run 22	Nominal	24.6	51.9	98.7	246	502	1000	2050	3970	5790	8260
Run 23	Nominal	27.3	43.6	107	271	465	1000	2140	3910	5640	8500
Run 24	Short	20.4	55.8	116	260	482	873	1970	4060	6780	11900
Run 25	Short	26.0	48.1	101	249	513	991	2100	3840	5680	8590
Run 26	Nominal	27.4	44.3	104	256	508	1000	2020	3930	5740	8380
Run 27	Nominal	27.0	48.6	98.4	251	502	996	2050	3960	5960	8030
		32.6	46.3	92.7	253	501	1050	1980	3860	6200	7950
Mean		26.3	48.5	102	254	497	988	2050	3940	5940	8740
Std Dev		3.41	4.00	6.91	8.53	15.9	50.2	56.8	69.1	383.0	1290.0
% CV		13.0	8.2	6.8	3.4	3.2	5.1	2.8	1.8	6.4	14.8
% Bias		5.2	-3.0	2.0	1.6	-0.6	-1.2	2.5	-1.5	-1.0	9.3
Total Error		18.2	11.2	8.8	5.0	3.8	6.3	5.3	3.3	7.4	24.1
n		8	8	8	8	8	8	8	8	8	8

^A=Anchor Point

(b) (4) Report

4.5.1.3 Precision

Validation samples were prepared in pooled rabbit serum collected from normal rabbits. The HQC = 4,800 ng/mL, MQC = 2,400 ng/mL, LQC = 150 ng/mL. ULOQ = 6,000 ng/mL, LLOQ = 50 ng/mL. Eight assays were run by three analysts on two days, the six sets of validation samples were run in duplicate on each plate. The %CV of the inter-assay results ranged from 5.1 to 15.7, which is acceptable. There appeared to be samples that had failed in the LLOQ and LQC because the %CV decreased when the failed samples were excluded but the line data were not available. Table 3 shows the results.

The %CV ranged from 1.1 to 8.7 for all levels of quality control, with approximately 83% of the samples recorded values <5%CV. Table 4 shows the cumulative precision results.

Table 3: Inter-Assay Accuracy and Precision
 Inclusive of Failed Data

Target (ng/mL)	LLOQ	LQC	MQC	HQC	ULOQ
50	150	2400	4800	6000	
Mean	48.7	153	2470	4900	6020
SD	7.63	12.3	220	351	308
%CV	15.7	8.0	8.9	7.2	5.1
%Bias	-2.6	2.0	2.9	2.1	0.3
n	48	48	48	48	48

Exclusive of Failed Data

Target (ng/mL)	LLOQ	LQC	MQC	HQC	ULOQ
50	150	2400	4800	6000	
Mean	50.3	152	2470	4900	6020
SD	4.62	11.7	220	351	308
%CV	9.2	7.7	8.9	7.2	5.1
%Bias	0.6	1.3	2.9	2.1	0.3
n	40	47	48	48	48

All concentrations are expressed as ng/mL.

(b) (4)

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Table 4: Cumulative results of tests for Intra-assay and Inter-assay Precision and Accuracy tests

Validation Sample	Nominal Conc. (ng/mL)	Intra-Assay Precision (% CV)	Intra-Assay Accuracy (% Bias)	Inter-Assay Precision (% CV)	Inter-Assay Accuracy (% Bias)
VS1-ULOQ	6,000	2.1 to 4.3	-6.0 to 6.3	5.1	0.3
VS2-HQC	4,800	1.8 to 6.9	-8.5 to 8.8	7.2	2.1
VS3-MQC	2,400	1.1 to 8.0	-11.3 to 14.2	8.9	2.9
VS4-LQC	150	1.1 to 6.4	-11.1 to 14.9	8.0	2.0
VS5-LLOQ	50.0	1.3 to 8.7	-33.7 to 19.1	15.7	-2.6

(b) (4)

4.5.1.4. Accuracy

Accuracy of quality control samples was calculated using the % Bias for each level of validation test sample. The acceptance level for the inter-and intra-assay was $\pm 20\%$ Bias except for the anchor points where a bias of $\pm 25\%$ was acceptable.

Table 5 shows that there was one failure event, this occurred in run 23 of the LLOQ level where the %Bias was -33.7% .

Table 5: Inter-assay and Intra-assay of the accuracy and precision tests results for the LLOQ by Run

	Run 20	Run 21	Run 22	Run 23	Run 24	Run 25	Run 26	Run 27
LLOQ	50.2	44.7	47.5	34.7	53.5	47.2	49.3	55.2
	51.4	45.1	44.3	30.4	50.7	45.3	54.1	58.2
	47.6	45.5	49.4	31.7	51.3	53.4	56.3	63.8
	48.9	45.4	45.6	35.5	51.7	51.6	53.2	58.2
	47.9	43.9	49.5	36.8	51.2	50.9	58.4	57.7
	49.8	45.0	47.3	29.9	49.8	51.5	54.0	64.3
Mean	49.3	44.9	47.3	33.2	51.4	50.0	54.2	59.6
SD	1.45	0.58	2.06	2.88	1.23	3.07	3.07	3.65
%CV	2.9	1.3	4.4	8.7	2.4	6.1	5.7	6.1
%Bias	-1.4	-10.1	-5.5	-33.7	2.7	0.0	8.4	19.1
n	6	6	6	6	6	6	6	6

(b) (4)

The accuracy of dilutions was tested using ETI-204 spiked into pooled rabbit serum to a concentration of 150,000 ng/mL. The two assays used to analyze this parameter were performed on two different days. The inter-run percent bias ranged from -9.3 to -10.0 % and the intra-assay bias ranged from -4 to 3.0%. The precision results ranged from 0.5 to 2.1% CV for the intra-assay and 8.8 to 13.6 %CV for the inter-assay precision.

Dilutional linearity was investigated by diluting an ETI-204 spiked sample concentration of 640,000 ng/mL at several ratios such as: 1:6.4 (100,000 ng/mL), 1:32 (20,000 ng/mL), 1:160 (4,000 ng/mL), 1:800 (800 ng/mL), 1:4,000 (160 ng/mL) and 1:20,000 (6.4 ng/mL), in pooled rabbit serum. The data from table 6 show that the results of the highest dilutions fell outside of the upper end of the standard curve. This is not surprising because the nominal values of Linearity 1, 2 and 3 were above the ULOD. The %CV of Linearity 4, 5, and 6 were 1.7, 0.9 and 1.0 respectively, and the variance from the nominal values was 16.0, 17.3 and 21.9 %, respectively. The acceptance criteria for these values were $\pm 20\%$ of the nominal value in

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conjunction with %CV of $\leq 20\%$. Linearity samples 4 and 5 were within the acceptable limit for difference from the nominal concentration. The difference between Linearity sample 6 and the nominal concentration was unacceptable, difference between the nominal and test result values was $> 20\%$. Linearity 7 and 8 with nominal concentrations of 32 and 6.4 ng/mL were lower than the LLOQ, therefore results from these samples, if they were accurate, would be expected to fall below the LLOQ – they did. This meant that only one of the samples was unacceptable. A pre-requisite for acceptability of this parameter was that the QC and standards must be acceptable. Therefore the dilutional linearity was acceptable i.e. diluted samples are expected to be accurate representations of the nominal value. The data for dilutional linearity are tabulated in table 6.

Table 6: Dilutional linearity of the ECL assay

Sample ID	Dilution Factor	Nominal Concentrations (ng/mL)	Mean Results (ng/mL)	Mean x Dilution Factor	% CV	% Difference from Nominal
Linearity 1	1	640,000	>ULOQ (>6000)	N/A	N/A	N/A
Linearity 2	6.4	100,000	>ULOQ (>6000)	N/A	N/A	N/A
Linearity 3	32	20,000	>ULOQ (>6000)	N/A	N/A	N/A
Linearity 4	160	4,000	4640	742400	1.7	16.0
Linearity 5	800	800	938	750400	0.9	17.3
Linearity 6	4000	160	195	780000	1.0	21.9
Linearity 7	20000	32	<BLOQ (<50.0)	N/A	N/A	N/A
Linearity 8	100000	6.4	<BLOQ (<50.0)	N/A	N/A	N/A

All concentrations are expressed as ng/mL.

(b) (4)

4.5.1.5. Specificity

Specificity of the matrix for the detection of the presence of ETI-204, was tested using the LLOQ concentration of 50 ng/mL in rabbit serum, with or without ETI-204. None of the samples that was unspiked showed the presence of ETI-204; while all, except one sample, that was spiked with 50 ng/mL of ETI-204, registered the presence of ETI-204 at a concentration approximating 50 ng/mL.

The results are shown in Table 7.

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Table 7: The Matrix effect of rabbit serum with and without the addition of 50 ng/mL of ETI-204

Unspiked Samples		Spiked at LLOQ (50 ng/mL)		
Matrix Lot Number	Mean Conc. (ng/mL)	Mean Conc. (ng/mL)	% Bias	% CV
RABBREC-37537-M	<BLOQ (<50.0)	55.7	11.4	1.9
RABBREC-37535-M	<BLOQ (<50.0)	51.2	2.4	3.5
RABBREC-37541-M	<BLOQ (<50.0)	54.1	8.2	8.6
RABBREC-37548-M	<BLOQ (<50.0)	55.5	11.0	0.5
RABBREC-37554-M	<BLOQ (<50.0)	<BLOQ (<50.0)*	-4.4	1.2
RABBREC-37577-F	<BLOQ (<50.0)	54.8	9.6	2.2
RABBREC-37562-F	<BLOQ (<50.0)	59.2	18.4	0.7
RABBREC-37568-F	<BLOQ (<50.0)	62.5	25.0	5.0
RABBREC-37571-F	<BLOQ (<50.0)	66.2	32.4	1.1
RABBREC-37579-F	<BLOQ (<50.0)	62.4	24.8	6.5

*Extrapolated value is 47.8 ng/mL.

(b) (4)

Specificity related to the effect of potentially cross-reacting antibodies was tested using mouse and human IgG. The applicant tested a concentration of 2,400 ng/mL for each analyte. Three aliquots of each sample were tested in duplicate. The samples contained equal concentrations of test antibody and ETI-204. Samples with the test IgG only were used as controls. The samples that contained both the test IgG and ETI-204 resulted in concentrations very close to the nominal value of 2,400 ng/mL. The results ranged from 2,210 to 2,440 ng/mL a range of -0.8% to 1.1 % from the nominal concentration. The %CV ranged from 1.0 to 3.3%. Therefore, from these results neither the mouse nor the human IgG affected the concentration of ETI-204 in the rabbit serum. However, in another assay also performed by (b) (4), the results shown in Table 8 indicate that human IgG does decrease the concentration of ETI-204. Six values are above the \pm 20 variability.

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Table 8: The Effect of Human IgG on the Quantitation of ETI-204

Sample #	Sample Name	Unspiked (ng/mL)	With 1000 ng/mL of Anti- Human IgG (ng/mL)	% Difference	With 1000 ng/mL of Anti-Rabbit IgG (ng/mL)	% Difference
1	TNJS11-130 410400000093 L33650 4 mg/kgA Serum-1 3 hour PTxA / Day 1 3h	10600	21000	98.1	10400	-1.9
2	TNJS11-130 410400000102 L33653 4 mg/kgA Serum-1 3 hour PTxA / Day 1 3h	5760	15900	176.0	6360	10.4
3	TNJS11-130 410400000111 L33645 4 mg/kgA Serum-1 3 hour PTxA / Day 1 3h	8990	21200	135.8	9980	11.0
4	TNJS11-130 410400000097 L33650 4 mg/kgA Serum-1 4 days PTxA / Day 4	5270	11600	120.1	5030	-4.6
5	TNJS11-130 410400000106 L33653 4 mg/kgA Serum-1 4 days PTxA / Day 4	45100	ALOQ>6000	N/A	49700	10.2
6	TNJS11-130 410400000115 L33645 4 mg/kgA Serum-1 4 days PTxA / Day 4	22300	84700	279.8	22800	2.2
7	TNJS11-130 410400000099 L33650 4 mg/kgA Serum-1 14 days PCA / Day 14	2610	2660	1.9	2600	-0.4
8	TNJS11-130 410400000108 L33653 4 mg/kgA Serum-1 14 days PCA / Day 14	3570	3790	6.2	3870	8.4
9	TNJS11-130 410400000117 L33645 4 mg/kgA Serum-1 14 days PCA / Day 14	6500	8650	33.1	6790	4.5
10	TNJS11-130 410400000201 L33646 16mg/kgA Serum-1 3 hour PTxA / Day 1 3h	53500	ALOQ>6000	N/A	48100	-10.1
11	TNJS11-130 410400000210 L33629 16mg/kgA Serum-1 3 hour PTxA / Day 1 3h	ALOQ>6000	ALOQ>6000	N/A	ALOQ>6000	N/A
12	TNJS11-130 410400000219 L33644 16mg/kgA Serum-1 3 hour PTxA / Day 1 3h	ALOQ>6000	ALOQ>6000	N/A	ALOQ>6000	N/A
13	TNJS11-130 410400000205 L33646 16mg/kgA Serum-1 4 days PTxA / Day 4	ALOQ>6000	ALOQ>6000	N/A	ALOQ>6000	N/A
14	TNJS11-130 410400000214 L33629 16mg/kgA Serum-1 4 days PTxA / Day 4	ALOQ>6000	ALOQ>6000	N/A	ALOQ>6000	N/A
15	TNJS11-130 410400000223 L33644 16mg/kgA Serum-1 4 days PTxA / Day 4	ALOQ>6000	ALOQ>6000	N/A	ALOQ>6000	N/A
16	TNJS11-130 410400000207 L33646 16mg/kgA Serum-1 14 days PCA / Day 14	5230	5540	5.9	5550	6.1
17	TNJS11-130 410400000216 L33629 16mg/kgA Serum-1 14 days PCA / Day 14	4860	5280	8.6	5150	6.0
18	TNJS11-130 410400000225 L33644 16mg/kgA Serum-1 14 days PCA / Day 14	3530	3720	5.4	3720	5.4

(b) (4)

The effect of the presence of protective antigen on the quantitation of ETI-204 in the serum of rabbits was measured using rPA63. Recombinant PA63 at concentrations of 20000, 10000, 5000, 2400, 1200, 600, 300, and 0 ng/mL were spiked into high, medium and low quality control samples. Both the spiked and unspiked controls were run on the same plate. Table 8 shows the results of this assay. There was interference in three values, LQC - 300 ng/mL, MQC - 1,200 ng/mL and HQC- 5,000 ng/mL.

The applicant therefore, investigated the effect of PA on low concentrations of ETI-204. Recombinant PA at concentrations of 300, 150, 75, 50, 25, 10, 5, and 0 ng/ml were spiked into rabbit sera to obtain a concentration similar to that of LQC (150 ng/mL), and tested. Table 9 shows the results. The applicant states that there was interference at the LQC 50 ng/mL level, however, this was not apparent from the data see Table 10. In conclusion, there was no definitive indication that the PA tested will affect the quantitation of ETI-204.

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Table 9: Results of tests to determine if Protective Antigen interferes with the Quantitation of ETI-204

Sample ID	ETI-204 (ng/mL)	rPA-63 (ng/mL)	Mean Conc. (ng/mL)	% CV	% Bias
LQC_rPA 0.0	150	0	158	1.3	5.3
LQC_rPA 0.3	150	300	37.7	5.1	-74.9
LQC_rPA 0.6	150	600	32.7	1.9	-78.2
LQC_rPA 1.2	150	1200	21.6	2.0	-85.6
LQC_rPA 2.4	150	2400	19.2	1.5	-87.2
LQC_rPA 5	150	5000	14.1	0.5	-90.6
LQC_rPA 10	150	10000	11.0	3.9	-92.7
LQC_rPA 20	150	20000	7.58	9.5	-94.9
MQC_rPA 0.0	2400	0	2480	2.0	3.3
MQC_rPA 0.3	2400	300	2360	1.8	-1.7
MQC_rPA 0.6	2400	600	2060	0.3	-14.2
MQC_rPA 1.2	2400	1200	1740	4.5	-27.5
MQC_rPA 2.4	2400	2400	1180	0.6	-50.8
MQC_rPA 5	2400	5000	335	0.6	-86.0
MQC_rPA 10	2400	10000	201	0.4	-91.6
MQC_rPA 20	2400	20000	141	3.0	-94.1
HQC_rPA 0.0	4800	0	4940	3.7	2.9
HQC_rPA 0.3	4800	300	4620	4.4	-3.7
HQC_rPA 0.6	4800	600	4720	3.6	-1.7
HQC_rPA 1.2	4800	1200	4230	1.8	-11.9
HQC_rPA 2.4	4800	2400	3950	2.3	-17.7
HQC_rPA 5	4800	5000	1960	2.9	-59.2
HQC_rPA 10	4800	10000	526	0.8	-89.0
HQC_rPA 20	4800	20000	333	2.3	-93.1

(b) (4)

All concentrations are expressed as ng/mL.

Table 10: Test to determine the specificity of the effect of PA on samples with low ETI-204 concentrations.

Sample ID	ETI-204 (ng/mL)	rPA-63 (ng/mL)	Mean Conc. (ng/mL)	% CV	% Bias
LQC 0 rPA	150	0	150	1.4	0.0
LQC 5 rPA	150	5	153	0.0	2.0
LQC 10 rPA	150	10	145	0.5	-3.3
LQC 25 rPA	150	25	144	0.5	-4.0
LQC 50 rPA	150	50	121	1.8	-19.3
LQC 75 rPA	150	75	105	0.0	-30.0
LQC 150 rPA	150	150	59.5	0.6	-60.3
LQC 300 rPA	150	300	27.9	3.8	-81.4

(b) (4)

The applicant in another study, tested the effect of hemolysis on the quantitation of ETI-204. The applicant used 140 mg/dl and 550 mg/dl of hemolyzed serum to make three samples each of HQC and LQC which were run in duplicate. The results are shown in Table 11. Sample LQC1 with a 20.7 % bias at hemolysis level of 140 mg/dL was unacceptable but the other levels were

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acceptable. This test showed that hemolysis at 140 mg/dl and 550 mg/dl did not affect the quantitation of ETI-204.

Table 11: The effect of hemolysis on the Quantitation of ETI-204

Sample ID	ETI-204 (ng/mL)	Hemolysis Level (140 mg/dL)			Hemolysis Level (550 mg/dL)		
		Mean (ng/mL)	%CV	%Bias	Mean (ng/mL)	%CV	%Bias
HQC-1	4800	4500	1.6	-6.2	4100	3.1	-14.6
HQC-2	4800	4420	0.3	-7.9	4420	2.7	-7.9
HQC-3	4800	4410	1.4	-8.1	4430	0.3	-7.7
LQC-1	150	181	0.4	20.7	162	3.9	8.0
LQC-2	150	178	0.8	18.7	167	1.3	11.3
LQC-3	150	175	0.8	16.7	159	0.9	6.0

(b) (4)

The applicant also tested levofloxacin (2,400 ng/mL) Table 12, to determine the effect on the MQC (2,400 ng/mL), lethal factor (20 , 200, 2000 ng/mL) on LQC (150 ng/mL), and on HQC (4,800 ng/mL) of ETI-204. Edema factor (4, 40, 400 ng/mL) was tested with HQC (4,800 ng/mL) of ETI-204, to determine their effect on ETI-quantitation.

Levofloxacin, edema factor, and lethal factor had no effect on the quantitation of ETI-204.

Table 12 : Effect of Levofloxacin on Quantitation of ETI-204

Sample ID	ETI-204 (ng/mL)	Levofloxacin (ng/mL)	Mean Conc. (ng/mL)	%CV	%Bias
LEVO 2400 1	0	2400	BLOQ	N/A	N/A
LEVO 2400 2	0	2400	BLOQ	N/A	N/A
LEVO 2400 3	0	2400	BLOQ	N/A	N/A
LEVO MQC 1	2400	2400	2320	1.8	-3.3
LEVO MQC 2	2400	2400	2210	3.5	-7.9
LEVO MQC 3	2400	2400	2570	2.2	7.1

Comment for specificity

The data resulting from tests to determine if PA interfered with the concentration of ETI-204 did not provide conclusive results. The preliminary results did not show a trend in concentration.

The supplementary test to determine if ETI-204 was affected at low concentrations was also not definitive. More tests should be performed in order to decisively determine if there is true interference by rPA in the quantitation of ETI-204.

Overall Comment

All of the parameters that were selected passed the acceptance criteria of the applicant.

The percentages coefficient of variation and the percentages of bias were very small, most of the results showed less than 5% CV, indicating good accuracy and precision of the test results. The applicant tested variety of interferents but only human IgG appeared to be tested in the quantitation of ETI-204

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4.6. Qualification of a Quantitative Assay to Measure *Bacillus anthracis* Bacteremia

4.6.1. *Bacillus anthracis* Bacteremia in Rabbits

(b) (4) QD-319-100029533, (b) (4) SOP - (b) (4) 054

This study was conducted by Elusys.

Bacteremia is one of the manifestations of infection with *Bacillus anthracis*. To detect bacteremia aliquots of blood are usually sampled from animals that were exposed to spores of the bacterium. The purpose of this assay is to quantitate the number of bacteria in the blood of an animal that has inhaled the spores of *Bacillus anthracis*.

For a quantitative bacteremia (qBact) assay, specific volumes of blood are sampled, the samples are cultured, and the colonies enumerated. The applicant used the Ames strain of *Bacillus anthracis* to perform the assay. The matrix was either whole rabbit blood or sterile buffered saline with 0.1% gelatin.

Whole blood of the rabbit was collect and placed in a tube of dipotassium ethylenediaminetetraacetic acid (K₂-EDTA) as an anticoagulant. The number of bacteria cultured, and used to spike blood was estimated by comparison with the Mc Farland standard (not specified). In plate counts of bacteria, the acceptable range for colony counts is 25 to 250 colonies /plate.

Safety is an important concern for culture of *Bacillus anthracis* which is a select agent, and must be handled with at least BSL-2 practices. The operations of the culture must be conducted in a BSL-2 or BSL-3 laboratory, using a Biological Safety Cabinet 11.

The purposes of the assay were to determine

- The rate of recovery of bacteria from whole blood or sterile saline
- The specificity of the assay to differentiate *Bacillus anthracis* from possible contaminants in the blood.

4.6.1.1. Acceptance criteria

- Colony counts that are less than 25 will be reported as the actual number of colonies isolated.
- If the colonies are < 10 the results will be reported as positive depending on the actual results of a culture, run in triplicate. If 2/3 of the plates in the culture have counts less than 10, the result will be reported as positive, if 2/3 have counts greater than 10 the actual average will be reported.
- For plates where the colonies are > 250 , if the colonies can be counted the actual counts will be reported.

Reference culture

- Must have no spores after suspension and culture (microscopic examination)
- The Optical Density at 600 nm (OD₆₀₀) must fall between 0.187 and 0.370
- McFarland standard must read between 1.6 and 2.9

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The following parameters will be tested:

Accuracy

- Linearity

Precision

- Percent coefficient of variation

Specificity

- Matrix effect

The study will be run by at least 2 analysts.

4.6.1.2. Accuracy and Precision

To test the accuracy and precision of this assay, six concentrations of *Bacillus anthracis* were diluted two-fold in rabbit blood or saline to obtain a concentration of (160,000, 80,000, 40,000, 20,000, 10,000 and 5,000 cfu/mL), then 0.10 mL of each dilution was cultured on solid medium. The recovered growth after 24 hours was enumerated. Table 1 shows the results of growth after incubation of 16 -24 hours. The %CV for samples spiked into blood ranged from 4.3 to 37.4. only one sample resulted in a %CV <10. In saline the range was 19.6 to 48%. Therefore the precision in both matrices was poor. The accuracy of the test was better than the precision, with differences between the target and the recovered concentration of bacteria ranging from 7.7 to 16.1%. In only one count was the difference between the original and cultured value less than 10%. The recovery rate was in all cases, less than the original value.

There was also a difference in growth between the two matrices, in 4/6 cultures the recovery was greater in the saline matrix. The results are shown in table 3.

Table 1: Summary of Accuracy and Precision for Spike and Recovery

<i>B. anthracis</i> Target Concentration** (cfu/mL)/ diluent	<i>B. anthracis</i> Geometric Mean Concentration* (cfu/mL)	Geometric Mean Sample Concentrations (cfu/mL)	Difference from Actual (%DFA)	Precision of Enumerations (%CV)	Difference of Blood from BSG (%)
5,000 Blood	4,480	3,760	-16.1	22.6	-2.9
5,000 BSG		3,870	-13.6	48.0	
10,000 Blood	8,970	7,610	-15.1	37.4	-1.8
10,000 BSG		7,750	-13.5	10.3	
20,000 Blood	17,900	15,200	-15.2	22.7	-1.4
20,000 BSG		15,400	-14.0	9.5	
40,000 Blood	35,900	39,500	10.1	19.8	23.7
40,000 BSG		31,900	-11.0	38.0	
80,000 Blood	71,900	66,400	-7.7	29.3	5.9
80,000 BSG		62,700	-12.8	19.6	
160,000 Blood	144,000	122,000	-15.3	4.3	-3.7
160,000 BSG		126,000	-12.1	22.5	

* Calculated concentration determined from dilution of enumerated reference culture. Value is the geometric mean of reference culture enumerations performed on 8/20/13 and 9/4/13.

** Calculated using the target bacterial concentration of the reference culture x dilution factor. In this example the reference culture is assumed to be 10,000,000 cfu/mL according to BBRC Method No. 174/Microbiology

Dilutional linearity. The accuracy data were used to determine the dilutional linearity.

Figure 1 shows the curves drawn from the expected load and the recovered load of bacteria.

There is a 1:1 relationship between the expected and recovered load of bacteria. A similar plot was seen with the bacteria grown in saline. This indicates that there is dilutional linearity.

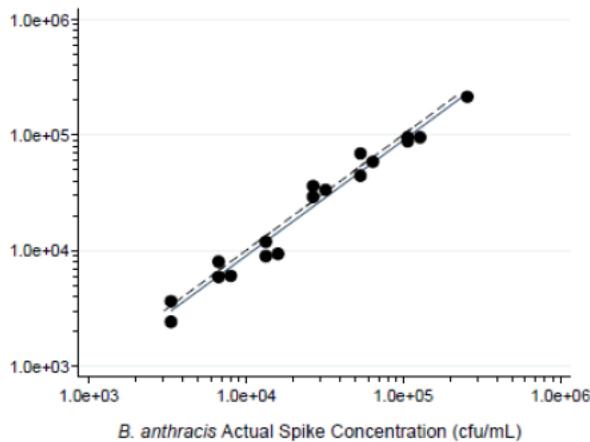
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Figure 1: Scatterplot of *Bacillus anthracis* in the Blood



4.6.1.3. Specificity

This parameter was designed to see if the *Bacillus anthracis* could be detected in blood that contained contaminating bacteria. *Bacillus atrophaeus* var. *globigii* a species of *Bacillus* that grows under the same conditions as *Bacillus anthracis*, but has different colonial morphology was used as the contaminating species. Figure 2 shows the mixed culture of the two *Bacillus* species on trypticase soy agar plates, after 16 – 24 hours of growth at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. From these pictures, only the difference in size and shape can be distinguished. Notice the small round colonies of *Bacillus atrophaeus* var. *globigii* as compared to the large spreading colonies of *Bacillus anthracis*.

Figure 2: Colonial morphologies of *Bacillus anthracis* and *Bacillus atrophaeus* var. *globigii* On Trypitase Soy agar



B. atrophaeus var. *globigii* on TSA



B. anthracis Ames on TSA (Elusys)



B. atrophaeus var. *globigii* and *B. anthracis*

To determine the specificity related to differing species, samples of rabbit blood were spiked with *Bacillus anthracis* reference strain to obtain a final concentration of 1,500, 750, and 375 cfu/mL. Spores of *Bacillus atrophaeus* var. *globigii* were also added to obtain a ratio of 1:10 and 10:1 (*Bacillus anthracis*:*Bacillus atrophaeus* var. *globigii*). The liquid culture was mixed

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thoroughly, plated in triplicate, incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and the colonies counted after 16 to 24 hours. The count of the inoculum was ascertained by making a dilution of the broth from the inoculum, diluting the mixture 10-fold, culturing and counting the colonies from the agar growth. The result was an average of the three similar dilutions. Table 2 shows the results.

The number of recovered *Bacillus anthracis* was less than expected, the decrease in colony forming units ranged from 57.5 to 87.8%, only in one of the target 1500 cfu/mL samples was there a slight increase in growth.

Therefore, in the presence of a competing species, the growth of *Bacillus anthracis* appeared to be reduced. Specificity in this case failed.

Table 2: Summary of the Specificity results

<i>B. anthracis</i> Target Concentration (cfu/mL)	<i>B. anthracis</i> Spike Concentration* (cfu/mL)	<i>B. atrophaeus</i> var. <i>globigii</i> Spike Concentration** (cfu/mL)	<i>B. anthracis</i> Geometric Mean Sample Concentrations (cfu/mL)	Difference from Actual Spike Concentration (%)
1,500	2,272	14,851.5	2,320	2.1
		1,485.2	1,770	-22.0
		148.5	1,930	-14.9
750	1,136	7,425.7	162	-85.8
		742.6	147	-87.1
		74.3	140	-87.7
375	568	3,712.9	122	-78.5
		371.3	104	-81.7
		37.1	242	-57.5

* Calculated from dilutions of enumerated reference culture | **Calculated dilutions of enumerated spore stock

Elusys

Comment:

This assay for the determination of the rate of recovery of bacteria from whole blood and sterile saline, and the specificity of the assay to differentiate *Bacillus anthracis* from possible contaminants in the blood did not meet its objectives. Possible reasons are:

- One of the possible reasons might be because the number of colony forming units was enumerated after incubation for 16 to 24 hours. Usually these types of cultures are incubated for 18 to 24 hours and may need up to 48 hours of incubation. If the colonies are counted after 16 hours some of the bacteria may not have grown. The colonies should have been recounted at 48 hours. The assay was not accurate, precise or specific.
- Another possible reason could have been the use of EDTA as the anticoagulant. The anticoagulant EDTA used in this study exhibits antimicrobial activity. One of the best anticoagulants used for blood culture is sodium polyanethol sulfonate (SPS), but this anticoagulant also has inhibitory effects on some species of bacteria. It would have been helpful to have the data that showed the recovery of *Bacillus atrophaeus* var. *globigii*. It is felt that the antimicrobial activity of EDTA did not play a significant role in the reduction of the bacterial growth from solid culture.
- Apart from anticoagulants that might inhibit the growth of bacteria there are other inhibitory substances that are naturally present in the blood that can inhibit the growth of *Bacillus anthracis*.
- Operator error. It is difficult to count colonies on a crowded plate especially if the growth is mixed.

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Lynette Y. Berkeley, Ph.D, M.T. (ASCP),
Microbiologist,
DAIP,
December 12, 2015

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

LYNETTE Y BERKELEY

12/15/2015

KERRY SNOW

12/16/2015

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Clinical Microbiology Review**

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Date Completed: 12/16/2015

Reviewer: Shukal Bala, Ph.D.

APPLICANT:

Elusys Therapeutics, Inc.
25 Riverside Drive
Pine Brook, NJ 07058

DRUG PRODUCT NAMES:

Proprietary: Anthim®
Nonproprietary: Obiltoxaximab; ETI-204
Chemical name: Not applicable
Antibody Class/Type: IgG1κ isotype humanized chimeric monoclonal antibody

Molecular weight: ~148 kDa

Empirical Formula:

(b) (4)

DRUG CATEGORY: Anti-protective antigen (PA) antibody

PROPOSED INDICATION:

Treatment of adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis* in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate.

PROPOSED DOSAGE FORM, ROUTE OF ADMINISTRATION AND DURATION OF TREATMENT:

Dosage form: Liquid solution (100 mg/mL)
Route of administration: Intravenous infusion over 90 minutes
Dosage: 16 mg/kg
Duration of treatment: Single dose

DISPENSED:

Rx

RELATED DOCUMENTS:

IND 12,285; PreIND [REDACTED] (b) (4) - [REDACTED] (b) (4) -
National Institutes of Allergy and Infectious Disease (NIAID), and DMF [REDACTED] (b) (4) (Type V)
– Lonza.

REMARKS

The subject of this BLA is Anthim® (obiltoxaximab; ETI-204) for the treatment of adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis* in combination with appropriate antibacterial drugs and for prophylaxis (pre-exposure and post-exposure) of inhalational anthrax. ETI-204, a monoclonal antibody, binds to protective antigen (PA) from the 3 strains (Ames, Sterne, and Vollum) of *B. anthracis*.

ETI-204 ([REDACTED] (b) (4) product) was effective in improving survival of mice infected with the Sterne strain of *B. anthracis* by the intra-tracheal route; treatment was administered at the time of infection. Lungs from the surviving animals at the end of the study were culture positive.

New Zealand White rabbits and cynomolgus monkeys exposed to a lethal dose of the spores of the Ames strain of *B. anthracis* via the inhalational route showed signs of anthrax. ETI-204 at a dose of 16 mg/kg was effective in improving survival of animals when treated based on a trigger for intervention (presence of PA by a screening electrochemiluminescent assay or an increase in temperature), or when administered at a fixed time post-exposure or up to 3 days prior to exposure. The survival decreased as the microbial burden (bacteremia and/or PA levels) increased or treatment was delayed.

Overall, the animal studies support the effectiveness of ETI-204 alone or in combination with an antibacterial drug for the treatment and prophylaxis (post-exposure and pre-exposure) of inhalational anthrax. ETI-204 treatment may also be beneficial when alternative therapies are not available or are not appropriate e.g., in the event of an infection due to a drug resistant strain of *B. anthracis*.

CONCLUSIONS AND RECOMMENDATIONS

From clinical microbiology perspective, this BLA submission is approvable pending an accepted version of the labeling. The changes to the proposed labeling are as follows:

- The changes proposed in the labeling:**

(Additions marked as double-underlined and deletions as striked out)

12.1 Mechanism of action

Obiltoxaximab is a monoclonal antibody that binds the PA of *B. anthracis*. [see (b) (4)
Microbiology (12.4)]

12.4 Microbiology

Mechanism of Action

Obiltoxaximab is a monoclonal antibody that binds free PA with an affinity equilibrium dissociation constant (Kd) of 0.33 nM. Obiltoxaximab inhibits the binding of PA to its cellular receptors; such an effect appears to be concentration dependent. Binding of PA to the cell surface receptor prevents the intracellular entry of the anthrax lethal factor and edema factor, the enzymatic toxin components responsible for the pathogenic effects of anthrax toxin.

Activity In Vitro and In Vivo

Obiltoxaximab binds *in vitro* to PA from the Ames, Vollum, and Sterne strains of *B. anthracis*. Obiltoxaximab binds to an epitope on PA that is conserved across reported strains of *B. anthracis*.

In Vitro studies in a cell-based assay, using murine macrophages, suggest that obiltoxaximab neutralizes the toxic effects of (b) (4) lethal toxin, (b) (4) a combination of PA + lethal facto (b) (4)

In vivo efficacy studies (b) (4) in NZW rabbits and cynomolgus (b) (4) challenged with the spores of *B. anthracis* by the inhalational route, showed a dose-dependent increase in survival (b) (4) following treatment with (b) (4). Exposure to *B. anthracis* spores resulted in increasing concentrations of (b) (4)-PA in the serum of (b) (4) NZW rabbits and cynomolgus (b) (4). (b) (4) treatment with obiltoxaximab there was a (b) (4) decrease in PA concentrations (b) (4) -in (b) (4) surviving (b) (4) animals. PA concentrations in placebo animals increased until they died. [see Clinical Studies (14.1)].

- Post marketing request:**

None.

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1. Executive Summary

Anthim® (obiltoxaximab; ETI-204) is a humanized chimeric immunoglobulin G₁ kappa (IgG1κ) monoclonal antibody (MoAb) against the protective antigen (PA) of *Bacillus anthracis* for the treatment and prophylaxis (post-exposure and pre-exposure) of inhalational anthrax. ETI-204 should also be beneficial when alternative therapies are not available or are not appropriate e.g., in the event of infection due to a drug resistant strain of *B. anthracis*. The applicant has proposed to market a single 16 mg/kg dose of ETI-204 alone or in combination with appropriate antibacterial drugs.

Mechanism of action

The *in vitro* studies showed that ETI-204 binds the PA with a dissociation constant (K_D) of 0.33 nM i.e., 48.8 pg/mL and neutralizes PA. ETI-204 blocked the receptor binding domain 4 of PA (PAD4) that binds to one of the anthrax toxin receptors, capillary morphogenesis gene 2 (CMG-2); such inhibition of binding of PA with the receptor was concentration dependent. The CMG-2 receptor has higher affinity for PA than tumor endothelial marker 8 (TEM-8), another anthrax receptor.

Binding of ETI-204 to PAD4 prevents the cell binding of PA63-edema factor (EF) and PA63-lethal factor (LF) complexes; this prevents the entry of EF and LF into the cytosol, thereby preventing the downstream deleterious effects of anthrax toxins, known to be the pathophysiological drivers of morbidity and mortality.

Activity in vitro

ETI-204 binds to PA antigen from the 3 strains (Ames, Sterne, and Vollum) of *B. anthracis*. However, the intensity of binding and the number of bands observed varied among the 3 strains although the concentration of protein loaded for western blot analysis was the same. One of the limitations of the experiment is that unpurified supernatants of the Ames, Sterne, and Vollum strains of *B. anthracis* were not tested.

ETI-204 was shown to inhibit the neutralizing activity of lethal toxin (LT) against murine macrophages (b) (4) *in vitro*.

Activity in mice

The (b) (4) product ((b) (4) anti-PA MoAb) of ETI-204 was effective in improving survival of mice infected with the Sterne strain, an avirulent non-capsulated strain, of *B. anthracis* by the intra-tracheal route; the (b) (4) anti-PA MoAb was administered at the time of infection. The surviving mice were euthanized on Day 15 and lung and spleen tissues collected were processed for measuring bacterial burden. The lungs were culture positive from all the 8 survivors. ETI-204 produced by (b) (4) cell line used for the manufacture of Baxter or Lonza products was not included for testing.

Efficacy in New Zealand White rabbits and cynomolgus monkeys (Clinical Microbiology)

The definitive efficacy studies were conducted in *B. anthracis* challenged juvenile New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*) and cynomolgus monkeys (*Macaca fascicularis*) because it would be unethical to deliberately expose healthy human volunteers to an infectious dose of *B. anthracis* spores.

Natural history studies: Characterization of an animal model i.e., understanding the natural history of disease, is important for understanding the efficacy of the products in the animal

models for the treatment of inhalational anthrax. The applicant referred to three published studies and four studies in the DMF [REDACTED] (b) (4) to support the choice of NZW rabbits and cynomolgus monkeys challenged with approximately 200X the 50% lethal dose (LD_{50}) spores of the Ames strain, a capsulated strain, of *B. anthracis* by aerosolization for measuring the efficacy of ETI-204. The studies show that both NZW rabbits and cynomolgus monkeys have well defined physiological and pathological responses to inhalational anthrax that are similar to humans. Due to the high mortality rate and rapid time to death, the NZW rabbit model is a more stringent model than cynomolgus monkeys. Gross lesions and histological findings observed in rabbits and cynomolgus monkeys were similar to those of inhalational anthrax in humans. Presence of bacteremia or PA occurs early, relative to some of the other indicators of infection. Detection of PA appears to be a useful trigger for intervention. Both NZW rabbits and cynomolgus monkeys infected with the Ames strain of *B. anthracis* by inhalational route with approximately 200X LD_{50} meet the essential data elements of an animal model and are useful models for evaluating treatment and prophylaxis against inhalational anthrax (for more details see sections 2.1 and 6.1).

Efficacy of ETI-204 alone or in combination with an antibacterial drug: The applicant performed 25 studies to evaluate the efficacy of ETI-204 administered as a single-dose alone or in combination with an antibacterial drug, IV or IM, for the treatment, post-exposure prophylaxis, and pre-exposure prophylaxis of inhalational anthrax in NZW rabbits and cynomolgus monkeys infected with the spores of the Ames strain of *B. anthracis* by aerosolized route. Of the 25 studies, the efficacy of ETI-204 was measured in combination with an antibacterial drug in 8 studies. The antibacterial drugs used for studies in rabbits were levofloxacin or doxycycline whereas ciprofloxacin was used in monkey studies. All studies, except 3 rabbit studies, were performed at [REDACTED] (b) (4) two studies (AR035 and AR037) [REDACTED] (b) (4) and one (AP-10-055) at [REDACTED] (b) (4) and one (AP-10-055) at the United States Army Medical Research Institute for Infectious Diseases (USAMRIID).

Study design: Animals were purchased from [REDACTED] (b) (4) for all the studies except for one rabbit study (Study AP-10-055). Experimentally naive animals were included in all the studies. Animals, in all studies except one (Study AR001), were exposed to 200X LD_{50} of *B. anthracis* via the inhalational route. Treatment with ETI-204 alone or in combination with an antibacterial drug was administered based on either trigger-to-treat i.e., after the onset of disease or at a fixed time point post-exposure or prior to exposure. In rabbits, PA positive findings by an electrochemiluminescent (ECL) assay or a significant increase in temperature were used as a biomarker for the trigger-to-treat. In monkeys, the trigger-to-treat was based on a PA positive finding by an ECL assay; an increase in temperature was not a reliable marker for trigger-to-treat due to diurnal rhythms.

The primary endpoint for all the ETI-204 monotherapy studies was an improvement in survival compared to placebo; for combination studies the survival rate was compared with the antibacterial drug. In most of the studies, animals were followed for microbial burden (bacteremia and PA levels) in the blood at different time intervals. In many studies, tissues collected at the time of necropsy in surviving and/or non-surviving animals were examined for the presence of bacteria by histology and/or cultures.

Microbiologic measurements included in the study were blood cultures and/or detection of PA by ECL assay as well as enzyme linked immunosorbent assay (ELISA). Culture is considered to be an established method for detection of bacteremia and is recommended as a biomarker for

therapeutic intervention. Other assays are experimental assays; the performance characteristics of these assays were reviewed by Dr Lynette Berkeley.

Blood cultures: Different methods used in different studies for measuring bacteremia included enriched blood cultures (qualitative), qualitative blood cultures, and/or quantitative blood cultures. For enriched cultures, blood was collected in sodium polyanethol sulfonate (SPS) whereas the anti-coagulant used for the other culture methods was ethylene diamine tetra acetic acid (EDTA).

Enriched (qualitative) cultures were performed by inoculating 1 mL of blood in brain heart infusion (BHI) broth or other appropriate culture broth at an approximate 1:10 dilution for a minimum of 24 hours and up to 64 hours at 37° C. A portion (~40 µL) of this broth culture was plated on blood agar plates to determine (qualitatively) the presence or absence of colony morphology consistent with *B. anthracis*. Other types of **qualitative blood cultures** were performed by inoculating either 10 µL or 40 µL of blood in EDTA onto tryptic soy agar (TSA) plates. **Quantitative blood cultures** were performed by plating of 100 µL of whole blood (collected in EDTA tubes) and a series of dilutions in triplicate on TSA plates. Blood samples were stored at room temperature until diluted and plated; typically samples were plated within 6 hours of collection with the exception of terminal samples which may have been held longer. The results were expressed as cfu/mL. The majority of the cfu counts were based on 2 of the 3 replicates. The lower limit of detection (LLOD) by quantitative culture method in different studies varied (see Appendix-1); the reason(s) for this are unclear. Overall, the studies showed that the enriched culture method was more sensitive compared to the other two culture methods. This could be due to a higher volume of blood used for enriched cultures compared to the other two methods as well as the anti-coagulant used. SPS was used as an anticoagulant for enriched cultures and EDTA for the other two methods. EDTA is known to have antibacterial properties and can decrease isolation of bacteria from blood.

Protective antigen: Detection of PA, in the sera from NZW rabbits and cynomolgus monkeys, by an **ECL assay** was used as a trigger-for intervention in some of the studies. As stated above, the performance characteristics of the ECL assay were reviewed by Dr Lynette Berkeley. The rabbit anti-PA polyclonal antibody was used as a capture antibody that detects the whole molecule of PA (PA83) as well as its fragments (PA63 and PA20). Overall the results support a LLOD of 4 ng/mL. However, the positive controls used were below the LLOD (1 ng/mL and 2 ng/mL for rabbit and monkey studies, respectively) and showed positive findings. The PA measurements, by the ECL assays, were not affected by the presence of antibacterial drugs *in vitro*, such as ciprofloxacin and levofloxacin. Presence of edema factor (EF), lethal factor (LF), or endogenous anti-PA antibodies in the serum may interfere with the detection of PA; the testing was limited to EF and LF at a concentration of 10 ng/mL. It would have been useful to test different concentrations of EF and LF. Cross-reactivity with other organisms such as *Bacillus cereus* was not examined.

PA levels, at different time points post-challenge were measured by **ELISA** in many of the NZW rabbit and cynomolgus monkey studies; the assays used in different studies varied (see Appendix-2). The ELISA assay used in some of the studies was based on rabbit anti-PA polyclonal antibodies which detected the whole molecule of PA (PA83) as well as its fragments; the PA measured in such an assay was termed as **total PA**. Like for the ECL assay, presence of EF, LF, or endogenous anti-PA antibodies in the serum may interfere with the detection of PA leading to false negative findings. In some of the other studies, ETI-204 was used as the capture

antibody; the PA detected by such a method was termed as **free PA**. The free PA assay does not detect PA20 fragment of the PA molecule as the capture antibody used in the assay is ETI-204 that binds to the PAD4 domain of PA63 fragment; also, the PA bound to ETI-204 or endogenous anti-PA antibodies will not be detected. The applicant states that the term ‘free PA’ implies PA not bound to ETI-204 and is a misnomer as the PA may still be complexed with other proteins such as LF or EF. The *in vitro* studies showed that the presence of EF, LF, or endogenous anti-PA antibodies in the serum may interfere with the detection of PA; such an effect may be concentration dependent. Cross-reactivity with other organisms such as *B. cereus* was not examined. There was no interference in the presence of ciprofloxacin or levofloxacin. As there was variability in the performance of the assays used in different studies, the LLOD, lower limit of quantitation (LLOQ), and upper limit of quantitation (ULOQ) varied among the assays (for details see Appendix-2). The studies suggest that ELISA is useful for comparing the levels of PA in circulation at different time points post-challenge and post-treatment. Some of the factors e.g., EF, LF, or anti-PA antibodies including ETI-204, are likely to be present in circulation and their concentration may vary at different time intervals during the study period; presence of one or more of these factors may lead to variability in PA levels at different time intervals.

The presence of PA, prior to challenge, was tested by the ECL assay and/or ELISA in animals, prior to challenge, in some of the studies; few rabbits and monkeys tested positive. It remains unclear whether such positive findings were false positives due to cross-reactivity with other organisms such as *B. cereus*, which is known to be common in animals and that the assays are not specific to PA of *B. anthracis*, or the animals had prior exposure to *B. anthracis*.

Blood culture is the most reliable method for the detection of bacteremia. However, the culture results can take up to 24 to 48 hours. Due to rapid progression of disease after inhalation of the spores of *B. anthracis*, PA positive findings, by a screening ECL assay, is an appropriate biomarker for therapeutic intervention. However, due to limitations of the performance of the ECL assay discussed above, efficacy analysis was based on animals that were bacteremic.

Measurement of immune parameters: The applicant measured anti-PA IgG antibodies by ELISA or ECL assay and toxin neutralizing antibodies (TNAs) in the sera of animals in some of the NZW rabbit and cynomolgus monkey studies. The purpose was to measure antibody response to challenge at different time intervals post-challenge. The assays used for measuring **anti-PA IgG antibodies** varied among the studies. In some assays, anti-rabbit IgG or anti-monkey IgG polyclonal antibodies were used as the capture reagents; these assays were designed to measure endogenous anti-PA IgG antibodies in rabbits and monkeys, respectively. In other assays, PA and non-specific Protein A/G were used as the capture reagents; although these assays were designed to measure concentrations of ETI-204, endogenous anti-PA IgG antibodies are detected as well. However, if the ETI-204 is known to be absent in the sera of animals e.g., prior to administration of ETI-204 or if ETI-204 is degraded/eliminated and unlikely to be present in circulation (ETI-204 half-life was ~3 to 4 days in NZW rabbits and ~5 to 12 days in cynomolgus monkeys), then the results would reflect the presence of host’s anti-PA IgG antibody response. The LLODs, LLOQs, and ULOQs varied for these assays for different animal studies (for details see Appendix-3). The antigen specificity of the IgG antibodies was not evaluated. The assays are appropriate for comparing the anti-PA IgG antibody levels at different time intervals post-challenge within a study. However, the use of these assays for screening animals prior to challenge is not appropriate. It would have been better to use a polyclonal antibody against *B. anthracis* lysate for screening of animals to ensure absence of any past-exposure; also, both IgM and IgG antibodies should have been measured.

Testing of animals for anti-PA antibodies, prior to challenge with the spores of *B. anthracis*, was performed at the test facility for some of the studies, during the quarantine period. The presence of anti-PA antibody positive findings was reported in few NZW rabbits and cynomolgus monkeys in some of the studies. The reason(s) for these antibody positive findings, prior to challenge, remain unclear. As discussed above, due to limited testing to evaluate the performance of the assays, especially specificity and cross-reactivity, these assays are not useful for screening animals prior to exposure.

TNA appears to be appropriate for measuring neutralizing antibodies (for details see Dr Berkeley's microbiology review). However, the assay does not distinguish between ETI-204 and the animals' endogenous antibody response. Based on the half-life of ETI-204 in infected rabbits and monkeys, the antibodies measured at Day 28 should be a reflection of host's antibody response and not ETI-204 administered.

Different products of ETI-204 (Lonza, Baxter, and [REDACTED]^{(b) (4)}) were used for measuring efficacy in different NZW rabbit and monkey studies (see Appendix-4).

Results: The studies indicate that ETI-204 at a dose of 16 mg/kg, pre-exposure, post-exposure, or after the onset of disease, is effective in improving survival and reducing microbial burden. Most of the treated animals that survived became blood culture and PA negative whereas non-surviving animals remained bacteremic. Few animals in the control group survived. The reason(s) for the survival of these animals remain unclear.

Variability in PA concentrations among the animals within a group in the same study was observed over time. As the assay used for testing within a study was the same such variability could be due to the intermittent release of PA from germinating bacteria, presence of interfering factors such as EF, LF, anti-PA antibodies, ETI-204, and/or the protective effect(s) of the inflammatory host cells such as macrophages/monocytes, neutrophils, and lymphocytes which should phagocytize the antigens that are released upon lysis of the cells.

Despite the variability in the microbiological assays used in different studies, there appears to be an inverse correlation between microbial burden (bacteremia and PA levels) at the time of treatment and survival. A delay in treatment decreased survival. The incidence and severity of clinical observations were less when ETI-204 was administered prior to exposure as compared to post-exposure or after the onset of disease.

A combination of ETI-204 with an antibacterial drug will be an added benefit especially when the treatment is delayed and the microbial burden is higher. This could be due to different mechanisms of action as the antibacterial drug targets the vegetative bacteria and ETI-204 neutralizes the PA in circulation thereby preventing the formation of edema and lethal toxins; this should inhibit *B. anthracis* growth and multiplication, and thus prevent subsequent mortality.

Bacteria were observed in most of the tissues from the non-surviving animals by microscopic examination as well as culture. However, no bacteria were observed in most of the tissues from the surviving animals; exceptions included presence of bacteria in the lung and sometimes lymph

nodes from some of the animals. Presence of residual bacteria in the lung is thought to reflect spores and not vegetative bacteria (Henderson *et al.*, 1956¹).

The measurement of anti-PA IgG antibodies and TNA levels was limited to surviving animals in some of the studies. High antibody levels were observed at the end of study in the surviving animals. Testing of anti-PA IgG antibodies in sera collected at terminal time point from non-surviving animals was limited to 13 animals in one study (AR0315); antibodies were detected in 2 animals treated at 24 hours post-challenge with either 4 mg/kg or 16 mg/kg of ETI-204.

The ciprofloxacin or levofloxacin minimum inhibitory concentrations (MICs) were determined for the inoculum used for aerosol challenge and isolates collected at terminal time point from animals in some of the ETI-204 + antibacterial drug combination studies. Treatment of rabbits with up to 50 mg/kg levofloxacin for 3 days or monkeys with 10 mg/kg or 26 mg/kg of ciprofloxacin for four days did not promote the growth of levofloxacin or ciprofloxacin resistant bacteria as no change in MICs was observed. After a short term treatment of 3 to 5 days, it is unlikely that resistance to drug would develop.

Effect of re-challenge: In a re-challenge study in NZW rabbits, administration of ETI-204 with or without concurrent levofloxacin resulted in detectable anti-PA antibodies on Day 28 post-challenge which persisted up to 9 months post-challenge. The animals were protected against re-challenge. ETI-204 administration did not interfere with development of anti-PA IgG antibodies or TNAs; presence of endogenous antibody response may help in clearance of circulating PA and decrease the effects of toxins of *B. anthracis*.

Overall, the studies show that the efficacy of different products of ETI-204 is similar.

For more details of efficacy of ETI-204 please refer to Sections 2 and 6 of this review.

2. Integrated Summary of Clinical Microbiology Studies

ETI-204 is intended to treat inhalational anthrax infection and human efficacy studies were not conducted because it would be unethical to deliberately expose healthy human volunteers to an infectious dose of *B. anthracis* spores. The definitive efficacy studies were conducted in *B. anthracis* challenged juvenile New Zealand White (NZW) rabbit (*Oryctolagus cuniculus*) and cynomolgus monkey (*Macaca fascicularis*) models. Characterization of the animal model is important for understanding the efficacy of the products in the animal models for the treatment of inhalational anthrax.

Microbiologic measurements in the animal efficacy studies include qualitative and quantitative blood culture, detection of protective antigen (PA) by electrochemiluminescent (ECL) assay and quantitation of PA levels by enzyme linked immunosorbent assay (ELISA). As stated above in Section 1, different quantitative culture methods as well as assays to measure PA levels in serum were used for different studies; the limit of detection of these assays varied (for details see Appendix-1 and 2).

¹ Henderson DW, Peacock S, and Belton FC. Observations on the prophylaxis of experimental pulmonary anthrax in the monkey. *J Hyg* (1956).54 (1): 28-36.

Culture is considered to be an established method for detection of bacteremia and is an appropriate biomarker for therapeutic intervention. However, the culture results can take up to 24 to 48 hours. Therefore, detection of PA by ECL assay was used as a biomarker for trigger-to-treat in both NZW rabbit and cynomolgus monkey studies; due to the limitations in the performance of these assays (for details see Section 1 above), the efficacy analysis was based on bacteremic animals.

2.1. Natural history studies (Animal model characterization)

The applicant did not conduct any natural history studies. However, the applicant does refer to three published studies as well as four studies sponsored by the National Institute of Allergy and Infectious Disease (NIAID, NIH) to support the choice of NZW rabbits and cynomolgus monkeys challenged with *B. anthracis* spores of the Ames strain by aerosolization for measuring the efficacy of ETI-204. The NIAID studies were submitted to the DMF [REDACTED]^{(b)(4)}. All the NIAID sponsored studies were [REDACTED]^{(b)(4)}. The Ames strain of *B. anthracis* used in the studies conducted at [REDACTED]^{(b)(4)} was derived from a subculture of *B. anthracis* received from the United States Army Medical Research Institute for Infectious Diseases (USAMRIID).

2.1.1. New Zealand White rabbits

NZW rabbits were infected with the spores of the Ames strain of *B. anthracis* by aerosolization with a 3-jet Collison nebulizer with a muzzle only exposure. Briefly, the concentrations of spores in the aerosol were determined by culture of samples collected from an impinger which sampled the aerosol that actually reached the rabbits. The actual inhaled dose for each rabbit was calculated using plethysmography data. The target 200X 50% lethal dose (LD_{50}) i.e., [REDACTED]^{(b)(4)} cfu was based on the study by Zaucha *et al.* (1998)² in 22 NZW rabbits. The aerosol inhaled dose for challenge of animals in the 3 studies conducted at [REDACTED]^{(b)(4)} varied from 168 and 450 times the LD_{50} .

All challenged rabbits exhibited a significant increase in body temperature, C-reactive protein (CRP), and neutrophil/lymphocyte (N/L) ratio as well as a significant decrease in white blood cells (WBC). All animals were bacteremic and succumbed to disease with an average time to death between 2 and 5 days following challenge (Table 1). Body temperature and disease-specific parameters (bacteremia and circulating PA) were detected relatively early in disease progression, whereas changes in nonspecific clinical parameters (hematology, CRP, and clinical signs of disease) were delayed. Detection of PA appears to be a useful trigger for intervention as culture results can take time. However, detection of bacteremia by culture was the most reliable method.

Gross lesions observed included red foci in the appendix, ovaries, and stomach, discoloration (red) of the lungs (representing hemorrhage), and enlargement of the spleen; these findings are consistent with those published by Zaucha *et al.* (1998)². Microscopic findings, graded semi-quantitatively, were consistent with inhalational anthrax and included presence of bacteria with appropriate morphology (large square ended bacilli) in vessels and extravascularly in tissues,

² Zaucha GM, Pitt LM, Estep J, Ivins BE, and Friedlander AM. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. *Arch Pathol Lab Med* (1998) 122: 982-992.

with necrosis (particularly of lymphoid tissues), hemorrhage, and fibrin accumulation with or without a component of suppurative inflammation.

The two unchallenged control rabbits, included in one study, did not exhibit clinical or physiological changes during the period of observation.

Overall, the lesions observed in juvenile rabbits were comparable to humans. Some of the differences include the relatively mild mediastinal lesions and a lower incidence of anthrax-related pneumonia in rabbits compared with humans. These differences may be attributed to the greater susceptibility of rabbits to anthrax. It is unclear if lesions in older rabbits will mimic more closely with humans.

All the three studies conducted at [REDACTED]^{(b) (4)} suggest that NZW rabbits show reproducible and well defined physiological and pathological responses to inhalational anthrax.

Table 1: Summary of the natural history studies in NZW rabbits and cynomolgus monkeys performed at [REDACTED] (b) (4)		
Parameters	NZW Rabbits [†] (N=31)	Cynomolgus Monkeys [‡] (N=12)
Baseline characteristics of animals		
Age	Not specified	5 years
Body weight (Range)	2.5-3.5 kg	2.7 -7.3 kg
B. anthracis strain, aerosolization, and inhaled dose		
B. anthracis strain	Ames	Ames
Spore lot	B23 and B30	B31
Aerosolization	Muzzle only	Head only
MMAD ¹ (Range)	1.13-1.19 µm [REDACTED] (b) (4)	1.13-1.18 µm [REDACTED] (b) (4)
Target LD ₅₀ (cfu)	200X	200X
Inhaled LD ₅₀ (Range)	168X – 450X	198X – 678X
Survival at Day 28		
Survival n/N (%)	0/31 (0)	2/12 (16.7)
Time to death (Range)	2-5 days	2-9 days (for the animals that died)
Time (hours) to increased temperature, bacteremia and PA positive in all animals		
SIBT ² Mean (Range)	13-69	Not reliable (confounded by the diurnal temperature patterns)
Bacteremia ³ (Range)	23-75	24-60
PA-ECL ⁴ (Range)	Not done	30-60
PA-ELISA ⁴ (Range)	24-48	24-60
Trigger for intervention	PA ^{+ve} or elevated temperature	PA ^{+ve}
Signs and symptoms	Lethargy, anorexia, tachypnea, seizure, and/or morbidity	Inappetence, stool abnormalities, posture changes, inactivity or lethargy, signs of respiratory abnormalities (e.g. coughing, wheezing, and labored respirations), unresponsiveness, seizures, and/or moribund (recumbent, weak, or unresponsive), fever.
Necropsy	Red foci in the appendix, ovaries, and stomach; discoloration (red) of the lungs (representing hemorrhage); enlargement of the spleen.	Body cavity effusions, red lung discoloration, liver foci, red or dark foci in the brain, enlargement and mottling of the ovary, and enlargement and/or dark color of multiple lymph nodes. Lesions of vascular damage (hemorrhage, edema and parenchymal necrosis) in multiple organ systems were typical of anthrax. Two of the ten animals in the challenged group showed lesions in the brain upon necropsy. Lesions were observed in many of the other tissues assessed.
Microscopic findings	Presence of bacteria (large square ended bacilli) in vessels and extravascularly in tissues, with necrosis (particularly of lymphoid tissues), hemorrhage, and fibrin accumulation with or without a component of suppurative inflammation.	Hepatic sinusoidal leukocytosis, pulmonary hemorrhage, mediastinal lymph node edema and lymphoid depletion, and splenic fibrin exudation and lymphoid depletion, presence of large rod-shaped bacteria consistent with <i>B. anthracis</i> in many of the organs

¹MMAD= The mass median aerodynamic diameter

²SIBT: To assess change in temperature trends, the average and standard deviation (SD) for individual baseline body temperature for each individual rabbit were determined. A significant increase in body temperature (SIBT) was dependent upon each rabbit's inherent variation in baseline body temperature readings and was defined as three consecutive readings (or two occurrences of two consecutive significant readings) that were greater than two times the SD of that rabbit's baseline average temperature.

³Blood cultures, 40 µL of blood was collected in EDTA tubes and cultured at 37°C for at least 48 hours.

⁴PA measured by ELISA (enzyme linked immunosorbent assay) in one rabbit study and by ECL assay and ELISA in the monkey study at [REDACTED] (b) (4)

[†]Based on 3 studies conducted at [REDACTED] (b) (4) except for PA and necropsy findings were based on one study only.

[‡]The animals utilized on this study were transferred from a previous study.

2.1.2. *Cynomolgus monkeys*

All the cynomolgus monkeys included in the study were used previously in another study (the study was not specified). Cynomolgus monkeys were infected with the spores of the Ames strain of *B. anthracis* by aerosolization with a 3-jet Collison nebulizer with a head only exposure; otherwise, the aerosolization procedure was similar to that used for NZW rabbits. The target 200X LD₅₀ [REDACTED]^{(b)(4)} cfu) was based on the study by Vasconcelos *et al.* (2003)³ in 12 juvenile cynomolgus monkeys. The aerosol inhaled dose for challenge in the study varied from 198 and 678 times the LD₅₀.

Six animals were utilized as unchallenged control animals and served to contrast the clinical and physiological signs and symptoms specific to anthrax observed in the challenged animals. Unchallenged control animals exhibited moderate changes in clinical parameters when manipulated at a frequency designed to monitor disease, beyond the normal monitoring of healthy study animals. There were distinct changes seen in challenged animals that were absent from unchallenged controls.

Two of the 12 challenged animals survived the period of observation of 28 days (Table 1). The reasons for their survival are unclear; these 2 animals were PA negative from Day 8 post-challenge. Immune status of the animals was not measured.

All the challenged animals were bacteremic and PA positive within 60 hours of challenge. One of the unchallenged animals was stated to be culture positive with less than 5 colonies at Day 28. The reason(s) for culture positive finding in an unchallenged animal remain unclear.

Fever (based on 6 consecutive significantly increased readings in temperature) was reported in 10 of the 12 animals. However, fever was not considered to be a reliable marker of disease as the results were confounded by the diurnal temperature patterns. Like for the studies in NZW rabbits, detection of PA appears to be an appropriate trigger for intervention. One unchallenged animal (# 23375) in the uninfected group was PA positive at 48 hours by the ECL assay; the applicant stated that this was due to a technical error. The reason(s) for PA positive finding in this animal are unclear. It would have been useful to perform blood cultures to ensure that animals are culture negative prior to challenge.

All challenged monkeys exhibited an increase in white blood cells (WBC) at Day 8, neutrophil and lymphocytes at about 48 to 54 hours post-challenge. However, due to general diurnal rhythms observed in unchallenged animals, these parameters may not be useful as a trigger for intervention or measuring efficacy of the therapeutic agent. C-reactive protein (CRP) levels increased between 22 and 66 hours post-challenge; however, an increase in CRP levels was observed in unchallenged animals. CRP is an acute phase reactant and could easily be affected by the frequent handling of the animals. It appears that these non-specific indicators may not be beneficial in defining illness compared to disease specific parameters.

Specific diagnostic parameters (bacterial cultures and circulating PA) were detected relatively early in disease progression, whereas the exhibition of clinical parameters of disease (increase in

³ Vasconcelos D, Barnewall R, Babin M, Hunt R, Estep J, Nielsen C, Carnes R, and Carney J. Pathology of inhalation anthrax in cynomolgus monkeys (*Macaca fascicularis*). *Laboratory Investigation* (2003) 83 (8): 1201-1209.

body temperature, changes in hematologic parameters, and outward clinical signs of disease) were delayed relative to confirming animals were infected.

Comments:

All the studies conducted at [REDACTED] (b) (4) suggest that both NZW rabbits and cynomolgus monkeys have well defined physiological and pathological responses to inhalational anthrax (Table 1) that are similar to humans. Due to the high mortality rate and rapid time to death, the NZW rabbit model is a more stringent model than cynomolgus monkeys. Lesions observed in rabbits and cynomolgus monkeys were similar to those of inhalational anthrax in humans.

Presence of bacteremia or PA occurs early relative to some of the other indicators of infection. Detection of PA appears to be a useful trigger for intervention as culture results can take approximately 24 to 48 hours. However, detection of bacteremia by culture is the most reliable method.

Both NZW rabbit and cynomolgus monkeys infected with the Ames strain by inhalational route with approximately 200X LD₅₀ meet the essential data elements of an animal model and are useful models for evaluating treatment and prophylaxis against inhalational anthrax.

2.2. Efficacy of ETI-204 – treatment (after the onset of disease) studies

The efficacy of different doses of ETI-204 administered intravenously (as a bolus) was measured in NZW rabbits (2 studies: AR021, and AR031) and cynomolgus monkeys (4 studies: AP201, AP202, AP203, and AP204). The objective was to evaluate efficacy of ETI-204 (either Baxter or Lonza products). The primary efficacy endpoint was survival at the end of the study (usually 28 days post-challenge).

Information on efficacy of ETI-204 was available from three other studies [two in rabbits (1030 and 1045) and one in monkeys (1056)]; these studies were sponsored by NIAID. The primary objective was to evaluate the efficacy of ETI-204 in combination with an antibacterial drug; ETI-204 was included as a comparator in these three studies.

All studies were conducted at [REDACTED] (b) (4) All animals were purchased from [REDACTED] (b) (4) experimentally naïve, and challenged with spores (target inhaled dose was 200X LD₅₀) of the Ames strain of *B. anthracis* as summarized above for the natural history studies.

2.2.1. New Zealand White rabbits

The efficacy of different doses of ETI-204 was evaluated in four studies (AR021, AR031, 1030 and 1045). All studies were randomized; all studies, except one (AR033), were open label; two of the studies (AR021 and AR033) were GLP studies (Table 2). Prior to challenge, all animals included in the study were PA negative by the ECL assay; of all the animals included in the studies, one rabbit in Study 1030 was PA positive (1.22 ng/mL) by ELISA. Blood cultures were negative for all animals included in studies AR021 and AR033. Otherwise, all animals were stated to be healthy. Three different spore lots used for challenge for the four studies were B31, B34, and B37 (Table 2). The baseline characteristics of the animals, and inhaled inoculum dose of spores were similar among the animals in the four studies (Table 2). The mass median

aerodynamic diameter (MMAD)⁴ ranged from 1.09 and 1.27 μm and was consistent with lower respiratory tract deposition.

Although the inhaled dose of spores and the mean LD₅₀ were similar among the animals in different studies, there were more animals in studies AR033 (54%) and 1045 (47%) with $\geq 200X$ LD₅₀ compared to studies AR021 (36%) and 1030 (27%). The trigger for treatment was positive PA finding by an ECL assay or a significant increase in body temperature (SIBT)⁵ in all studies except Study 1045; if no trigger was observed, all animals were treated at a fixed time post-challenge (72 hours) in AR021 or AR033. All animals in Study 1045 were treated at 72 hours post-challenge. A majority of the animals were bacteremic, PA positive, or showed an increase in temperature at the time of initiation of treatment.

All animals were followed for time to death, clinical observations, and microbiological parameters. The tissues from non-survivors and/or survivors were processed for histological evaluations and/or culture. The toxin neutralizing antibodies (TNAs) were measured in serum from the surviving animals in one study (1030).

Results:

Effect of treatment on survival: Overall, the studies suggest that ETI-204 at a dose of 8 mg/kg and 16 mg/kg was effective in improving survival compared to the control group (Table 2). The efficacy of ETI-204 in the animals treated with either 8 mg/kg or 16 mg/kg in Study AR033 was similar. All the non-surviving animals died within 10 days of challenge.

One rabbit (K99373 in Study AR021) in the control group that survived was treated based on SIBT as a trigger; this animal was PA negative by the ECL assay and culture positive at the time of trigger. However, at 24 hours post-treatment, the rabbit was culture negative and remained negative for the duration of the study. No bacteria were observed by culture in any of the tissues. The reason for survival of one rabbit in the control group is unclear.

Effect of treatment on microbial burden: All rabbits which survived to the end of the study became PA negative by Day 8 post-challenge, and remained negative for the duration of the study. PA levels were detectable in more of the vehicle group of animals between 8 and 48 hours post-treatment, compared to the ETI-204-treated animals.

All rabbits which survived to the end of the study became culture negative by eight days post-challenge and remained culture negative for the duration of the study. Of the rabbits that died, almost all were culture positive when tested at the terminal time point.

⁴ The mass median aerodynamic diameter (MMAD) is determined by averaging the cumulative median size from the aerosol size distributions obtained from the aerosol particle sizer for all aerosol exposures.

⁵ SIBT was defined as a temperature reading \geq a two standard deviation (SD) increase from (daily) baseline temperature either three consecutive times or two consecutive times twice (measured hourly).

Table 2: Overview of efficacy studies (monotherapy) of inhalational anthrax in NZW rabbits and cynomolgus monkeys									
Parameter	NZW rabbits				Cynomolgus monkeys				
	AR021 (b) (4)	AR033	1030	1045 ^{\$}	AP202	AP201	AP203	AP204	1056 (b) (4)
Site									
Randomized	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Blinded	No	Yes	No	No	Yes ¹	Yes	Yes	Yes ¹	No
GLP	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No
Baseline characteristics of the animals									
Age (Range)	7-8 months	7-19 months	4 months	5 months	2.7-5 years	2.9-5.1 years	3.0 - 5 years	2.6-3.3 years	2.0-4.0 years
Body weight (kg)	2.9 - 3.5	3.2-4.0	2.4 - 2.7	2.5-3	2.2-4.6	2.9 - 5.1	3.0 - 4.8	2.5-3.3	2.3-3.7
Target and inhaled dose of <i>B. anthracis</i> spores									
Target LD ₅₀	200	200	200	200	200	200	200	200	200
Inhaled dose LD ₅₀ Mean±SD	180.4±55.9	200.7±35.4	180.3±59.1	196.5±51.2	257.1±49.6	199.4±59.8	288.6±71.2	212.2±43.5	194.4±61.2
Inhaled spores (cfu×10 ⁷)	(b) (4)								
≥200 LD ₅₀ (%)	35.5	54.3	27.3	46.9	84.0	39.5	93.8	58.3	43.8
Spore lot	B31	B37	B34	B34	B37	B35	B37	B36	B35
ETI-204 product and dose: Survivors in all animals n/N (%)									
ETI-204 Product	Baxter	Baxter	Baxter	Baxter	Baxter and Lonza	Baxter	Lonza	Baxter	Baxter
ETI-204 0 mg/kg	1/10 (10)	0/14 (0)	0/6 (0)	0/6 (0)	0/17 (0)	2/14 (14.3)	2/16 (12.50)	1/16 (6.3)	0/8 (0)
ETI-204 1 mg/kg	4/10 (40)*	4/14 (28.6)*	ND	ND	ND	ND	ND	ND	ND
ETI-204 4 mg/kg	13/17 (76.5)	6/14 (42.9)*	ND	ND	ND	11/14 (78.6)	ND	4/16 (25.0)	ND
ETI-204 8 mg/kg	ND	10/14 (71.4)*	12/16 (75)*	7/11 (63.6)	ND	11/15 (73.3)	1/16 (6.25)	ND	4/8 (50)*
ETI-204 16 mg/kg	16/17 (94.1)*	9/14 (64.3)*	ND	ND	6/17 (35) ^{†, *} 5/16 (31) ^{‡, *}	ND	ND	8/16 (50.0)	ND
ETI-204 32 mg/kg	ND	ND	ND	ND	ND	ND	6/16 (37.5)	ND	ND
ETI-204 product and dose: Survivors based on bacteremic animals prior to treatment n/N (%)									
ETI-204 0 mg/kg	0/9 (0)	0/13 (0)	0/6 (0)	0/3	0/17 (0)	2/14 (14.3)	2/16 (12.5)	1/16 (16.3)	0/8
ETI-204 1 mg/kg	3/8 (38)	2/12 (16.7)*	ND	ND	ND	ND	ND	ND	ND
ETI-204 4 mg/kg	11/15 (73)*	3/11 (27.3)*	ND	ND	ND	10/13 (76.9)*	ND	4/16 (25.0)	ND
ETI-204 8 mg/kg	ND	9/13 (69)*	8/12 (66.7)*	5/9 (55.6%)*	ND	11/15 (73.3)*	1/16 (6.3)	ND	4/8 (50.0)*
ETI-204 16 mg/kg	8/9 (89)*	8/13 (62)*	ND	ND	6/17 (35) ^{†, *} 5/16 (35) ^{‡, *}	ND	ND	7/15 (46.7)*	ND
ETI-204 32 mg/kg	ND	ND	ND	ND	ND	ND	5/15 (33.3)	ND	ND

¹Baxter; [‡]Lonza product; ^{\$}No vehicle administered; ND=Not done; NA= Not applicable. 'N' represents number of animals tested and 'n' that survived.

¹Not fully blinded

PTT=Prior to treatment; *Statistically significant compared to control group (for details see statistics review by Dr Xianbin Li, PhD)

Effect of microbial burden on survival: Microbial burden (bacteremia and PA levels) was measured in Study AR033. There appears to be a trend towards a decrease in survival in animals with higher microbial burden prior to treatment.

Tissue bacterial assessments: Bacteria were observed by histology and culture of most of the tissues (spleen, lymph nodes, lungs, and other organs) from the non-surviving animals. Slightly more bacteria were evident in organs from control animals as compared to the non-surviving animals treated with ETI-204. However, no bacteria were observed in most of the tissues from a majority of the animals that survived. Culture is more sensitive than histological examination for detection of bacteria in tissues.

In one study (AR033), the cerebral spinal fluid (CSF) was processed for bacterial cultures. The CSF from a majority (22/26) of the non-survivors was culture positive; of these, 13 animals were in the control group, 3 treated with 1 mg/kg ETI-204, 2 treated with 4 mg/kg ETI-204, 2 treated with 8 mg/kg ETI-204, and 2 treated with 16 mg/kg ETI-204. The CSF from all the surviving rabbits was culture negative.

Clinical Observations: Abnormal clinical signs were observed starting approximately 48 hours post-challenge and were consistent with those observed in the natural history studies summarized above. Animals that succumbed to disease showed a characteristic progression of signs that followed from unremarkable to observations of lethargy, respiratory abnormalities, and morbidity. Animals that survived until scheduled sacrifice were documented as lethargic with signs of respiratory abnormalities and reduced food consumption within the first few days post-challenge. The animals returned to normal observations generally between Days 5 and 11 post-challenge with occasional stool abnormalities or reduced food consumption.

Necropsy and Histopathology: Gross lesions in rabbits dying post-challenge were similar to those summarized above for natural history studies. No gross lesions were reported for the surviving animals that were terminated at study completion for gross and microscopic evaluation.

Microscopic findings were similar to those summarized above for natural history studies and present in all rabbits that died or became moribund during the study. As stated above, slightly more bacteria were evident in organs from the animals in the control group as compared to the animals treated with ETI-204. There were no other qualitative differences in lesions of anthrax among the control and treated groups. No significant microscopic lesions were present in the rabbits that survived until the end of the study; however, in one study (1030), minimal chronic inflammation in the lung and variable clusters of hemosiderin or eosinophilic granular debris-laden macrophages within the spleen were reported suggesting inflammation/infection of these animals prior to Day 28.

Neuropathological evaluations: Neuropathological evaluation was performed by microscopic evaluation of the brain in some of the surviving and non-surviving animals from one study (AR033). The applicant states that of the 15 animals that died, nine died with either no microscopic changes or with just scattered hemorrhage and/or areas of intravascular bacteria suggesting that these animals died prior to the bacteria leaving the blood stream due to

septicemia without any notable morphologic response. Six of the non-surviving animals had notable microscopic changes. These changes included observations of an inflammatory response in four animals and extravascular bacteria/notable hemorrhage in two animals without a visible inflammatory response; however, there was a lack of a dose response. The lack of a dose response may be due to biologic variation. However, these findings should be interpreted with caution due to a small number of animals examined.

Role of immune response in conferring protection: The immune response parameters measured were limited to the measurement of anti-PA IgG (Study AR034) and TNAs using murine macrophages (J774A.1 cells) (Studies 1030 and AR034) in the serum from animals that survived. Both anti-PA IgG antibodies and TNAs were present in the animals that survived. It would have been useful to measure antibodies in non-surviving animals to evaluate the role of antibodies in conferring protection.

Comments:

Overall, the studies suggest that ETI-204 at a dose of 16 mg/kg is effective in protecting against death due to anthrax when administered intravenously after the onset of disease. There appears to be a trend towards a decrease in survival in animals with higher microbial burden (bacteremia and PA levels) prior to treatment.

2.2.2. *Cynomolgus monkeys*

The efficacy of different doses of ETI-204 (Baxter or Lonza product) was evaluated in five randomized studies (AP201, AP202, AP203, AP204, and 1056). All studies, except one (Study 1056), were blinded and GLP studies (Table 2). The baseline characteristics of the animals were similar among the animals in different studies. Prior to challenge, all animals included in the studies were culture negative as well PA negative by the ECL assay; of all the animals included in the study, two monkeys, one in Study AP201 and another in Study AP204, were PA positive by ELISA; PA concentration was >30 ng/mL and 10.7 ng/mL, respectively. Otherwise all animals were stated to be healthy.

The MMAD ranged from 1.09 and 1.27 μm and was consistent with lower respiratory tract deposition. Although the target LD₅₀ was same (200X LD₅₀) for all the studies, there was variability in the LD₅₀ for the inhaled inoculum among different studies (Table 2). The LD₅₀ was ≥ 200 in approximately 84% of the animals in Study AP202, 94% in Study AP203, 58% in Study AP204, 44% in Study 1056 and 40% in Study AP201 (Table 2). Three different spore lots (B35, B36, and B37) were used for challenge (Table 2). PA by ECL assay was used as a trigger for intervention; SIBT was not used as a trigger for intervention because of diurnal temperature rhythms in monkeys. If no trigger was observed, all animals were treated at a fixed time post-challenge, at approximately 54 hours.

All animals were followed for time to death, clinical observations, and microbiological parameters as was done for the rabbit studies. The tissues from non-survivors and/or survivors were processed for histological evaluations and/or culture. The anti-PA IgG antibodies and/or TNAs were measured prior to challenge in two studies (AP202 and 1056); all animals included

in the study were antibody negative; one animal that tested antibody positive in Study AP202 was not included in the study.

Results:

A majority of the animals were PA positive by ECL assay and/or bacteremic at the time of treatment. Bacteremia and PA levels, at the time of treatment, were the highest for animals in studies AP202 and AP203 compared to other studies (Figure 1). There was a positive correlation coefficient (0.723; p-value <0.0001) between bacteremia and PA levels (Figure 2). It is possible that different sensitivities of the assays (quantitative culture methods and PA ELISA) would affect such a correlation; however, the overall trend should be the same.

Figure 1: Cynomolgus monkeys - Bacteremia and PA levels prior to treatment by study and treatment

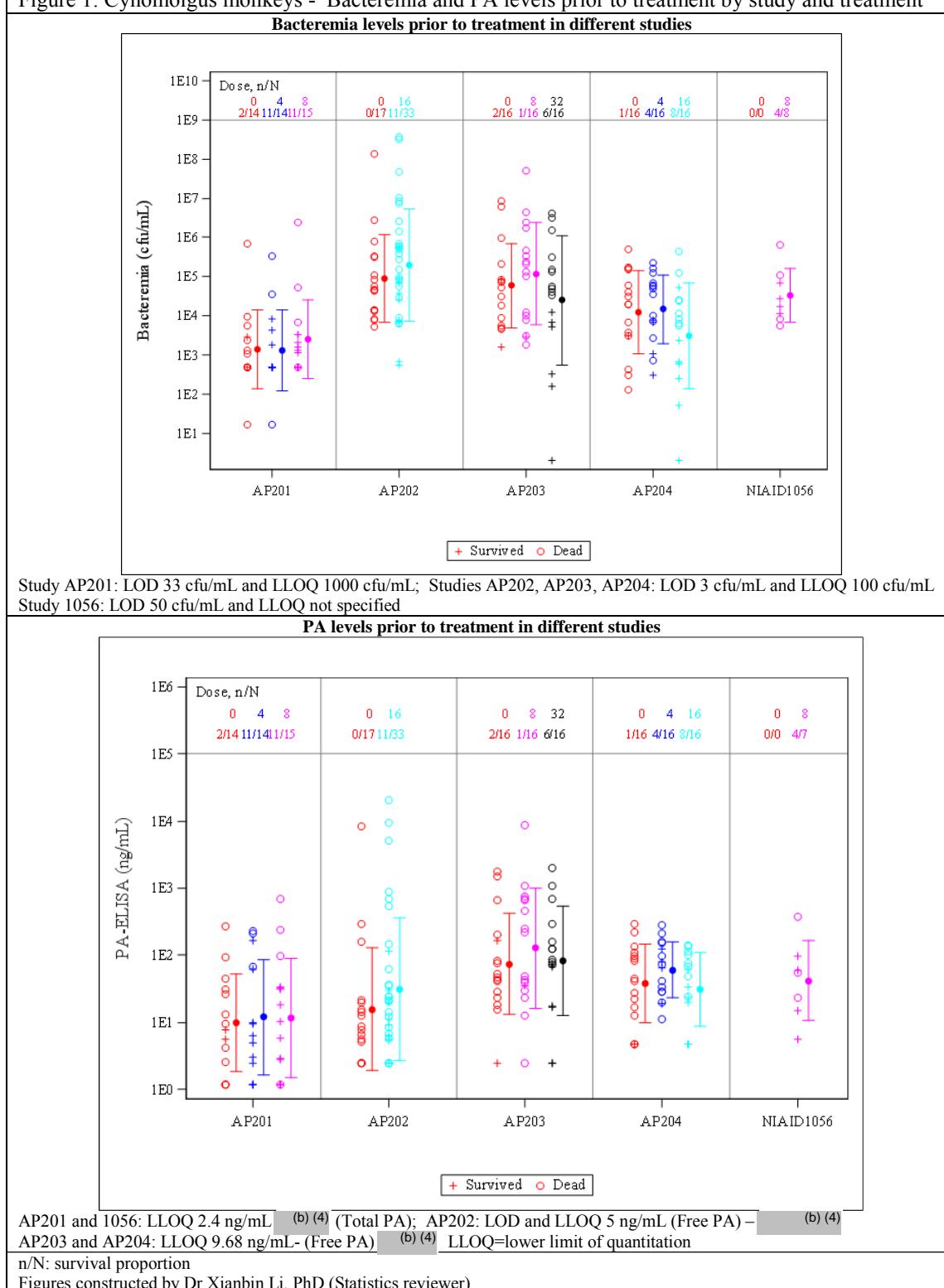
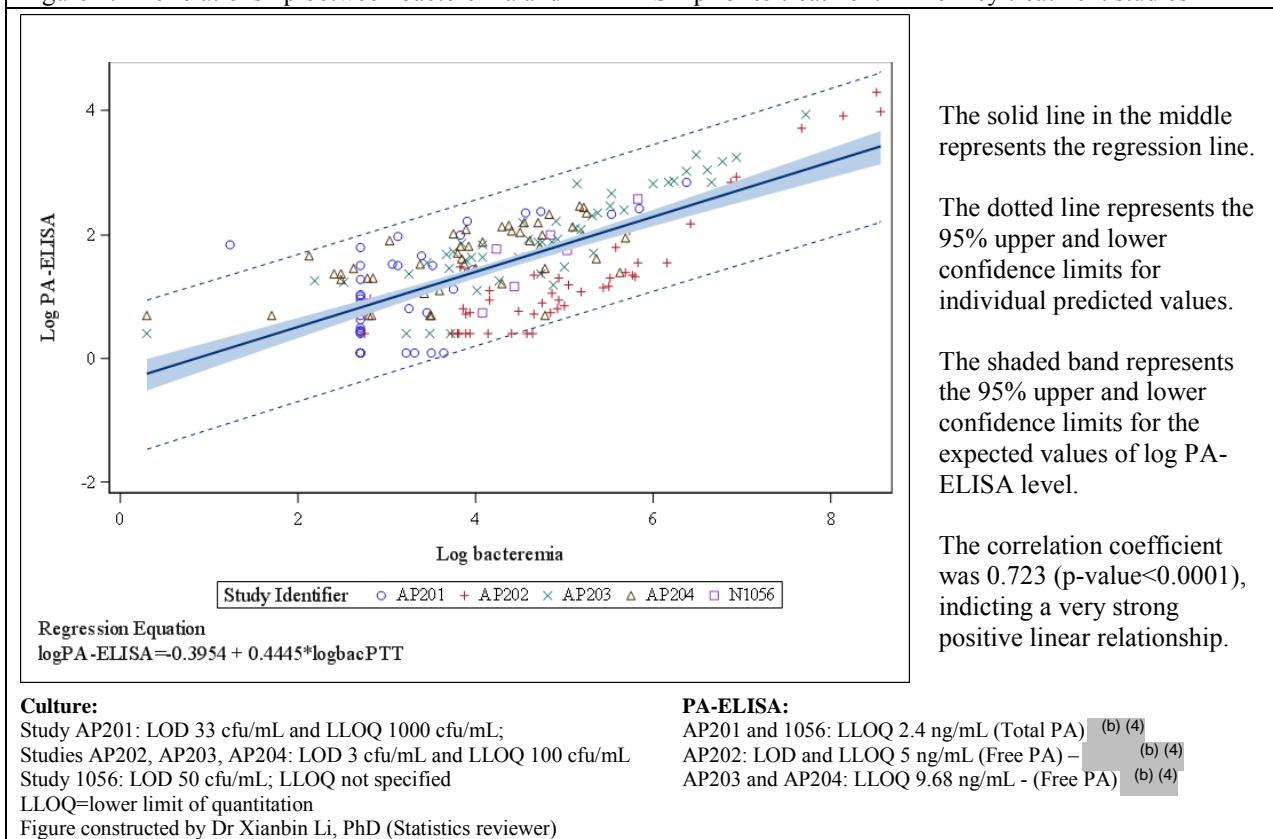


Figure 2: The relationship between bacteremia and PA-ELISA prior to treatment in monkey treatment studies



Effect of treatment on survival: An increase in survival after treatment with the Baxter product of ETI-204 was observed in four of the five studies; the efficacy at doses of 4 mg/kg and 8 mg/kg of ETI-204 in Study AP201 was similar (Table 2). In Study 1056, 8 mg/kg dose of ETI-204 (Baxter product) improved survival in about 50% of the monkeys. However, in Study AP203, the 8 mg/kg and 32 mg/kg doses of ETI-204 were not effective in improving survival.

The efficacy of Lonza and Baxter products of ETI-204 at a dose of 16 mg/kg was compared in Study AP202. Although survival was lower (35%) for animals in Study AP202 compared to other studies (Study AP201, AP204 or 1056) both Lonza and Baxter products of ETI-204 were equally effective in improving survival compared to the untreated control animals. A majority of the treated non-surviving animals died within 8 days of challenge in all studies.

A majority of the control group animals died by Day 7 post-challenge; five animals (two in Study AP201, two in Study AP203, and one in Study AP204) in the control group survived the period of observation; some of the microbiological findings for these five animals are summarized below:

- Study AP201: Two animals (C37762 and C39097) that survived were PA positive and bacteremic at the time of trigger. The PA levels at Day 7 post-challenge were 49 ng/mL (C37762) and 78 ng/mL (C39097); both monkeys became PA negative by Day 14. One animal was culture negative by Day 4 and another by Day 14. No bacteria were observed in the spleen and bronchial lymph node cultures at the time of necropsy.

- Study AP203: Two animals (C49041 and C49058) that survived were PA positive by the ECL assay and bacteremic at the time of treatment. PA by ELISA was below the lower limit of quantitation (LLOQ) for one animal (C49058) at the time of trigger; however, animal was PA positive between 15 minutes and 96 hours post-treatment. Animal C49041 was PA positive by ELISA at the time of trigger. Both animals were PA and culture negative at Day 7 post-treatment and remained negative until the end of study. Bacteria were observed by culture in the brain from one animal (C49041) but not from animal C49058; all other tissues (bronchial lymph nodes, kidney, liver, lung, and spleen) tested were culture negative.
- Study AP204: One animal (C42861) that survived was PA positive by ECL assay and bacteremic at the time of treatment; however, PA by ELISA was below the LLOQ. Animal became PA negative and culture negative by Day 7; lung, collected at the time of necropsy, was culture positive whereas other tissues were culture negative.

Recently, the applicant tested for anti-PA IgG antibodies in the serum from the five control animals that survived; these sera were collected prior to challenge and stored. Testing was done at [REDACTED] ^{(b)(4)}. No anti-PA IgG antibodies were detected [the assay's lower limit of quantitation (LLOQ) 100 ng/mL], prior to challenge, in these five survivors. Anti-PA antibody response post-treatment was not measured in these five animals.

The reason(s) for survival of the five control animals are unclear.

Effect of treatment on microbial burden: Treatment with ETI-204 decreased bacteremia and PA levels. In Study AP203, PA was undetectable in a majority of the animals within 15 minutes of treatment.

A majority of the surviving animals in all studies were culture and PA negative by Day 7 post-treatment whereas a majority of the control group animals remained bacteremic.

Effect of microbial burden on survival: As stated above, there was considerable variability in bacteremia and PA levels prior to treatment among the animals in different studies (Figure 1). The bacteremia levels were similar for animals in studies AP202 and AP203; PA levels were higher for animals in Study AP203 compared to Study AP202 (Figures 1 and 2). It is possible that higher microbial burden (bacteremia and PA levels) in studies AP202 and AP203 are associated with decreased survival. In Study AP201, the bacteremia and PA levels were lower and higher proportion of animals survived compared to other studies. There was an inverse relationship between microbial burden (bacteremia or PA levels) at the time of initiation of treatment and survival time. As stated above, it is possible that different sensitivities of the assays (quantitative culture methods and PA ELISA) would affect such a correlation; however, the overall trend should be the same.

Bivariate analysis of survival with bacteremia and PA-ELISA was performed to explore the relationship between bacteremia and PA levels versus survival based on animals in all the studies (for more details see Dr Xianbin Li's statistics review). At each ETI-204 dose, increased bacteremia levels were associated with decreased proportion of animals that survived (Table 3). No animals with bacteremia level of $\geq 10^6$ cfu/mL survived; only one animal in Study AP202 with bacteremia level of 10^5 cfu/mL survived.

Table 3: Survival by bacteremia level prior to treatment in monkey treatment studies

Bacteremia level (cfu/mL)	ETI-204 dose				
	0 mg/kg N=63	4 mg/kg N=30	8 mg/kg N=39	16 mg/kg N=49	32 mg/kg
<10 ⁴	4/28 (14.3%)	15/20 (75.0%)	12/19 (63.2%)	12/18 (66.7%)	5/5 (100%)
10 ⁴ - <10 ⁶	1/31 (3.2%)	0/10 (0%)	4/15 (26.7%)	7/23 (30.4%)	1/8 (12.5%)
≥10 ⁶	0/4	0	0/5	0/8	0/3

Table constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Increased PA levels prior to treatment, in each dose group, were associated with decreased proportion of animals that survived (Table 4).

Table 4: Survival by PA levels prior to treatment

PA level (ng/mL)	ETI-204 dose				
	0 mg/kg N=60	4 mg/kg N=30	8 mg/kg N=38	16 mg/kg N=45	32 mg/kg N=16
<10	3/18 (16.67%)	8/8 (100%)	7/9 (77.8%)	7/13 (53.9%)	2/2 (100%)
10 - <50	0/22 (0%)	2/8 (25.0%)	7/14 (50.0%)	6/16 (37.5%)	2/2 (100%)
50 or higher	1/20 (5.0%)	5/14 (35.7%)	2/15 (13.3%)	2/16 (12.5%)	2/12 (16.7%)

Table constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Please note that these findings are applicable when sample sizes are larger than or greater than 9 (for more details see Dr Xianbin Li's statistics review).

Effect of spore lot of the Ames strain used for challenge on survival: It is interesting to note that the spore lot used for challenge of animals in Studies AP202 and AP203 was B37 whereas for the other studies it was B35 or B36 (Table 2). As stated above, the survival rate was lower in studies AP202 and AP203 compared to other studies. It does not appear that there were any differences among the different spore lots used for challenge.

Tissue bacterial assessments: Tissue bacterial assessments were performed by either culture and/or histology in some of the studies. In surviving animals, no bacteria were observed in any of the tissues, by histology; by culture, no bacteria were observed in a majority of the tissues examined except lung and rarely brain (Study AP202 and Study AP203) as well as lymph nodes (AP204). However, bacteria were observed in a majority of the tissues from non-survivors. Detection of bacteria by culture was more sensitive than by microscopic observations.

In one study (AP204), the applicant did examine the stored (refrigerated) lung tissue, for the presence of spores or vegetative bacteria, by heat treatment of the tissue suspensions at 60°C for 60 minutes. The applicant concluded that the spores were present in the lung of surviving animals at Day 28 or Day 56. However, the results should be interpreted with caution as the refrigerated samples, prior to heat shock, were not processed for culture. The possibility of loss of viability of vegetative bacteria after long term storage cannot be ruled out. The applicant states that the presence of bacteria in the lung of surviving animals is probably a reflection of spores; previous studies (§ 5(b)(4) Study No. 1121- G924204; not a part of this BLA; Henderson *et al.*, 1956¹) have shown that spores can be found in the lung up to 56 days after challenge in surviving nonhuman primates.

Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these observations were consistent with those reported in the natural history studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is common with laboratory housed nonhuman primates) after Day 22 post-challenge. No change in body weight was reported in animals that survived to the end of the study.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge were similar to those summarized above for natural history studies. No gross lesions were reported for the surviving animals that were terminated at study completion.

Histopathology was performed in all studies except Study AP202. Microscopic lesions, in monkeys that were found dead or were euthanized in moribund condition were similar to those summarized above for natural history studies. Monkeys that survived until the end of study had no findings attributable to anthrax or ETI-204 administration. Inflammatory reaction was more in animals treated with ETI-204 compared to the control animals that died. Such an effect may be due to immune response to *B. anthracis* infection.

Neuropathological evaluations: Neuropathological evaluations were performed in three studies (AP201, AP203, and AP204) in select surviving and non-surviving monkeys. The areas of the brain that were most affected were those with the greatest surface area (cerebrum and cerebellum) and therefore with the most exposure to the meninges. All of the non-surviving animals had intravascular and/or extravascular bacteria, suggesting the cause of death in these animals was a bacterial infection. Only ETI-204-treated non-surviving animals had evidence of an inflammatory response, and always in association with extravascular bacteria. None of the deaths were attributed to ETI-204. Hemorrhage and extravascular bacteria were noted in control animals, but inflammation was limited to the ETI-204 treated animals.

Role of immune response in conferring protection: Anti-PA IgG antibodies, TNA, or any other measure of assessing immune response, post-challenge or after administration of ETI-204, was not performed.

As stated above, the immune parameters measured prior to challenge, were limited to the measurement of anti-PA IgG antibodies by ELISA and/or TNAs in two of the studies (AP202 and 1056); all animals included in the study were anti-PA IgG antibody negative. However, as stated above, the applicant did test the sera collected prior to challenge from five control group animals in different studies that survived the period of observation. All the five animals were stated to be negative for anti-PA IgG antibodies. The reason(s) for their survival is unclear.

Comments:

Overall, the studies suggest that ETI-204 at a dose of 16 mg/kg is effective in improving survival; efficacy of the Lonza product is similar to the Baxter product of ETI-204. Animals with lower bacteremia or PA levels prior to treatment are more likely to survive.

2.3. Efficacy of ETI-204 in combination with an antibacterial drug:

The efficacy of ETI-204 in combination with an antibacterial drug was measured in experimentally naïve NZW rabbits and cynomolgus monkeys. Animals for all studies, except one (Study AP-10-055), were purchased from [REDACTED] (b) (4) NZW rabbits for the Study AP-10-055 were purchased from [REDACTED] (b) (4). All animals were challenged with the spores (target inhaled dose was 200X LD₅₀) of Ames strain of *B. anthracis* as summarized above for the natural history and ETI-204 monotherapy studies. All studies were randomized (except one – Study AP-10-055), open-label, and non-GLP (except one - Study AR007) studies (Table 5).

2.3.1. New Zealand White rabbits

The efficacy of ETI-204 in combination with levofloxacin was evaluated in 5 studies (1030, 1045, AR028, AR034, and AR007) and doxycycline was used in one study (AP-10-055). Of the 5 studies that evaluated the efficacy of ETI-204 in combination with levofloxacin, a human equivalent dose of levofloxacin (50 mg/kg) was tested in 4 studies (1030, 1045, AR034, and AR007) and about 8 times lower dose (6 mg/kg for 3 days) of levofloxacin was tested in Study AR028. Levofloxacin was administered for 3 days in all studies except Study AR007 where levofloxacin was administered for 5 days. A low (less than human equivalent) dose of doxycycline was tested in Study AP-10-055.

All the studies to evaluate the efficacy of ETI-204 in combination with levofloxacin were conducted at [REDACTED] (b) (4) and 3 different spore lots (B24, B34, and B39) were used for aerosolization (Table 5). Study AP-10-055 was conducted at USAMRIID; the spore lot used in this study was not specified.

Prior to challenge, presence of PA by ECL and/or ELISA, was measured in some of the studies. Two animals (one in Study 1030 and one in Study AR034) were PA positive by ELISA; one animal in Study AP10-055 was PA positive by the ECL assay (Table 5). Bacterial cultures were performed in three studies (Studies AR028, AR034, and AP-10-055); all animals were culture negative. One animal in Study AR034 was anti-PA IgG antibody positive; four of the surviving animals in Study AR028 were antibody positive prior to challenge.

Although the target dose of spores was similar in different studies, there was lot of variability in the inhaled dose and number of animals with LD₅₀ of ≥200. The average LD₅₀ among different studies varied between 176 and 379; the percentage of animals with ≥200X LD₅₀ varied from 65% to 91% when the average inhaled dose was ≥219. In two studies (1030 and 1045), the LD₅₀ was 176 and 196 and the number of animals with ≥200X LD₅₀ was 30% and 46%, respectively. The MMAD ranged from 1.1 and 1.22 µm for the five studies performed at [REDACTED] (b) (4) this was consistent with lower respiratory tract deposition. MMAD for Study AP-10-055 was not specified.

Table 5: Overview of efficacy studies of ETI-204 in combination with an antibacterial drug of inhalational anthrax in NZW rabbits and cynomolgus monkeys							
Parameter	NZW rabbits					Cynomolgus monkeys	
	1030	1045	AR028	AR034	AR007 (b) (4)	AP-10-055 USAMRIID	1056 (b) (4)
Study site						USAMRIID	2469 (b) (4)
Randomized	Yes	Yes	Yes	Yes	Yes	No	Yes
Blinded	No	No	No	No	No	No	No
GLP	No	No	No	No	Yes	No	No
ETI-204 product	Baxter	Baxter	Baxter	Lonza	(b) (4)	Baxter	Baxter
Baseline characteristics of the animals							
Age (Range)	4 months	5 months	6-9 months	8 months	4 months	Not specified	2.0-4.0 years
Body weight (Range)	2.4 – 2.7 kg	2.5-3.1 kg	2.8-3.6 kg	2.8-4.2 kg	2.2-2.7 kg	Not specified	2.2-4.1 kg
PA-ECL n/N	0/54	0/54	ND	ND	ND	1/24	0/48
PA-ELISA n/N	1/54 (1.9%)	0/54	0/84	1/68 (1.5%)	ND	ND	0/48
Anti-PA IgG n/N	ND	ND	4/45 (8.9%)*	1/68 (1.5%)	ND	ND	0/48
TNA n/N	0/16*	ND	ND	0/60	ND	ND	0/48
Blood cultures (<i>B. anthracis</i>)	ND	ND	0/84	0/68	ND	0/24	0/48
Target and inhaled dose of <i>B. anthracis</i> spores							
Target LD ₅₀	200	200	200	200	200	200	200
Inhaled dose LD ₅₀ Mean±SD	175.6±49.1	196.3±43.6	219±44	218.5±47.6	274.4±50.6	378.8±225.2	185±67
Inhaled spores (cfu×10 ⁷)	(b) (4)						
≥200 LD ₅₀ (%)	29.6	46.3	69.0	64.7	91.2	70.8	29.2
Spore lot	B34	B34	B39	B39	B24	Not specified	B35
PTT=Prior to treatment; Ciprofloxacin 10 mg/kg [§] . Ciprofloxacin humanized dose in non-human primates is approximately 75 mg based on its exposure equivalent to 500 mg; therefore, based on a body weight of 2.9 kg in Study AP1056, 75 mg fixed dose will be 25.9 mg/kg.							
PA-ELISA: PA concentration was 1.22 ng/mL for one animal in Study 1030; for the animal in Study AR034 PA concentration was 29.3 ng/mL.							
*Only surviving animals tested. 'N' represents number of animals tested and 'n' represents number of animals positive							

Time of treatment initiation, with a combination of ETI-204 and antibacterial drug or the antibacterial drug alone, varied in different studies:

- Studies AR034: 30 hours post-challenge.
- Study AP-10-055: based on trigger to treat - PA positive finding by the ECL assay; if no trigger was observed, all animals were treated at a fixed time-point i.e., 30 hours post challenge.
- Studies 1045 and AR028: 72 hours post-challenge (delayed).
- Study 1030: 96 hours post-challenge (delayed).
- Study AR007: 9 hours post-challenge.

All animals were followed for time to death, clinical observations, and microbiological parameters. The tissues from non-survivors and/or survivors were processed for histological evaluations and/or culture. The anti-PA IgG antibodies and/or TNAs were measured prior to challenge and post-challenge in three studies (AR028, AR034 and 1030).

Results:

Effect of treatment on survival: The results showed that ETI-204 (4 mg/kg, IV or 8 mg/kg, IM) in combination with a human equivalent dose of levofloxacin (50 mg/kg for 3 days) in Study AR007, was more effective than levofloxacin alone in improving survival when administered at 9 hours post exposure for 5 days (Table 6). When ETI-204 at a dose of 8 mg/kg or 16 mg/kg IV in combination with levofloxacin (50 mg/kg) or levofloxacin alone was administered at approximately 30 hours (Study AR034) or 72 hours (Study 1045) post-challenge, the proportion of animals surviving in the two groups (i.e., treated with ETI-204 in combination with levofloxacin or levofloxacin alone) was similar. However, when treatment was delayed to 96 hours (Study 1030), ETI-204 (8 mg/kg, IV) in combination with levofloxacin (50 mg/kg) was more effective than levofloxacin alone; the number of animals was small as the treatment was delayed and majority of animals died before treatment was administered (Table 6). The lower survival rate in animals treated with human equivalent dose of levofloxacin (50 mg/kg) in Study AR007 in comparison to other studies could be due to shorter duration of treatment with the antibacterial drug alone administered prior to germination of spores to the vegetative form as well as shorter duration of treatment. Anti-bacterial treatment early after spore challenge has been observed to lead to persistence of spores and disease development following cessation of therapy.⁶

In Study AR028, the proportion of surviving animals was similar among the animals treated, at 72 hours post-challenge, with either ETI-204 (16 mg/kg, IV) in combination with a low dose of levofloxacin (6.5 mg/kg) or levofloxacin alone.

In Study AP-10-055, the efficacy of ETI-204 (8 mg/kg, IV) was measured in combination with a low dose of doxycycline. Although treatment with the combination of ETI-204 and doxycycline

⁶Gutting BW, Nichols TL, Channel SR, Gearhart JM, Andrews GA, Berger AE, Mackie RS, Watson BJ, Taft SC, Overheim KA, and Sherwood RL. Inhalational anthrax (Ames aerosol) in naive and vaccinated New Zealand rabbits: characterizing the spread of bacteria from lung deposition to bacteremia. Frontiers in Cellular and Infection Microbiology (2011) 2 (Article 87): 1-12.

showed a trend towards improved survival compared to doxycycline alone, such differences were not statistically significant.

Overall, the studies suggest that a combination of ETI-204 with an antibacterial drug is an added benefit especially when the microbial burden is high, low doses of either antibacterial drug or ETI-204 are used for treatment, or treatment is delayed.

Table 6: Combination studies - Summary of survival results

Study and ETI-204 product	Antibacterial Drug		ETI-204 Dose (mg/kg; Route)	Treatment Initiation (hours)	PA-ELISA (ng/mL) GM [†] (95%CI)	Survival % (# survived / # treated)		Difference and 95%CI (ETI-204 and antibacterial drug)
	HED*	Drug name				ETI-204 + antibacterial drug	Antibacterial drug	
Rabbits								
(b) (4) AR007 (b) (4) MoAb (b) (4)	HED ¹	Levofloxacin	4 IV	9 ± 3	NA	89% (8/9)	33% (4/12)	56% (11, 82)
			8 IM			100% (9/9)		67% (27, 90)
1030 Baxter	HED ¹	Levofloxacin	8 IV	96±1 (delayed)	84.8 (24, 303)	100% (4/4)	40% (2/5)	60% (-9, 95)
						100% (3/3) [‡]	50% (2/4) [‡]	50% (-30, 93)
1045 Baxter	HED ¹	Levofloxacin	8 IV	72±1	7.9 (4, 17)	82% (9/11) [‡]	78% (7/9) [‡]	4% (-36, 44)
AR034 (Phase I) Lonzza	HED ¹	Levofloxacin	16 IV	30	5.9 (5, 7)	95% (19/20)	100% (20/20)	-5% (-26, 11)
						94% (16/17) [‡]	100% (18/18) [‡]	-6% (-29, 11)
AR028 Baxter	< HED ²	Levofloxacin	16 IV	72±4 (delayed)	31.7 (20, 71)	68% (23/34)	58% (22/38)	10% (-12, 32)
						68% (23/34) [‡]	59% (22/37) [‡]	9% (-14, 30)
AP-10-055 Baxter	< HED ³	Doxycycline	8 IV	PA positive or at 30 hours	N/A	90% (9/10)	50% (5/10)	40% (-2, 72)
						89% (8/9) [‡]	56% (5/9) [‡]	33% (-14, 30)
Cynomolgus macaques								
1056 Baxter	< HED ^{4, §}	Ciprofloxacin	8 IV	PA positive + 24±12 (delayed)	235.3 (160, 345)	62% (8/13) [‡]	15% (2/13) [‡]	46% (4, 77)
2469 Baxter	< HED ^{4, §}	Ciprofloxacin	8 IV	PA Positive + 24±12 (delayed)	310.1 (193.6, 496.6)	57% (8/14) [‡]	31% (4/13) [‡]	26% (-14, 60)

*HED=human equivalent dose:

¹Levofloxacin 50 mg/kg for 3 days except for Study AR007- levofloxacin was administered for 5 days

²Levofloxacin 6.5 mg/k for 3 days

³Doxycycline 2 mg/kg, b.i.d. for 3 days

⁴Ciprofloxacin 10 mg/kg for 4 days

[†]GM: geometric mean for quantitative PA by PA-ELISA prior to treatment

[§]Ciprofloxacin 10 mg/kg - Ciprofloxacin humanized dose in non-human primates is approximately 75 mg based on its exposure equivalent to 500 mg; therefore, based on a body weight of 2.9 kg in Studies AP1056 and 2469, 75 mg fixed dose will be 25.9 mg/kg.

[‡]Represent bacteremic animals prior to the time of treatment

USAMRIID study: no electronic data provided. Doxycycline administered at 2 mg/kg bid, for 3 days.

Adapted from a Table constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on microbial burden: Treatment with a combination of ETI-204 and levofloxacin or levofloxacin alone decreased both bacteremia and PA levels. In most of the studies, the surviving animals treated with ETI-204 or ETI-204 + levofloxacin were PA negative by the ECL assay between 24 and 96 hours post-treatment; whereas a majority of the animals were culture negative by Day 7. There appears to be a trend towards a decrease in time to PA negative finding earlier in ETI-204 treated animals compared to levofloxacin treated animals; however, conversion of culture positive findings to culture negative appears to occur faster after treatment with an antibacterial drug. The changes in bacteremia levels over time were similar in the ETI-204+levofloxacin or levofloxacin treated animals.

Effect of microbial burden on survival: Based on information available from one study (Study AR028), increased quantitative bacteremia levels and PA levels at the time of treatment appear to be associated with decreased probabilities of survival. These observations are similar to those observed in the ETI-204 treatment studies summarized above in Section 2.2.

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the surviving animals irrespective of the treatment. However, bacteria were observed in almost all the tissues from all the animals in the control group and some of the tissues from animals treated with ETI-204 or levofloxacin that died.

Cultures: Tissues collected from non-surviving animals in two studies (Study AR028 and AR034) and some of the surviving animals in one study (AR007) were processed for cultures. Bacteria were observed in the brain, liver, lymph nodes, lung, and spleen of the majority of the animals in the control group and in most of the non-surviving animals treated with levofloxacin or levofloxacin + ETI-204. No bacteria were observed in the tissues from the surviving treated animals.

Clinical, necropsy, and histopathological observations: Clinical, necropsy, and histopathological findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above. Generally, surviving animals, treated with levofloxacin alone or the combination of levofloxacin and ETI-204, returned to normal between 7 and 10 days post-challenge with the exception of food consumption and sporadic documentation of decreased appetite or diarrhea. In one study (Study AR034) animals were observed until month 9 post-challenge; all animals remained normal except for occasional stool abnormalities.

Neuropathological examination was performed in select survivors and non-survivors in Study AR028. The neuropathologist concluded that animals treated with levofloxacin with or without ETI-204 and succumbed to the anthrax infection were more likely to mount an inflammatory response as compared to the untreated animals. However, survivors did not have microscopic changes with exception of one combination treated animal with mild hemorrhage in/near meninges interpreted to be unrelated to ETI-204. Overall, the applicant concluded that study survivors did not have any CNS lesions (morphologic changes) due to ETI-204.

Role of immune response in conferring protection: The immune response parameters measured were limited to the measurement of TNAs and/or anti-PA IgG antibodies by ELISA in surviving animals in three studies.

Toxin neutralizing antibodies: TNAs were measured in the serum from surviving rabbits in two studies (1030 and AR034) by the TNA assay using murine macrophages as the target cells; in both studies the TNAs were measured prior to challenge and at Day 28. In Study AR034, TNAs were measured at Month 2 in addition to Day 28 post-challenge. All the animals at Day -1 were TNA negative. The results showed that neutralizing antibodies were present post-challenge in all animals, in both studies, except one animal (L40821) treated with levofloxacin. This observation was consistent with the lack of an anti-PA IgG level in this animal through 6 months after primary challenge. There were no significant differences among the treatment groups for TNA. Antibody response in non-survivors at different time interval during the study was not measured.

Anti-PA IgG antibodies: Anti-PA IgG antibodies were measured in the serum from surviving animals in two studies (AR028 and AR034) prior to challenge and post-challenge by an ELISA or ECL assay. In Study AR034, antibody levels were measured at Days 7, 14, and 28 as well as Months 2, 3, 4, 5, and 6 post-challenge by ELISA whereas in Study AR028, the antibody levels were measured at Day 25 post-challenge by the ECL assay. Both ELISA and ECL assay detect ETI-204 as well as any endogenous anti-PA antibodies formed as the methods use PA as a capture reagent and protein A/G as the detection reagent. As the half-life of ETI-204 in NZW rabbits is approximately 3 to 4 days (for details see Clinical Pharmacology review), the results at later time points, especially at Day 28, may be a reflection of endogenous anti-PA antibody levels.

In Study AR028, the anti-PA antibodies were detected in all the surviving animals on Day 25.

In Study AR034, a majority of the animals were anti-PA IgG antibody positive between Day 7 and Month 7 post-challenge. At Days 7, 14, and 28 post-challenge, the anti-PA IgG levels in animals treated with levofloxacin were lower than animals treated with ETI-204 or ETI-204 + levofloxacin. As ETI-204 is also detected in the anti-PA IgG ELISA, the higher levels of anti-PA IgG antibodies in animals administered ETI-204 in comparison to animals administered levofloxacin alone, may be a reflection of endogenous IgG + ETI-204; the anti-PA IgG in animals treated with levofloxacin is a reflection of endogenous anti-PA IgG. The applicant states that by Day 28 post-challenge, the ETI-204 levels were either below the limit of detection or reduced compared to previous time points. Therefore, antibody levels at Day 28 and after reflect anti-PA IgG levels.

It should be noted that some of the animals (four in Study AR028 and one in study AR034) were anti-PA IgG antibody positive prior to challenge. Although the antibody titers prior to challenge were much lower (≥ 40 -fold) compared to post-challenge, the reason(s) for antibody positive findings prior to challenge remain unclear. The possibility of false positive findings or cross-reactivity with other organisms cannot be ruled out.

One of the limitations of these studies is that antibody levels in non-surviving animals post-challenge and post-treatment were not measured. It is unknown if the death of non-surviving animals was due to absence of antibody response.

Comments:

*Overall, the studies suggest that treatment with ETI-204 in combination with an antibacterial drug following exposure to *B. anthracis* is effective in improving survival and decreasing microbial burden. Bacteria were observed by microscopic examination and/or cultures of many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation.*

TNAs and anti-PA antibodies were present in the animals that survived. It would have been useful to measure TNA in non-surviving animals.

*Treatment with ETI-204 did not prevent induction of an antibody response in surviving animals. The pre-study serum samples from some of the rabbits had positive anti-PA antibodies before exposure to *B. anthracis* spore aerosols. The reason for this is unknown, but strongly suggests that the assay was not specific for anti-PA antibodies.*

2.3.2. Cynomolgus monkeys

The efficacy of ETI-204 (8 mg/kg, IV) in combination with a low dose ciprofloxacin (10 mg/kg for 3 days) was evaluated in 2 studies (1056 and 2469). Both studies were open-label non-GLP studies and conducted at [REDACTED] ^{(b) (4)} (Table 5). All animals were PA negative (by both ELISA and ECL assay) as well as culture negative on Day-7 i.e., prior to challenge. The anti-PA IgG antibodies by ELISA and/or TNAs were measured prior to challenge and at different time points post-challenge in both studies. In Study 1056, all animals were antibody negative (both TNA and anti-PA antibodies). In Study 2469, 12 of the animals were positive for anti-PA IgG antibody positive by ELISA; all animals were TNA negative (Table 5).

The spore lots of the Ames strain of *B. anthracis* used for challenge were B35 and B36 (Table 5). The MMAD ranged between 1.13 and 1.22 µm in the two studies and was consistent with lower respiratory tract deposition. The average inhaled dose was 185X and 223X LD₅₀ and approximately 29% and 67% of the animals had ≥200X LD₅₀. An increase in challenge dose was associated with higher bacteremia levels at the time of treatment. Treatment with ETI-204 (Baxter product) was delayed by 24 hours after PA positive finding by an ECL assay in both studies. All animals were followed for time to death, clinical observations, and microbiological parameters. The tissues from non-survivors and/or survivors were processed for histological evaluations and/or culture.

Results:

Effect of treatment on survival: The results showed that ETI-204 at a dose of 8 mg/kg, IV in combination with a low dose of ciprofloxacin was more effective in improving survival compared to levofloxacin alone; however, such differences were statistical significant in Study

1056 but not in Study 2469. Levofloxacin was less effective in Study 1056 compared to Study 2469. No antagonism was observed (Table 6).

Effect of treatment on microbial burden: Treatment with ETI-204 in combination with ciprofloxacin decreased bacteremia and PA levels. The mean time to resolution of bacteremia was shorter in animals treated with ciprofloxacin + ETI-204 or ciprofloxacin alone compared to ETI-204 monotherapy. The mean time to resolution of PA was shorter in animals treated with ciprofloxacin + ETI-204 or ETI-204 alone compared to ciprofloxacin alone. A majority of the terminal samples from animals that did not survive until Day 28 were bacteremic and PA positive.

Effect of microbial burden on survival: Higher bacteremia levels at the time of treatment were associated with decreased survival. Association between PA levels and survival was unclear.

Clinical, necropsy and histopathological observations: The clinical signs of disease as well as necropsy and histopathological findings were similar to those summarized above for the natural history and treatment studies. In treated animals that survived to the end of the study, most of these abnormal observations were not observed. Findings within the brain/meninges tended to predominate in animals treated with ciprofloxacin and/or ETI-204 (similar incidence and severity among treatment groups). Widespread intravascular bacteria were observed more often in untreated animals. Although some of the brain findings were observed in untreated animals that succumbed to infection, they were less severe. There were no anthrax-related microscopic findings in animals that survived until the end of the study.

Tissue bacterial assessments: Bacteria were observed by microscopic examination in many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation. Cultures of tissues were not performed.

Role of immune response in conferring protection: Post-challenge, no immune response parameters were measured in any of the studies.

As stated above, all animals included in the study were tested for anti-PA IgG antibodies by ELISA and TNA prior to challenge. Of all the animals included in the studies, 12 were anti-PA IgG antibody positive by ELISA in one study (2469) but none of the animals were TNA positive.

There does not appear to be any correlation between presence of anti-PA IgG antibodies prior to challenge and response to challenge or treatment. For example, two control animals survived and only one of those animals tested positive for anti-PA IgG (Animal A12335); nine anti-PA IgG positive animals that were randomized to treatment groups, three succumbed to challenge. The reason for anti-PA IgG positive findings in experimentally naïve animals is unclear. The possibility of cross-reactivity or false positive findings by the anti-PA IgG ELISA was not examined and cannot be ruled out.

Comments:

The studies suggest that treatment with ETI-204 in combination with an antibacterial drug following exposure to *B. anthracis* is effective in improving survival and decreasing microbial burden. Bacteria were observed by microscopic examination in many of the tissues from the treated animals that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation. Overall, the studies suggest that ETI-204 has a complementary mechanism of action to antibacterial drugs and therefore, this may be an added benefit.

2.4. Post-exposure prophylaxis

The post-exposure prophylaxis of [REDACTED] (b) (4) MoAb), Baxter, or Lonza products of ETI-204 was measured in NZW rabbits and cynomolgus monkeys. All the animals (NZW rabbits and cynomolgus monkeys) were purchased from [REDACTED] (b) (4)

2.4.1. New Zealand White rabbits

The post-exposure prophylaxis of [REDACTED] (b) (4) MoAb), Baxter, or Lonza product of ETI-204 in NZW rabbits was measured in 5 studies (AR004, AR012, AR0315, AR035, and AR037). Additional information was available from two other studies (AR007 and AR034); the primary objective of these two studies was to evaluate the efficacy of ETI-204 in combination with an antibacterial drug at a fixed time point post-exposure and ETI-204 was included as a comparator (Tables 5 and 7).

All studies were randomized and open label; four studies (AR007, AR012, AR035, and AR037) were GLP studies whereas others (AR004, AR034, and AR0315) were non-GLP studies (Tables 5 and 7). All studies, except two (AR035 and AR037), were conducted at [REDACTED] (b) (4) studies AR035 and AR037 were conducted at [REDACTED] (b) (4) (w) (4).

Prior to challenge, anti-PA IgG antibodies were measured in some of the studies. Three rabbits in Study AR037 were anti-PA IgG antibody positive; all animals were culture negative for *B. anthracis* (Tables 5 and 7). Study design was similar in all studies expect that the time of administration of ETI-204 varied. Animals were challenged with the Ames strain of *B. anthracis* as summarized above; the target LD₅₀ was 200X LD₅₀ for all studies. The average inhaled LD₅₀ ranged from 143-286; the number of animals with ≥200 LD₅₀ varied between 10% and 91% among different studies (Tables 5 and 7). The MMAD varied from 0.95 and 1.18 μm for the studies conducted at [REDACTED] (b) (4). The MMAD varied from 0.60 to 3.46 μm for the studies conducted at [REDACTED] (b) (4); the MMAD range was higher compared to that for the studies conducted at [REDACTED] (b) (4)

Treatment with ETI-204, either a fixed dose or based on body weight, was initiated at 9, 18, 24, 36, and/or 48 hours post-exposure in different studies by either IV or IM route.

Table 7: Overview of post-exposure prophylaxis studies of inhalational anthrax in NZW rabbits and cynomolgus monkeys

Parameter	NZW rabbits					Cynomolgus monkeys		
	AR004	AR012	AR0315	AR035	AR037	AP107	AP301	AP307 (b) (4)
Site								
Randomized	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Blinded	No	No	No	No	No	No	Yes	No
GLP	No	Yes	No	Yes	Yes	Yes	Yes	No
ETI-204 Product	(b) (4)	Baxter	Baxter	Lonza	Lonza	Baxter	Lonza	Lonza
Baseline characteristics of the animals								
Age (Range)	3-4 months	3.8 months	6-7 months	6-7 months	6-7 months	2-5 years	2.6-4.6 years	3-4 years
Body weight (kg)	2.0-2.8	2.6±0.1	2.9±0.2	3.3±0.2	3.5±0.3	2.5±0.3	2.8±0.2	3.2±0.5
Anti-PA IgG n/N	ND	ND	0/58	0/38	3/58 (5.2%)	ND	ND	ND
Blood culture (<i>B. anthracis</i>) n/N	ND	ND	0/58	0/38	0/58	0/40	ND	ND
Target and inhaled dose of <i>B. anthracis</i> spores								
Target LD ₅₀	200	200	200	200	200	200	200	200
Inhaled dose LD ₅₀ Mean±SD	182.8±63.1	200.5±64.3	236.0±33.7	285.5±82.2	143.1±44.6	314.9±78.3	401.7±152.9	204.5±67.6 (b) (4)
Inhaled spores (cfu×10 ⁷)	Not specified in the data sets							
≥200 LD ₅₀ (%)	41.7	39.3	91.4	89.5	10.3	97.6	97.6	50.0
Spore lot	Not specified	B30	B37	Ames strain obtained from the (b) (4)		B39	B39	B39

N represents number of animals tested and n represents number of animals positive; ND = not done

Results:

Effect of treatment on survival: The studies show that ETI-204 is effective in improving survival; the survival rate varied with the dose and time of administration of ETI-204 (Table 8). The post-exposure prophylaxis was measured by administering ETI-204 at a fixed time point in four studies (AR007- 9 hours, AR012 - 24 hours, AR034 -30 hours, and AR037- 24 hours). When ETI-204 was administered at a dose of ~4 mg/kg IV or ~8 mg/kg IM, at 9 hours post-exposure (Study AR007), all the treated animals survived. In Study AR012, treatment was delayed to 24 hours post-exposure; ETI-204 at a dose of ~4 mg/kg and ~8 mg/kg, IV, was effective in improving survival in 50% and 58% of the animals; however, when administered IM, the proportion of animals that survived was lower. Additionally, a higher dose (~15 mg/kg) of ETI-204 was less effective (Table 8). In Study AR037, ETI-204, administered IM, was effective in improving survival time in about one-third of the animals at either of the doses (8, 16 or 32 mg/kg); a dose-dependent effect was not observed (Table 8). The reason for lower survival and no dose-dependent effect in this study is unclear.

The effect of a delay in administration of ETI-204 was measured in three studies (AR004, AR0315, and AR035). In Study AR004, ETI-204 at a dose of ~4 mg/kg IV was administered at 24, 36, or 48 hours; ETI-204 was more effective when administered 24 hours post-exposure compared to the later time points (Table 8). At 24 hours, none of the animals were bacteremic and all of the animals that became bacteremic at Day 2 ultimately died. In Study AR035, ETI-204 16 mg/kg administered IM at 18 or 24 hours post-challenge was effective in improving survival of 60% of the animals (Table 8). None of the animals survived when ETI-204 was administered at 36 hours post-exposure.

All rabbits in the control group died within 7 days of challenge.

A decrease in survival with a delay in treatment is probably due to an increase in microbial burden post-challenge over time. Blood from all the surviving animals until the end of study was culture negative. All the animals that died or found moribund were bacteremic.

Clinical, necropsy and histopathological observations: The clinical signs of disease as well as necropsy and histopathological findings were similar to those summarized above for treatment studies. No alteration in body weights were reported during the 14 or 34 day period of observation.

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Table 8: Survival in post-exposure prophylaxis studies in NZW rabbits (Table constructed by Dr Xianbin Li, PhD)

Study	Route	Hours post challenge	ETI-204 mg	n/N (%) Survival	95% CI Adjusted 95% CI	Unadjusted one-sided p-value	
AR004 Elusys Day 28	IV	48	0	0/9 (0)			
		24	10 ^{\$}	8/10 (80.0)	0.80 [0.402, 0.975] [0.303, 0.986]	0.0001* (0.0083)	
		36	10 ^{\$}	5/10 (50.0)	0.50 [0.084, 0.813] [-0.017, 0.856]	0.010 (0.0083)	
		48	10 ^{\$}	3/7 (42.9)	0.429 [0.012, 0.816] [-0.084, 0.865]	0.0226 (0.0083)	
AR007 (b) (4) Day 34	IV	9	0	0/9 (0)			
	IV		10 ^{\$}	9/9 (100)	1 [0.629, 1]	<0.0001* (0.0125)	
	IM		20 ^{\$}	9/9 (100)	1 [0.629, 1]	<0.0001* (0.0125)	
AR012 Elusys Day 14	IM	24	0	0/9 (0)			
	IV		2.5 ^{\$}	1/9 (11.1)	0.111 [-0.224, 0.483] [-0.436, 0.610]	0.4073	
			10 ^{\$}	6/12 (50)	0.50 [0.094, 0.789] [-0.057, 0.859]	0.0074	
			20 ^{\$}	7/12 (58.3)	0.583 [0.187, 0.848] [-0.018, 0.904]	0.0026* (0.0036)	
	IM		5 ^{\$}	1/9 (11.1)	0.111 [-0.224, 0.483] [-0.436, 0.610]	0.4073	
			10 ^{\$}	3/9 (33.3)	0.333 [-0.071, 0.701] [-0.238, 0.794]	0.049	
			20 ^{\$}	5/12 (41.7)	0.417 [0.034, 0.725] [-0.134, 0.806]	0.0186	
			40 ^{\$}	4/12 (33.3)	0.333 [-0.066, 0.655] [-0.217, 0.749]	0.051	
AR0315 Baxter Day 28	IM	24	0	0/10 (0)			
		18	4 mg/kg	11/12 (91.7)	0.917 [0.535, 0.998] [0.425, 1]	<0.0001* (0.0063)	
		24	4 mg/kg	5/12 (41.7)	0.417 [0.065, 0.723] [-0.058, 0.786]	0.0131 (0.0063)	
		18	16 mg/kg	11/12 (91.7)	0.917 [0.535, 0.998] [0.425, 1]	<0.0001* (0.0063)	
		24	16 mg/kg	8/12 (66.7)	0.667 [0.290, 0.901] [0.172, 0.934]	0.0005* (0.0063)	
AR034 Phase I Lonza Day 28	IV	30	0	0/8			
			16 mg/kg	13/20 (65)	0.65 [0.156, 0.846] [0.300, 0.969]	0.0008* (0.0125)	
AR035 Lonza Day 28	IM	18	0	0/10 (0)			
		18	16 mg/kg	6/10 (60)	0.60 [0.213, 0.878] [0.119, 0.912]	0.0018*	
		24	16 mg/kg	6/10 (60)	0.60 [0.213, 0.878] [0.119, 0.912]	0.0018*	
		36	16 mg/kg	0/8 (0)	0 [-0.309, 0.369] [-0.387, 0.480]	0.5	
AR037 Lonza Day 28	IM	24	0	0/10			
			8 mg/kg	5/16 (31.3)	0.313 [-0.019, 0.587]	0.33	
			16 mg/kg	5/16 (31.3)	0.313 [-0.019, 0.587]	0.33	
			32 mg/kg	5/16 (31.3)	0.303 [-0.019, 0.587]	0.33	

^{\$}ETI-204 dose was a fixed dose: 2.5 mg= ~1.25 mg/kg; 5 mg =~2 mg/kg; 10 mg =~4 mg/kg; 20 mg =~8 mg/kg; 40 mg= ~15 mg/kg

*Statistically significant at the specified significant level with Bonferroni adjustment for multiple comparisons

Reference ID: 3881961

Tissue bacterial assessments: No incidences of bacteria were observed by culture and/or histology in any of the tissues tested from surviving animals. However, bacteria were observed in the organs from non-surviving animals in all groups.

Role of immune response in conferring protection: The immune response parameters measured were limited to the measurement of anti-PA IgG antibodies by ELISA in surviving animals in three studies (AR035, AR037 and AR0315). The assay used for studies AR035 and AR037 was the same as that for the combination studies summarized above and captured both ETI-204 and endogenous anti-PA IgG antibodies. Therefore, the reported anti-PA IgG value for a given sample represents residual ETI-204, if any, and endogenous anti-PA IgG antibodies. The LLOQ was 50 ng/mL. However, the assay used in Study AR0315 was different and the lower limit of detection (LLOD) was 1 µg/mL (for details see Dr Berkeley's microbiology review).

Despite the differences in the assays, high antibody levels were observed in all the surviving animals at Day 28 in all three studies. Testing of anti-PA IgG antibodies in sera collected at terminal time point from non-surviving animals was limited to 13 animals in one study (AR0315); antibodies were detected in 2 animals treated at 24 hours post-challenge with either 4 mg/kg or 16 mg/kg of ETI-204.

In Study AR037, 3 animals (one in the control group and 2 treated with ETI-204 8 mg/kg) were anti-PA IgG positive prior to challenge; all three animals died within 4 days of challenge. Additionally, 3 animals (one treated with 8 mg/kg and two treated with 16 mg/kg of ETI-204) were antibody positive at the time of death or were moribund; these positive findings may be a reflection of ETI-204 as the method used for detection of anti-PA IgG antibodies detects ETI-204 as well as endogenous anti-PA IgG antibodies; also the antibody response after primary challenge at Day 4 is likely to be IgM and not IgG. The antibody titers in the two animals (#3003 and #4010), treated with 16 mg/kg ETI-204, were high (~50,000 ng/mL).

The study suggests development of humoral response in surviving animals after treatment with ETI-204. However, it would have been useful to test all animals for IgM and IgG anti-PA antibodies as well as antibodies against a crude lysate of bacteria at different time points to understand the contribution of humoral response in conferring protection.

Comments:

Overall, the studies suggest that ETI-204 is effective in preventing death due to anthrax when administered post-exposure in NZW rabbits; the earlier the post-exposure treatment was administered, the survival rate was higher.

2.4.2. Cynomolgus monkeys

The post-exposure prophylaxis of Baxter or Lonza products of ETI-204 in cynomolgus monkeys was measured in 3 studies (AP301, AP307, and AP107). All studies were randomized; two were blinded (AP301 and AP305) and one (AP107) was an open-label study; two studies (AP107 and AP 301) were GLP studies (Table 7).

All studies were conducted at [REDACTED] (b) (4) and the study design was similar. Animals were challenged with the spores (spore lot no. B39) of the Ames strain of *B. anthracis* as summarized above; the target LD₅₀ was 200X LD₅₀ for all studies. The average inhaled LD₅₀ was variable and ranged from 205-402; the number of animals with ≥200 LD₅₀ varied between 50% and 98% among different studies. The MMAD varied between 1.14-1.30 µm (Table 7). Treatment with

ETI-204 was initiated at 18 or 24 hours post-exposure in different studies by either IV or IM route.

Results:

Effect of treatment on survival: In Study AP107, a dose-dependent effect of ETI-204 on survival was observed when administered IV (2 and 4 mg/kg) but not when administered IM (4 and 8 mg/kg) at 24 hours post-exposure (Table 9). In study AP307, ETI-204 at a dose of 16 mg/kg administered IM at 24 hours post-exposure was effective in improving survival (Table 9). In Study AP301, treatment with 8 or 16 mg/kg of ETI-204, IM, was administered at 18, 24, or 36 hours post-exposure; when intervention was delayed to 36 hours post-challenge, the proportion of animals that survived decreased (Table 9). When the treatment was delayed, dose also played a role in survival. For example, at 36 hours 8 mg/kg ETI -204 resulted in no survivors (0/6) while 16 mg/kg ETI-204 protected about 50% of the animals (3/6). All of the control animals died by Day 5 post-exposure. The efficacy of Baxter and Lonza products of ETI-204 was similar.

Effect of treatment on microbial burden: The animals that died were bacteremic. All the animals that survived were culture negative until terminal sacrifice (Day 28 or 56).

Effect of microbial burden on survival: The effect of treatment time on survival depended on the prior to treatment bacteremia levels. All deaths occurred in animals that were confirmed bacteremic prior to treatment. All animals that were culture negative prior to treatment including 2 animals in the 36 hour treatment group survived.

Tissue bacterial assessment: Most of the tissues from all of the animals that died on study were culture positive or bacteria were observed by microscopic examination. No bacteria were observed in most of the tissues from the animals that survived; exceptions included positive cultures of lymph nodes from some of the animals that survived.

Clinical, necropsy and histopathological observations: The clinical signs of disease as well as necropsy and histopathological findings were similar to those summarized above for treatment studies summarized above.

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Table 9. Survival rates in post-exposure prophylaxis studies in cynomolgus monkeys

Study	Route	Hours post challenge	ETI-204 mg/kg	n/N(%) Survival	Difference [95% CI] [Adjusted 95% CI]	One-sided p-value (sig. level)
AP107 Baxter Day 30 survival	IV or IM	24	0	1/6 (16.7)		
	IV	24	2	4/9 (44.4)	0.278 [-0.295, 0.641] [-0.391, 0.765]	0.210 (0.0063)
	IV	24	8	6/8 (75.0)	0.583 [0.018, 0.902] [-0.130, 0.941]	0.020 (0.0063)
	IM	24	4	6/8 (75.0)	0.583 [0.018, 0.902] [-0.130, 0.941]	0.020 (0.0063)
	IM	24	8	5/9 (55.6)	0.389 [-0.158, 0.777] [-0.292, 0.835]	0.087 (0.0063)
AP301 Lonza Day 28 or 56 survival ¹		18	0	0/6 (0)		
	IM	18	8	6/6 (100)	1 [0.471, 1] [0.438, 1]	0.0012 (0.0042)
	IM	18	16	6/6 (100)	1 [0.471, 1] [0.438, 1]	0.0012 (0.0042)
	IM	24	8	5/6 (83)	0.83 [0.230, 0.996] [0.196, 0.998]	0.0032 (0.0042)
	IM	24	16	5/6 (83)	0.83 [0.230, 0.996] [0.196, 0.998]	0.0032 (0.0042)
	IM	36	8	0/6 (0)		1.0000 (0.0042)
	IM	36	16	3/6 (50)	0.5 [-0.037, 0.882] [-0.069, 0.893]	0.0345 (0.0042)
AP307 Lonza Day 28 survival	IM	24	0	1/10 (10)		
		24	16	13/14 (93)	0.83 [0.431, 0.976] [0.347, 0.987]	0.001 (0.0083)
		36		6/14 (43)	0.33 [-0.068, 0.643] [-0.155, 0.699]	0.053
		48		4/14 (29)	0.18 [-0.234, 0.504] [-0.320, 0.570]	0.203

¹ Survival assessed after spore challenge (28 days) except for the 16 mg/kg IM dose in AP301 which was assessed at 56 days after spore challenge.

Sig.: Significance.

*Statistically significant at the specified significant level with Bonferroni adjustment for multiple comparisons.

Table constructed by Dr Xianbin Li, PhD (Statistics reviewer)

2.5. Pre-exposure prophylaxis

The efficacy of [REDACTED] ([REDACTED] MoAb) or Lonza products of ETI-204 was measured in NZW rabbits and in cynomolgus monkeys. All animals (rabbits and monkeys) were purchased from [REDACTED] and all the studies were conducted at [REDACTED] ([REDACTED])

2.5.1. New Zealand White rabbits

The efficacy of [REDACTED] ([REDACTED] anti-PA MoAb) product was measured in two randomized, open label, non-GLP studies (AR001 and AR003). In both studies, the age and body weights of the animals were similar (Table 10). The target LD₅₀ was lower (100X LD₅₀) for the animals in Study AR001 compared to the Study AR003 (200X LD₅₀). The LD₅₀ was ≥200 in approximately 14% and 81% of the animals in Study AR001 and Study AR003, respectively. The MMAD ranged from 1-2 µm.

A fixed single dose of ETI-204 was administered either IV or IM approximately 30 to 45 hours prior to exposure.

Table 10: Overview of pre-exposure prophylaxis studies of inhalational anthrax in NZW rabbits and cynomolgus monkeys

Parameter	NZW rabbits		Cynomolgus monkeys
	AR001	AR003	AP305
Site			(b) (4)
Randomized	Yes	Yes	Yes
Blinded	No	No	Yes
GLP	No	No	Yes
Baseline characteristics of the animals			
Age	13-17 weeks	13-17 weeks	2.4-4 years
Body weight	2.3 kg	2.5 kg	2.6 kg
Anti-PA IgG	ND	ND	ND
Target and inhaled dose of <i>B. anthracis</i> spores			
Target LD ₅₀	100	200	200
Inhaled dose (LD ₅₀)	162±47	287±81	221±78
Mean±SD			
≥200 LD ₅₀	14%	81%	53%
Spore lot	Not specified		B39
ETI-204			
Product used	(b) (4) anti-PA MoAb	(b) (4) anti-PA MoAb	Lonza
Dose (Route)	10 mg* (IV)	1.25, 2.5, 5, 10 mg* (IV) 20 mg* (IM)	16 mg/kg (IM)
Time of treatment	30-45 minutes	35 minutes	Day -3, -2, and -1

*Fixed single dose administered: 1.25 mg= ~ 0.5 mg/kg; 2.5 mg= ~ 1 mg/kg; 5.0 mg= ~ 2 mg/kg;

10.0 mg= ~ 4 mg/kg; 20.0 mg= ~ 8 mg/kg

IV=intravenous; IM=intramuscular; ND = not done

Results:

Effect of treatment on survival and microbial burden: ETI-204 ([REDACTED] anti-PA MoAb) was effective in improving survival when administered IV or IM, about 30-45 minutes prior to treatment in both studies (Table 11). A dose-dependent improvement in survival was observed when ETI-204 was administered, IV, approximately 30 to 45 minutes prior to exposure in Study AR003 (Table 11). ETI-204 doses of 10 mg (4 mg/kg) administered IV or 20 mg (8 mg/kg) administered IM were most effective in improving survival (Table 11).

All the surviving-treated animals, in Study AR001, were blood culture negative for every time point analyzed during the course of the study (Table 11). However, the presence of bacteria was

reported by culture of some tissues collected at the time of necropsy from 3 of the surviving treated animals (lymph node - one animal; lung - two animals).

In Study, AR003, a majority of the surviving ETI-204-treated animals were blood culture negative at all the time point analyzed during the course of the study; only 2 rabbits that survived (#K49261 treated with a dose of 5 mg IV and #K49273 treated with 20 mg IM) were bacteremic within 48 hours. Of the 14 treated rabbits that died, 8 were bacteremic at least at one of the time point post-challenge.

Of all of the tissues processed for bacterial cultures from the 26 surviving animals, presence of bacteria was reported in the lung from nine animals; there were no incidences of positive cultures in any of the spleen and lymph nodes examined (Table 11). Tissues from non-surviving animals were not processed.

All of the control group animals died within 4 days of challenge in both studies; all the rabbits were bacteremic, at least, at one time point between Days 2 and 5 (Table 11). Tissues from non-surviving animals were not examined for the presence of bacteria.

(b) (4) anti- PA MoAB [†]	Route	n/N (%) Survival	Unadjusted P-value	Proportion (%) of animals*	
				Bacteremic at least one time point post-challenge	Surviving animals with bacteria in tissues [‡]
Study AR001: (b) (4) 30-45 minutes prior to a targeted 100 LD₅₀ exposure					
0 mg	IV	0/5 (0)		5 (100)	ND
10 mg		9/9 (100)	0.0001	0 (0)	3/9 (33)
Study AR003: (b) (4) 35 minutes prior to a targeted 200 LD₅₀ exposure					
0 mg	IV	0/8 (0)		8 (100.0)	ND
1.25 mg		1/8 (12.5)	0.402	6 (75.0)	0/1 (0)
2.5 mg		5/8 (62.5)	0.004	1 (12.5)	1/5 (20.0) Lung [§]
5 mg		5/8 (62.5)	0.004	4 (50.0)	0/5 (0)
10 mg		7/8 (87.5)	0.0003	0 (0)	4/7 (57.1) Lung [§]
20 mg	IM	8/8 (100)	<0.0001	1 (12.5)	4/8 (50.0) Lung [§]

[†]Fixed single dose administered; 1.25 mg= ~ 0.5 mg/kg; 2.5 mg= ~ 1 mg/kg; 5.0 mg= ~ 2 mg/kg;
10.0 mg= ~ 4 mg/kg; 20.0 mg= ~ 8 mg/kg. IV=intravenous; IM=intramuscular

*Represent qualitative culture results

[‡]Represent culture results in tissues; tissues from non-survivors not done

[§]Lymph nodes, lungs and spleen processed; only lungs were culture positive

** There was no adjusted CI, because of no multiple comparisons

Statistical analysis by Dr Xianbin Li, PhD (for details see statistics review)

2.5.2. Cynomolgus monkeys

Study AP305 was a randomized, blinded, placebo-controlled GLP study (Table 10). The target LD₅₀ was 200X LD₅₀ and the average MMAD ranged from 1.21-1.22 µm. The LD₅₀ was ≥200 in approximately 53% of the animals. The efficacy of ETI-204 Lonza product, administered IM, was measured by administering on Day-3, -2, and -1 prior to exposure.

Results:

Effect of treatment on survival and microbial burden: ETI-204 at a dose of 16 mg/kg, administered IM, between Days-3 and -1 was effective in improving survival in all the treated animals; one of the control group animal survived (Table 12).

Bacteremia, in all the placebo group animals, was detectable and increased by 54 hours after spore challenge in both survivors and non-survivors. A majority of the treated animal remained culture negative for the duration of the study. The bacterial load between 1 and 7 days post-treatment was lower in treated animals compared to the animals in the control group. In addition, the bacteremia in the ETI-204-treated animals was transient and only present in low numbers; complete resolution of bacteremia occurred by 7 days post-challenge in all monkeys that survived to scheduled termination.

No bacteria were detected in any of the tissues processed for culture or histological examination in treated animals (Table 12).

Table 12: Survival at Day 28 in pre-exposure (30–45 minutes) prophylaxis studies in cynomolgus monkeys (Study AP305)								
ETI-204		n/N (%) Survival	Unadjusted P-value	Proportion of culture positive animals in blood* or tissues				
Dose	Day administered			Blood (at least one time) post- challenge*	Tissues by culture (microscopy)			
				Surviving animals				
0		1/10 (10)		10 (100%)	0/1 (0/1)	8/9 (9/9)		
16 mg/kg	-3	15/15(100)	<0.0001	2 (13.3%)	0/15 (0/15)	NA		
16 mg/kg	-2	14/14(100)	<0.0001	3 (21.4%)	0/14 (0/14)	NA		
16 mg/kg	-1	14/14(100)	<0.0001	1 (7.1%)	0/14 (0/14)	NA		

NA-not applicable
 *Represent results by quantitative culture
 Table constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Clinical, necropsy and histopathological observations: The clinical signs of disease as well as necropsy and histopathological findings although similar to those summarized above for treatment studies were reported in less number of animals and were less severe.

Comments:

*Overall, the studies in rabbits and monkeys suggest that ETI-204 prevents the development of inhalational anthrax when administered prior to challenge with *B. anthracis* spores.*

Presence of PA in serum was not assessed in the pre-exposure prophylaxis studies.

2.6. Response to re-challenge

The effect of re-challenge with *B. anthracis* spores of the Ames strain was evaluated in one NZW rabbit study (Study AR034). The objective was to investigate whether ETI-204 administration can prevent the development of a protective endogenous antibody response to PA. The study was performed in two phases. In phase I, rabbits were treated with either ETI-204, levofloxacin, or a combination of ETI-204 and levofloxacin at approximately 30 hours post-exposure and followed for up to 9 months post-exposure. As discussed above (for details see section 2.3 “Efficacy of ETI-204 in combination with an antibacterial drug”), the results suggest that levofloxacin (20/20; 100%) or a combination of ETI-204 + levofloxacin (19/20; 95%) were more effective in improving survival compared to ETI-204 (13/20; 65%) monotherapy (Tables 6 and 8). All the control animals succumbed to *B. anthracis* infection. All the animals that died were bacteremic and PA positive whereas all the animals that survived were culture and PA negative by Day 7 post-challenge.

The 52 survivors from Phase I study and 12 experimentally naïve animals (approximate age- and weight-matched) were re-challenged with an average 301 (\pm 69) LD₅₀ equivalents of *B. anthracis* spores via aerosol exposure nine months after the primary challenge; the LD₅₀ was >200 in all animals in the second exposure whereas after primary challenge the LD₅₀ was >200 in 65% of the animals. All animals were culture negative prior to re-challenge; only one animal was PA positive prior to re-challenge. Phase II part of the study was blinded to prevent potential bias in the study conduct and outcome assessment. No treatment was administered during Phase II. Animals were followed for up to 21 days after second challenge. A majority of the re-challenged animals survived until Day 21:

- 13/13 (100%) treated with ETI-204 in phase I.
- 19/20 (95%) treated with levofloxacin.
- 17 of 19 (89.5%) treated with the combination of ETI-204 + levofloxacin in phase I.

A majority of the animals remained culture as well as PA negative after re-challenge; bacteremia and PA positive findings in survivors were transient and present in small number (10%) of the animals. All animals that survived the re-challenge did not have a positive culture of the tissues assessed at the time of necropsy; however, all of the animals that died during phase II had a positive *B. anthracis* tissue culture result for at least three of the tissues tested. All naïve control animals that were challenged in phase II were bacteremic and PA positive; the bacteremia and PA levels at 24, 72, and 120 hours post- secondary challenge were greater than the animals in either of the treatment groups. Only one animal (control) that died post-challenge had gross lesions in Phase II; lesions were consistent with acute *B. anthracis* infection.

Role of immune response in conferring protection: The immune response parameters measured were limited to the measurement of anti-PA IgG antibodies by ELISA and TNAs in surviving animals. A majority of the animals were anti-PA IgG antibody positive between Day 7 and Month 7 post-challenge as well as at all-time points tested after re-challenge. The TNAs at Day 28 after primary challenge as well as 7 days before and Day 21 after second exposure were detected in surviving animals. TNA and anti-PA IgG antibody titers increased at Day 21 post-second challenge compared to Day-7. There was no difference in antibody levels among the animals in the three treatment groups. Overall, the study suggests development of antibody response in animals surviving after primary challenge; treatment with ETI-204 does not interfere with the development of humoral immune response. TNA and anti-PA IgG antibodies were not measured in non-surviving animals.

Clinical observations: Following second challenge through Day 7 post-challenge, some of the treated animals in all groups were documented as lethargic (5/49 survivors; 2/3 non-survivors) with signs of respiratory abnormalities (2/49 survivors; 2/3 non-survivors), stool abnormalities (17/49 survivors; 1/3 non-survivors), and reduced food consumption (24/49 survivors; 3/3 non-survivors). The animals returned to normal observations generally between 7-11 days of re-challenge with occasional stool abnormalities or reduced food consumption noted.

Necropsy and Histopathology: After re-challenge, only one animal in the control group that died had gross lesions as reflected by red foci in the large intestinal sacculus rotundus and appendix; these lesions are typical of anthrax in rabbits. Histopathology was not performed.

Comments:

Overall, the study shows that ETI-204 administration does not prevent the development of the endogenous antibody response.

3. Introduction and Background

The subject of this BLA is Anthim® (obiltoxaximab; ETI-204) for the treatment of adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis* in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate. ETI-204 is a monoclonal antibody (MoAb) against the protective antigen (PA) of *B. anthracis*. Raxibacumab, another anti-PA MoAb, is approved for the treatment of adult and pediatric patients with inhalational anthrax due to *B. anthracis* in combination with appropriate antibacterial drugs, and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate.

ETI-204 is intended to treat inhalational anthrax infection and definitive human efficacy studies were not conducted because it would be unethical to deliberately expose healthy human volunteers to an infectious dose of *B. anthracis* spores. The definitive efficacy studies were therefore conducted in anthrax-challenge models in animals (§21 CFR 601.90) and are summarized in the clinical microbiology section of this review.

3.1. ETI-204

ETI-204 is a humanized chimeric MoAb against the PA of *B. anthracis*. ETI-204 contains human constant region sequences and deimmunized murine variable region sequences generated from the murine MoAB clone 14B7, known to bind to domain 4 of PA (PAD4) responsible for the binding of PA to cell surface receptors.^{7, 8} The applicant states that a single-chain variable fragment (scFv), 1H, containing 3 amino acid mutations within the immunoglobulin light-chain variable region (VL) exhibited affinity for PA that was >50-fold greater than that of wild-type 14B7 scFv. Structural studies confirmed that 1H's binding site on PAD4 is identical to that of 14B7 scFv (for details see Product Quality review). ETI-204 contains 1H affinity-enhanced immunoglobulin heavy-chain variable region (VH) and VL genes which are fused to human $\nu 1$ and κ constant regions, respectively. Additionally, the VH and VL segments were altered to modify human T-cell stimulatory motifs to reduce the potential for immunogenicity.⁹

Over the course of development, two different cell lines ((b) (4) and (b) (4) were utilized by the applicant for the manufacture of ETI-204. The (b) (4) cell line was utilized to manufacture ETI-204 at (b) (4); (b) (4) was utilized to manufacture ETI-204 at Baxter Healthcare and Lonza Biologics. (b) (4)

The ETI-204 manufactured by the commercial manufacture process at the Lonza Portsmouth facility was stated to be comparable to that manufactured at the Baxter Hayward HealthCare facility. The applicant proposes to market the Lonza product of ETI-204.

⁷ Little SF, Leppla SH, and Cora E. Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. *Infect Immun* (1988) 56(7):1807-13.

⁸ Little SF, Novak JM, Lowe JR, Leppla SH, Singh Y, Klimpel KR, Lidgerding BC, and Friedlander AM. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiology* (1996) 142 (Pt 3):707-15.

⁹ Mohamed N, Clagett M, Li J, Jones S, Pincus S, D'Alia G, Nardone L, Babin M, Spitalny G, and Casey L. a high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized *Bacillus anthracis* spore challenge. *Inf Immun* (2005) 73 (2): 795-802.

Table 13: Comparison of cell lines utilized for the manufacture of ETI-204

Details	Elusys Cell Line	(b) (4)
Production Facility		
Host Cell		
Expression Vectors		
Vector Configuration		
Antibody gene sequence		
NS0 represent	(b) (4)	non-secreting GS-NS0 myeloma cells

3.2. Biology of anthrax

B. anthracis, a Gram positive, toxin-producing, encapsulated, spore-forming, facultative anaerobic bacillus, can cause three forms of anthrax depending on the route of exposure: cutaneous, gastrointestinal, and inhalational.

Inhalational anthrax disease results from entry of the *B. anthracis* spores via the respiratory tract and deposition in the lung. In the alveolar spaces, macrophages phagocytize the spores and then migrate to regional lymph nodes. The organisms proliferate in the lymph nodes, rather than as a primary focus in the lungs. The appearance of organisms in the lymphatics draining the lungs and the establishment of infection in the intra-thoracic lymph nodes precedes the development of bacteremia after aerosol exposure. As the phagocytic capacity of the lymph node is overwhelmed, vegetative organisms pass through efferent lymphatics, infect successive lymph nodes, and ultimately enter the blood stream through the thoracic duct (Figure 3).

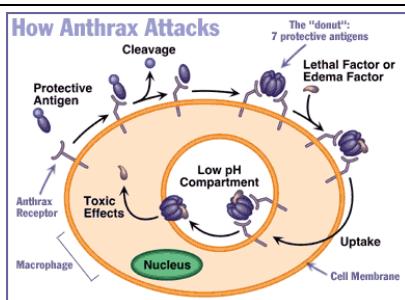
In the macrophages, the spores germinate within phagosomes and produce anthrax toxin, which comprises of three protein components: lethal factor (LF), edema factor (EF), and protective antigen (PA). Individually, these components are harmless; however, they combine to form toxins, namely lethal toxin (LT, a combination of PA and LF) and edema toxin (ET, a combination of PA and EF). All three components of anthrax toxin: PA, LF, and EF would be present during established infection. PA has been shown to be present on the surface of spores and anti-PA antibody can bind the PA on the surface of spores¹⁰ in addition to binding the PA in circulation.

¹⁰ Cote C, Rossi CA, Kang AS, Morrow PR, Lee JS, and Welkos SL. The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. *Microbial Pathogenesis* (2005) 38: 209-225.

Figure 3: Diagrammatic representation of interactions between *B. anthracis* and the host during inhalational anthrax

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Source: Passalacqua KD and Bergman NH. *Bacillus anthracis*: interactions with the host and establishment of inhalational anthrax. Future Microbiology (2006) 1 (4): 397-415.



Source:
http://whyfiles.org/shorties/089_anthrax_receptor/

Disease progression: Once the spores germinate and the anthrax bacteria start replicating, the typical course of inhalation anthrax progresses in a biphasic manner. Patients experience a prodromal phase of localized infection with flu-like symptoms. The transition from localized infection to systemic illness (fulminant phase) develops abruptly with high fever, dyspnea, diaphoresis, and shock; at this point, blood cultures are typically positive for the characteristic Gram-positive spore-forming bacilli. Without treatment, the condition progresses rapidly and is considered uniformly and rapidly fatal. Patients who present with signs of fulminant disease may die despite administration of antibacterial drugs because the latter have no activity against anthrax toxin. Significant levels of accumulated anthrax toxin can still drive the progression of disease by causing hemorrhagic meningitis and hemodynamic alterations, including severe hypotension with subsequent tissue hypoperfusion, hypoxia, and metabolic acidosis.

In humans, the incubation period of inhalational anthrax is difficult to estimate because of the highly variable length of the incubation period; based on previous outbreaks the time from estimated exposure to onset of illness may range from 4 to 6 days.¹¹ Studies suggest that humans

¹¹ Jernigan DB, Raghunathan PL, Bell BP, et al. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. *Emerg Infect Dis* (2002) 8:1019-1028.

and nonhuman primates appear to be intermediate in susceptibility to anthrax, between the rabbits (more susceptible) and dogs or pigs (more resistant).^{12,13}

In rabbits weighing 2.5 to 3.5 kg, a particle size of 1-2 µm results in about 7% of the aerosol being deposited in the lower respiratory tract (lungs, bronchi, and trachea) and about 9.5% of the aerosol being swallowed. Thus, the respiratory tract may not be the only route of infection during aerosol exposures and this may be more significant in rabbits since herbivores are highly susceptible to gastrointestinal anthrax. In monkeys, it is known that at a particle size of 1-2 µm relatively more particulates are deposited in nonhuman primate lungs than in humans, and the overall distribution of particles within the respiratory tract and stomach is about equal.

Virulence Factors: The major virulence factors for *B. anthracis* are the capsule and anthrax toxins. The *B. anthracis* capsule enables the vegetative form of *B. anthracis* to avoid phagocytosis. All three components of anthrax toxin (PA, LF, and EF) are present during established infection. PA, LF, and EF are encoded by the pXO1 plasmid. Another plasmid, pXO2, contains the genes for the synthesis of a poly-c-D-glutamic acid capsule. The expression of the genes encoding the capsule are under the control of two regulatory gene products, anthrax toxin activator (AtxA) and anthrax capsule activator (AcpA), that are also located on the pXO1 and pXO2 plasmids. AtxA and AcpA respond to as yet undefined environmental cues.¹²

PA is the receptor-binding component that allows intracellular entry of LF and EF. LF is a zinc metalloprotease that cleaves and inactivates mitogen-activated protein kinase kinases (MAPKKs), key signal transduction molecules required for effective host responses against bacterial pathogens as well as cellular functions. EF is a calmodulin-dependent adenylate cyclase that induces edema in various tissues. There has been experimental utility in examining the independent effects of EF and LF, each in combination with their shared receptor-binding component, PA. LT targets a variety of cell types, including immune cells (macrophages, dendritic cells, neutrophils, and lymphocytes), leading to disruption of immune responses, and thereby facilitating infection. LT also has toxic effects on endothelial cells, leading to loss of barrier function, which is thought to be a major factor underlying the pathology induced by LT. The LT, despite its name, is not directly lethal to most cells in culture. Instead, it disrupts a wide variety of cellular functions that require MAPKK signaling (e.g., cell cycling and cytokine production). Similarly, ET has wide-ranging effects through its enzymatic activity that results in increased cellular cyclic AMP, a critical cellular signaling molecule. In addition to mediating edema, ET has immunomodulatory effects and perturbs endocrine function.¹⁴ The secreted *B. anthracis* toxins are thought to be responsible for the morbidity and high mortality rates characteristic of anthrax infection despite appropriate antimicrobial therapy.

PA (monomeric), secreted as an 83 kDa protein, is a pore-forming protein that requires post-translational processing and assembly to become functional. Before PA can translocate LF or EF inside a target host cell, it binds to one of two anthrax toxin receptors (ATRs) on the cell surface, either capillary morphogenesis gene 2 protein (CMG-2) or tumor endothelial marker 8 (TEM-8),

¹² Hirsh DC and Biberstein EL. *Bacillus*. In: Hirsch DC, MacLachlan N.J., Walker R.L., eds. Veterinary Microbiology. Ames, IA: Blackwell Publishing (2004) 168-174.

¹³ Jaax NK and Fritz DL. Anthrax. In: Conner DH, Chandler F.W., Schwartz D.A., *et al.*, eds. Pathology of Infectious Diseases. Stamford, CT: Appleton and Lange (1997) 391-406.

¹⁴ Moayeri M and Leppla SH. Cellular and systemic effects of anthrax lethal toxin and edema toxin. *Mol Asp Med* (2009). 30 (6): 439-455.

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which are widely expressed on the majority of mammalian cells. Both receptors are Type 1 transmembrane proteins with an extracellular von Willebrand factor A domain (integrin inserted domain; VWA/I) that contains a metal ion-dependent adhesion site (MIDAS) that interacts with PA. The PA binding site is thought to be conserved between the two receptors with 60% identity within the VWA/I domain and 40% overall amino acid identity.¹⁵

Monomeric PA is cleaved by a host protease, such as furin, to remove an N-terminal 20 kDa fragment. There is some controversy as to whether this happens only on the cell surface after PA binds to the receptor or whether the cleavage can also occur before PA binds to the cellular receptor. In either case, seven 63 kDa fragments of PA then assemble into a heptamer ring on the cell surface leading to the formation of binding sites for up to three molecules of LF and/or EF. The toxin complex is internalized by endocytosis, a pore is formed in the acidic environment of the endosome, and LF and EF are extruded into, and exert their effects in the cytosol (Figure 4).¹⁶ PA plays key roles in multiple stages of inhalational anthrax disease pathogenesis. During early stages of disease, PA is essential for systemic bacterial dissemination and infection of peripheral tissues, and for suppression of innate and adaptive immunity.^{17,18,19,20,21} During fulminant anthrax, PA in combination with LF or EF contributes directly to tissue cytotoxicity, and impairment of organ functions and hemodynamics (Liu *et al.*, 2014¹⁸).

¹⁵ Banks DJ, Ward SC, and Bradley KA. New insights into the functions of anthrax toxin. 2006. *Expert Rev Mol Med* 8 (7):1-18.

¹⁶ Xu L and Frucht DM. *Bacillus anthracis*: a multi-faceted role for anthrax lethal toxin in thwarting host immune defenses. *Internat J Biochem Cell Biol* (2007) 39: 20-24.

¹⁷ Lovchik JA, Drysdale M, Koehler TM, Hutt JA, and Lyons CR. Expression of either lethal toxin or edema toxin by *Bacillus anthracis* is sufficient virulence in a rabbit model of inhalational anthrax. *Inf Immun* (2012) 80 (7): 2414-2425.

¹⁸ Liu S, Moayeri M, and Leppla SH. Anthrax lethal and edema toxins in anthrax pathogenesis. *Trends in Microbiology* (2014) 22 (6) 317-325.

¹⁹ Lowe DE and Glomski IJ. Cellular and physiological effects of anthrax exotoxin and its relevance to disease. *Frontiers in Cellular and Infection Microbiology* (2012) 2 (Article 76): 1-13.

²⁰ Tournier JN, Quesnel HA, Aurélie C, and Vidal DR. Contribution of toxins to the pathogenesis of inhalational anthrax. *Cellular Microbiology* (2007) 9 (3): 555-565.

²¹ Agrawal A, Lingappa J, Leppla SH, Agrawal S, Jabbar A, Quinn C, and Pulendran B. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* (2003) 424: 329-334.

Figure 4: Diagrammatic representation of the entry of anthrax toxin into cells
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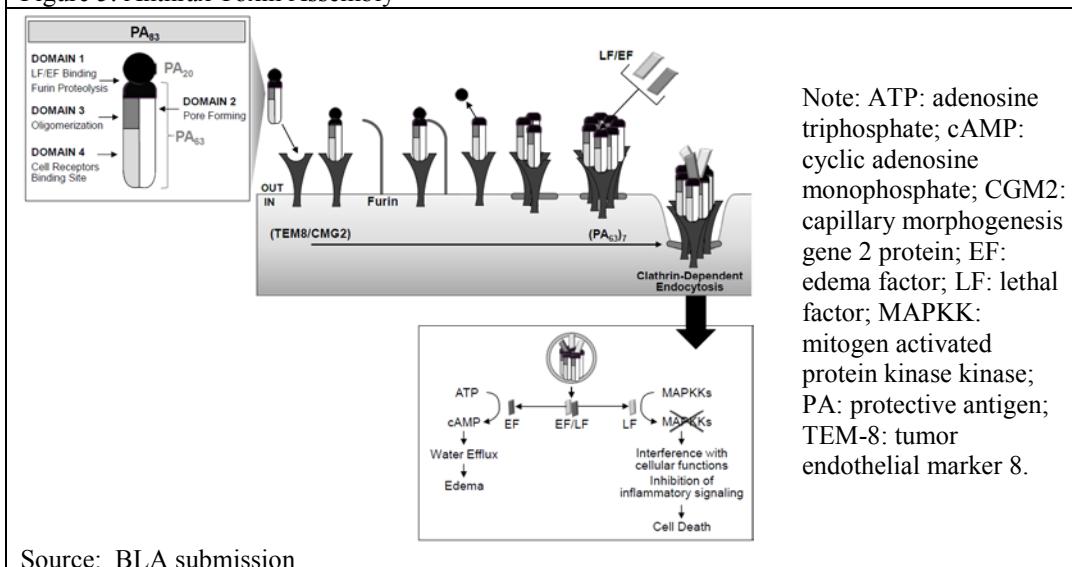
PA₈₃ binds one of two cellular receptors, TEM8/ATR or CMG2, which have been reported to be associated with the LRP6 co-receptor. After binding, PA₈₃ is cleaved by cellular proteases, such as furin, and the small PA₂₀ fragment is released. PA₆₃ then forms a ring-shaped heptameric pre-pore, which can simultaneously bind up to three molecules of LF and/or EF. The toxin/receptor complex is then internalized. The endocytic vesicles are subsequently acidified, initiating a conformational change of the PA heptamer which converts it from the pre-pore into a mature pore that allows entry of EF and/or LF into the cell cytoplasm. LF is a protease targeting specific MAPKs. EF is an adenylate cyclase that increases cAMP formation in cells.

Source: Xu, L and Frucht, DM. *Bacillus anthracis*: a multi-faceted role for anthrax lethal toxin in thwarting host immune defenses. *Internat J Biochem Cell Biol* (2007) 39: 20-24.

Because of PA's central role in toxin assembly and intoxication of target cells, PA neutralization is considered to be effective in preventing the establishment and progression of disease subsequent to inhalational exposure to anthrax spores (Mohamed *et al.*, 2005⁹). Studies by Little *et al.*, (1996)⁸ and Leysath *et al.* (2009)²² show that binding of 14B7 MoAb to domain 4 of PA (PAD4) prevents the cell binding of PA63-EF and PA63-LF complexes thereby preventing the entry of EF and LF into the cytosol and the downstream deleterious effects of anthrax toxin (Figure 5). As stated above, ETI-204 is a chimeric antibody containing the enhanced versions of 14B7's VH and VL genes fused to human v1 and K constant regions. This homology between 14B7 and ETI-204 suggests the potential for ETI-204 to inhibit binding of PA63 to the cell surface receptor.

²² Leysath CE, Monzingo AF, Maynard JA, Barnett J, Georgiou G, Iverson BL, and Robertus JD. Crystal structure of the engineered neutralizing antibody M18 complexed to domain 4 of the anthrax protective antigen. *J Mol Biol* (2009) 387: 680-693.

Figure 5: Anthrax Toxin Assembly



4. Non-clinical Microbiology Studies

4.1. Mechanism of action

4.1.1. Binding of ETI-204 to protective antigen

The binding of ETI-204 as well as murine 14B7 and chimeric 14B7 MoAbs to PA was measured using Biacore 3000 instrument that works on the principle of surface plasmon resonance and utilizes polarized light; by this minute changes in optical resonance resulting from association or dissociation from an immobilized ligand can be detected. In order to measure the affinity of anti-PA MoAbs for PA, rabbit-anti-human and rabbit-anti-mouse polyclonal antibodies were immobilized onto a sensor chip surface for capturing either ETI-204 (humanized antibody) or murine MoAbs, respectively.²³ The protein-protein interaction results were expressed as kinetic constants (K_{on} , K_{off}) as well as equilibrium disassociation constant (K_D). Based on these measurements, the ETI-204 with the highest affinity for PA (lowest K_D) was selected for further development; the affinity of ETI-204 for PA ($K_D = 0.33$ nM i.e., 48.8 pg/mL) was about 10-fold higher than murine 14B7 or chimeric 14B7 (Table 14); the applicant states that such binding was similar to PA binding to its receptor (K_D 0.17 – 1 nM).

Table 14: Kinetic constants for murine 14B7, chimeric 14B7, and ETI-204

Sample mAb	k_{on} (M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	K_D (nM)	
			Mean	%CV
Murine 14B7	5.23×10^5	1.96×10^{-3}	3.74	7.1
Chimeric 14B7	4.35×10^5	1.53×10^{-3}	3.51	17.1
ETI-204	4.57×10^5	1.50×10^{-4}	0.33	9.9

%CV – coefficient of variation; k_{on} – association rate constant; k_{off} – dissociation rate constant; K_D – dissociation constant; mAb – monoclonal antibody; nM – nanomolar

Comments:

ETI204 binds PA with a $K_D = 0.33$ nM i.e., 48.8 pg/mL.

4.1.2. Binding of ETI-204 to anthrax toxin receptors

The ability of ETI-204 to compete with downstream binding of PA83 and PA63 to host cell ATRs i.e., CMG-2 and TEM-8, was investigated by a PA ELISA assay.²⁴ Briefly, the capture ATR's were coated onto microtiter plate wells at 2 and 65 times the concentration of either ETI-204 or polyclonal anti-PA antibody (pAb) used as the capture. PA83 or PA63 were first allowed to bind to the capture reagents, and then increasing concentrations (0.05, 0.1, 0.5, 1, and 5 molar ratio) of ETI-204 added to compete with that binding. EDTA was added to prevent protease cleavage and degradation of PA83. Rabbit anti-PA serum was utilized as the primary detection reagent, and HRP conjugated goat anti-rabbit as the secondary detection reagent to assess the performance of the capture reagents in binding PA83, and interference of binding by ETI-204. The results showed a high level of recovery with little interference with addition of ETI-204 when pAb was used as the capture agent (Figure 6); such a recovery may be due to the specificity range of the pAb capture, as the pAb likely recognized PA at various epitopes resulting in little interference with addition of ETI-204 at up to a 5:1 molar ratio. However, with the ETI-204 as the capture agent, the recovery was reduced, even at a 0.05:1 molar ratio of added ETI-204, suggesting the high specificity of ETI-204 as a capture reagent. TEM-8 showed no ability to capture PA83 in this format: therefore, ETI-204 interference could not be assessed. ETI-204 showed dose-dependent interference with CMG-2 binding of PA83 starting at a 0.05:1 molar ratio, confirming that ETI-204 blocks the receptor-binding domain of PA (PAD4).

Comments:

- *ETI-204 blocked the receptor binding domain in a dose dependent fashion, as clearly seen in the inhibition of binding of PA83 in the presence (+) EDTA to CMG2.*
- *The CMG-2 has 1000-fold higher affinity for PA than TEM-8; therefore, TEM-8 was not an effective capture, even at 65X molar excess of ETI-204 capture in this assay. CMG2 captured PA83 + of EDTA > PA83 in the absence (-) of EDTA > PA63.*
- *Binding of PA63 on the pAb coated wells was inhibited by ETI-204.*
- *CMG-2 was a better capture than TEM-8 in this assay; TEM-8 did not capture at low or high concentrations.*

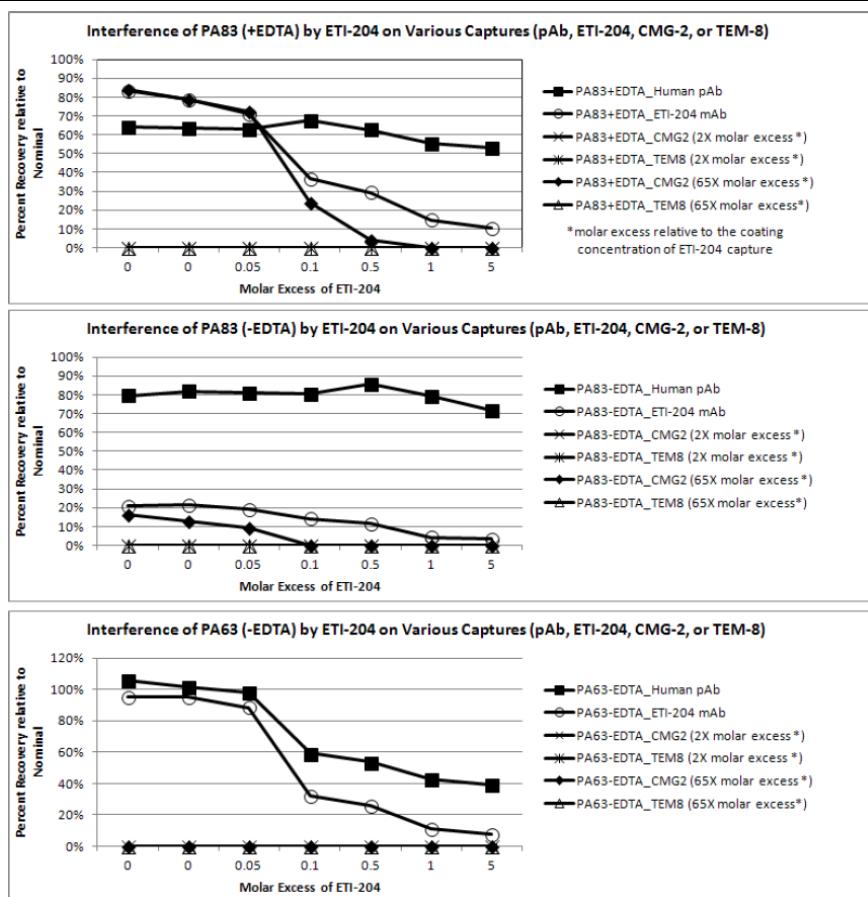
²⁴

(b) (4) Study No. 1169-100001621: Research and development of an enzyme linked

immunosorbent assay (ELISA) for the detection of *Bacillus anthracis* protective antigen in rabbit and non-human

Reference ID: 9861581 specimens (March 28, 2013).

Figure 6: Interference by ETI-204 with PA83 binding to polyclonal antibody (pAb), ETI-204, CMG-2 or TEM-8 coated plates



The interference of PA83 by ETI-204 on various captures, including pAb, ETI-204, CMG-2, and TEM-8, was evaluated. EDTA was present to prevent PA83 degradation.

Source: BLA submission (Study No. 1169-100001621)

4.2. Activity *in vitro*

4.2.1. Binding to the protective antigen from three strains of *Bacillus anthracis*

The *in vitro* activity of ETI-204 was measured against the PA in supernatants collected from the Ames (obtained from C. Rick Lyons, University of New Mexico), Sterne 34 F2 (Colorado Serum Company), and Vollum (BEI Resources) strains of *B. anthracis*.²⁵ Briefly, the Ames and Vollum strains were grown under BSL-3 conditions; the Sterne strain was grown under BSL-2 conditions. *B. anthracis* bacterial culture purity was ascertained on Tryptic Soy Agar (TSA) plates incubated for 16 to 24 hours at 37°C. The bacterial culture suspensions (10 mL) in PBS adjusted to a McFarland 0.5 standard was added to 500 mL of Tryptic Soy Broth (TSB) and cultures incubated with shaking for 20 hours. The cultures were centrifuged and supernatants collected and filtered. The supernatants from the Ames and Vollum strains of *B. anthracis* were checked for sterility before removing from the BSL-3 facility. All supernatants were concentrated using sterile Amicon Ultra-15 centrifugal filter device (Millipore; a cut-off of 30,000 molecular weight), and processed for PA purification by incubating with A/G magnetic beads cross-linked with ETI-204, using the Pierce Crosslink Magnetic IP/Co-IP kit, for about 1 hour. The PA was eluted from the beads. The negative controls included for testing were TSB

²⁵Study FY13-011 – PA production and Analysis report.

(b) (4)

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and ovalbumin; a positive control, purified PA (List Biological Laboratories, Inc.) was also included. All controls were processed using the same kit used for processing of supernatants from the three bacterial strains of *B. anthracis*. The eluted samples were processed for measuring protein concentration, polyacrylamide gels electrophoresis (PAGE), and western blot using ETI-204 and anti-human antibody tagged with horse radish peroxidase. The polyacrylamide gels were also stained with Coomassie blue. The results showed ETI-204 binds the PA antigen (PA83 and its cleave products) from the 3 strains tested (Figure 7). Although the concentration of protein loaded for analysis was the same, the intensity of binding and the number of bands observed varied among the 3 strains. The Coomassie blue staining results are ambiguous as no stained bands were observed for any of the samples tested other than ovalbumin control and the standard molecular weight ladder. The applicant states that this could be due to the low concentration of PA loaded that was close to the limit of detection for Coomassie staining.

Comments:

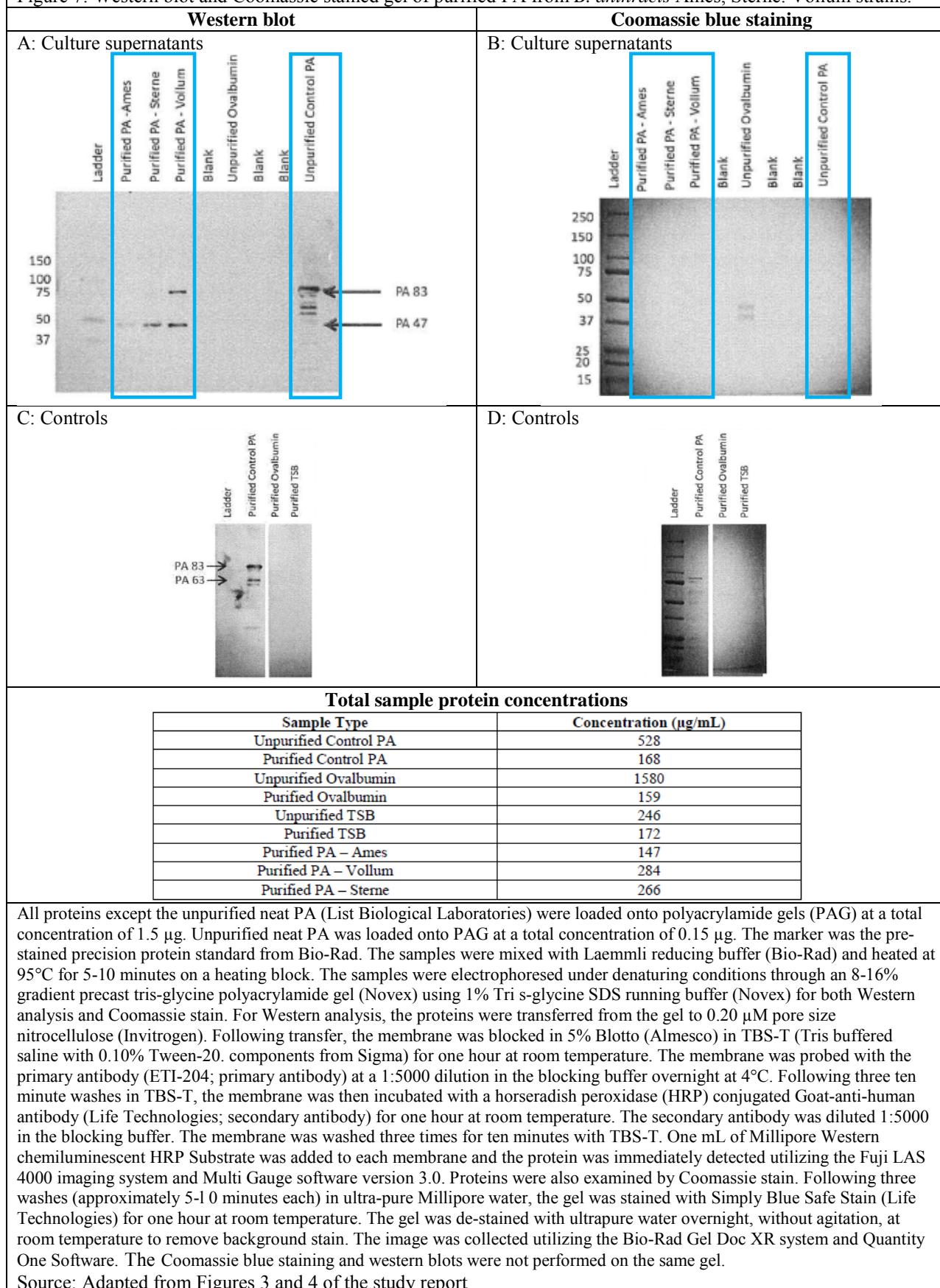
- *The results showed ETI-204 binding to PA antigen from the 3 strains (Ames, Sterne, and Vollum) strains of *B. anthracis* tested. However, the intensity of binding and the number of bands observed varied among the 3 strains although the concentration of protein loaded for analysis was the same.*
- *One of the limitations of the experiment is that unpurified supernatants from the Ames, Sterne and Vollum strains of *B. anthracis* were not tested.*

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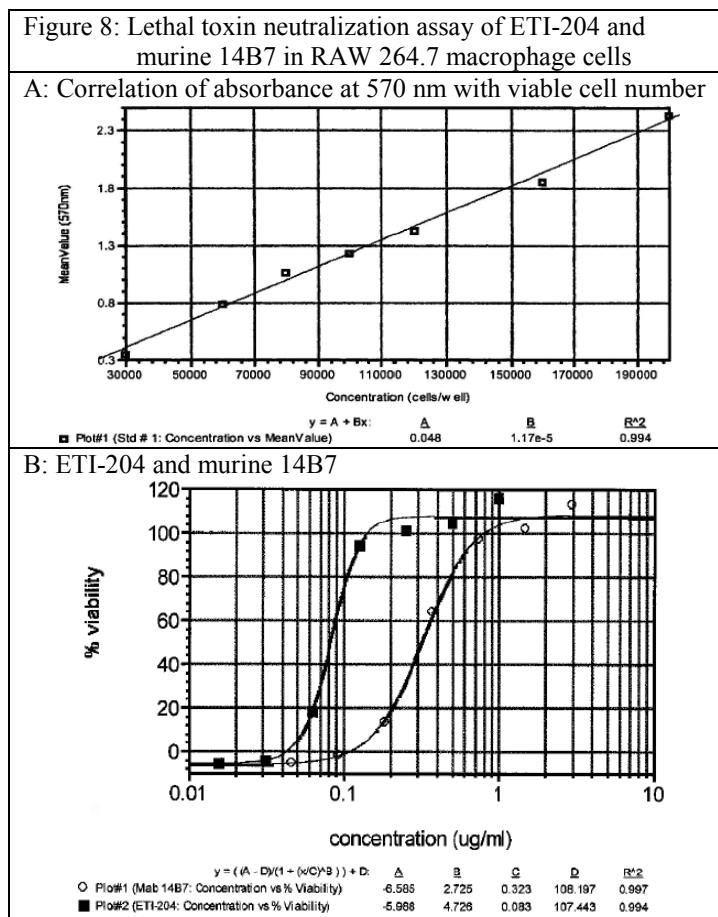
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Figure 7: Western blot and Coomassie stained gel of purified PA from *B. anthracis* Ames, Sterne, Vollum strains.



4.2.2. Lethal toxin neutralization assay

The neutralizing ability of the ETI-204 and murine 14B7 was investigated in a toxin neutralization assay (TNA).²⁶ Briefly, 2.0×10^6 RAW 264.7 (murine macrophages) cells in 1 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin were seeded in a 96-well tissue culture plate, and incubated overnight. LT (List Biologicals Laboratories) + ETI-204 or 14B7 at different concentrations (0.01, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$) or medium alone were incubated for 1 hour at 37°C; these were then added to the cells and incubated at 37°C for 4 hours. The cells were stained by addition of [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)] and incubated for 1 hour at 37°C. At the end of the incubation period, cells were lysed and solubilized by addition of 20% SDS in 50% N, N-dimethylformamide (DMF) at pH 4.7 and incubated overnight at 37°C. MTT is converted into a blue colored, water-insoluble formazan salt by the metabolic activity of viable cells. The insoluble formazan was solubilized; the intensity of the blue signal was proportional to the number of viable cells (Figure 8A). The intensity was measured by a colorimeter at a wavelength of 570 nm on a microwell plate reader and the percentage survival of cells calculated. Although both MoAbs improved survival of RAW 264.7 cells incubated with LT compared to the vehicle control (i.e., medium alone), 4-fold lower concentration of ETI-204 was required to reach 50% of maximal survival (EC₅₀) compared to murine 14B7 (Figure 8B). The higher affinity of ETI-204 for PA correlated with higher neutralizing activity.



The TNA is used to evaluate potency of the drug substance; 50% effective concentration was stated to be 105 ± 35 ng/mL.²⁷

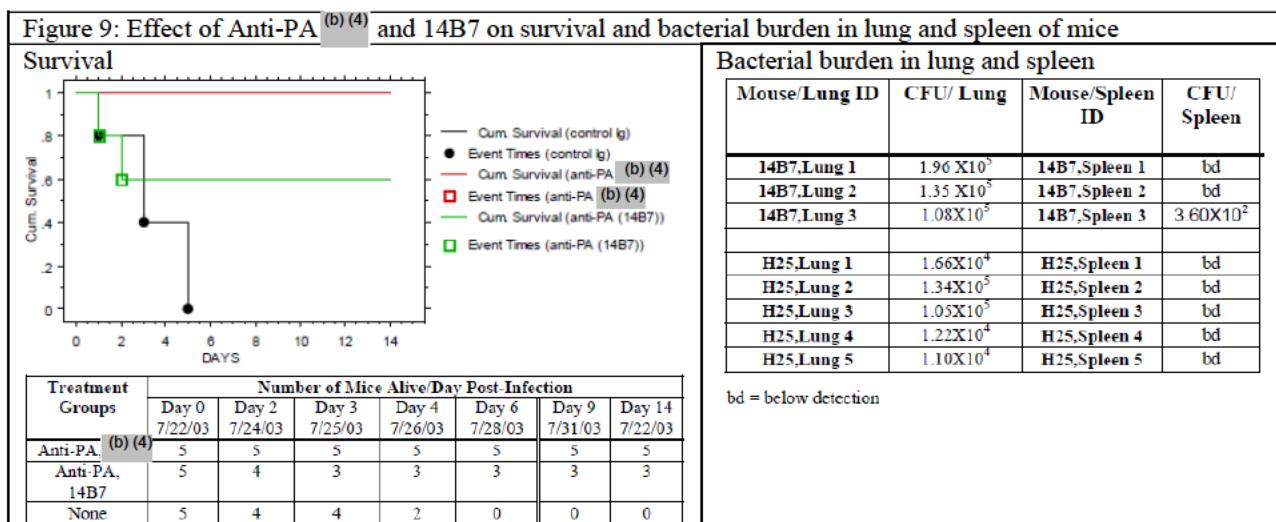
Comments:

- ETI-204 was effective in neutralizing LT in the toxin neutralization assay.

4.3. Activity *in vivo*

4.3.1. Mice

The protective effect of (b) (4) (produced from (b) (4) cells line, manufactured at (b) (4)) and 14B7 (murine MoAb clone) anti-PA MoAb treatment was measured in a murine pulmonary infection model.²⁸ Briefly, 11 to 12 weeks old DBA/2 mice were administered anti-PA MoAb immediately prior to infection with (b) (4) spores (LD₁₀₀ dose) of the Sterne strain of *B. anthracis* by the intra-tracheal route. The actual number of colony forming units (cfu) deposited in the lung was evaluated by sacrificing two mice following infection and homogenizing the lungs that were then cultured on sheep blood agar plates; the time when these mice were sacrificed was not specified. The spores in lungs of these 2 mice varied from 5.61×10^5 and 7.61×10^5 (mean 6.61×10^5 spores). The remaining animals were followed for survival for 14 days. The results showed (b) (4) MoAb to be more protective than 14B7 in protecting mice when administered immediately prior to exposure (Figure 9). All the control group of mice died within 6 days of exposure. The surviving mice were euthanized on Day 15 and lung and spleen tissues collected were processed for measuring bacterial burden. The lungs were culture positive from all the 8 survivors. However, spleen was culture positive from one mouse administered the murine MoAb (14B7).



Comments:

The (b) (4) product (MoAb produced by (b) (4) cell line), administered at the time of infection with the Sterne strain of *B. anthracis*, is effective in improving survival of mice. ETI-204 produced by (b) (4) cell line used for the manufacture of Baxter or Lonza products was not included for testing.

²⁷ BLA, Module 3.2.S.3. Elucidation of structure and other characteristics.

4.3.2. Rabbits and monkeys

The efficacy of ETI-204 was measured in NZW rabbits and cynomolgus monkeys infected with the Ames strain of *B. anthracis* by inhalational route. As the efficacy of ETI-204 cannot be investigated in controlled clinical trials in humans for ethical reasons and field efficacy studies are not feasible, the definitive efficacy studies were therefore conducted in anthrax-challenge models in animals (§21 CFR 601.90) and are summarized in the clinical microbiology section of this review (for details see Section 6).

5. Overview of Clinical Pharmacology

The pharmacokinetics (PK) of ETI-204 in healthy human volunteers, was stated to be linear over the dose range of 4 to 16 mg/kg following single IV dosing. The peak ETI-204 concentrations (C_{max}) and area under the curve (AUC_{inf}) were $400 \pm 91.2 \mu\text{g/mL}$ and $5170 \pm 1360 \text{ mcg}\cdot\text{day}/\text{mL}$, respectively following single dose (16 mg/kg) IV administration of ETI-204; the half-life was about 20 days (Table 15). After intramuscular (IM) administration of a single 16 mg/kg IM dose, the C_{max} ($105 \pm 22.5 \mu\text{g/mL}$) was lower and delayed and AUC_{inf} ($3200 \pm 755 \text{ mcg}\cdot\text{day}/\text{mL}$) lower compared to intravenous administration at the same dose.

Table 15: A comparison of ETI-204 after IV administration, PK parameters in rabbits, monkeys and healthy human volunteers

A: Rabbits and healthy human volunteers (Mean±SD)				
	Healthy Rabbits	Spore-Challenged Rabbits that Survived		Healthy Humans
	Study AR010	Study AR033		Studies AH105, AH104, and AH110 ^a
	10 mg/kg (n = 10)	16 mg/kg (n = 9)		16 mg/kg (n = 269)
C_{max} ($\mu\text{g/mL}$)	342 ± 112	391 ± 65.5		392 ± 89.3
$t_{1/2}$ (d)	4.17 ± 2.66 ^b	1.60 ± 0.48 ^c		20.1 ± 5.03 ^d
AUC _(0-inf) ($\mu\text{g}\cdot\text{d}/\text{mL}$)	1200 ± 243 ^b	958 ± 67.1 ^c		5050 ± 1290 ^e
CL (mL/d/kg)	8.72 ± 2.03 ^b	16.8 ± 1.18 ^c		3.43 ± 0.964 ^e
V_z (mL/kg)	46.5 ± 24.4 ^b	38.0 ± 9.35 ^c		95.3 ± 24.3 ^e
V_{ss} (mL/kg)	53.9 ± 17.3 ^b	43.2 ± 7.52 ^c		80.1 ± 19.6 ^f

B: Monkeys and healthy human volunteers (Mean±SD)				
	Healthy Monkeys	Spore-Challenged Monkeys that Survived		Healthy Humans
	Study AP116	Study AP204	Study AP202	Studies AH105, AH104, and AH110 ^a
	10 mg/kg (n = 6)	16 mg/kg (n = 8)	Baxter ETI-204 16 mg/kg (n = 6) Lonza ETI-204 16 mg/kg (n = 5)	16 mg/kg (n = 269)
C_{max} ($\mu\text{g/mL}$)	292 ± 21.4	459 ± 80.1	336 ± 73.1	392 ± 89.3
$t_{1/2}$ (d)	12.4 ± 4.03 ^b	5.13 ± 2.11	7.17 ± 2.68	20.1 ± 5.03 ^c
AUC _(0-inf) ($\mu\text{g}\cdot\text{d}/\text{mL}$)	2520 ± 641 ^b	1860 ± 169	2110 ± 345	5050 ± 1290 ^d
CL (mL/d/kg)	4.18 ± 1.05 ^b	8.66 ± 0.739	7.82 ± 1.64	3.43 ± 0.964 ^e
V_z (mL/kg)	71.3 ± 13.2 ^b	63.0 ± 22.6	76.3 ± 21.0	95.3 ± 24.3 ^d
V_{ss} (mL/kg)	NR	60.3 ± 12.5	56.2 ± 16.4	80.1 ± 19.6 ^e

AUC_(0-inf): area under the concentration versus time curve from time 0 extrapolated to infinity; CL: systemic clearance; C_{max} : maximum concentration; d: day; SD: standard deviation; $t_{1/2}$: half-life; V_z : volume of distribution in the terminal phase; V_{ss} : steady-state volume of distribution.
^aAH110 includes subjects who received ETI-204 alone (n = 20) and with IV (400 mg single dose) and oral (750 mg twice daily for 8 days) ciprofloxacin (n = 18).
^bn = 8.
^cn = 6.
^dn = 257.
^en = 255.
^fn = 226.

Source: BLA submission

In New Zealand White (NZW) rabbits challenged with the Ames strain of *B. anthracis*, a dose dependent increase in ETI-204 concentration was reported. The peak serum concentrations of ETI-204 were achieved within 0.25 hours of administration and declined with terminal half-life values between 0.83 and 4.42 days. The systemic exposure (C_{max} , AUC_{inf}) was dose proportional; the C_{max} and AUC_{inf} , and $t_{1/2}$ values were lower in infected animals compare to humans (Table 15A).

In cynomolgus monkeys challenged with the Ames strain of *B. anthracis*, the ETI-204 C_{max} in serum were achieved as early as the 15 minute post-treatment time in animals treated with 4, 16 or 32 mg/kg IV ETI-204, respectively. This was followed by a decline and a secondary increase. The elimination half-lives did not appear to be affected by the secondary increase. The C_{max} , AUC_{inf} , and $t_{1/2}$ values were lower in infected animals compare to humans (Table 15B).

The applicant states that no ETI-204 was detected in pre-dose samples or in the control group of animals. After peaking, the serum ETI-204 concentrations in the 8 and 32 mg/kg dose groups declined.

In Study AP202, the pharmacokinetics of ETI-204 16 mg/kg dose was shown to be similar between the Lonza and Baxter products.

Comments:

- *The systemic exposure (C_{max} and AUC_{inf}) of ETI-204 in humans is similar or greater compared with New Zealand White rabbits and cynomolgus monkeys administered the same dose (single 16 mg/kg IV dose).*
- *The half-life of ETI-204 in NZW rabbits is ~3 to 4 days and cynomolgus monkeys ~5 to 12 days; in humans the half-life of ETI-204 is longer (15 to 23 days). For more details see Clinical Pharmacology review.*

6. Clinical Microbiology (Natural History and Animal Efficacy) Studies

The efficacy of ETI-204 cannot be investigated in controlled clinical trials in humans for ethical reasons and field efficacy studies are not feasible. The efficacy of ETI-204 was measured in two animal models, NZW rabbits and cynomolgus monkeys, under the Food and Drug Administration's (FDA) Animal Rule regulations (601.90) and in accordance with the FDA guidance (FDA, 2009²⁹; FDA, 2014³⁰, FDA, 2015³¹).

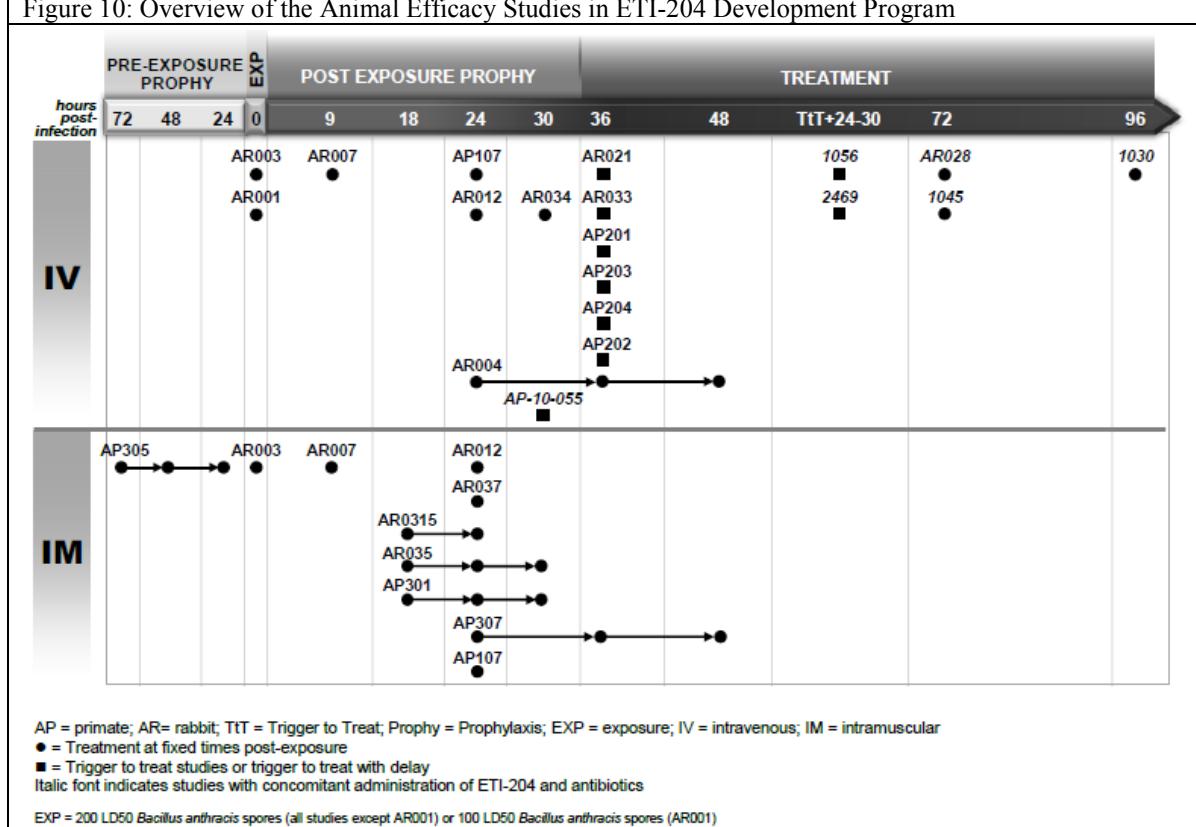
The applicant performed several studies to evaluate the efficacy of ETI-204 as a single-dose IV or IM for the treatment, post-exposure and pre-exposure prophylaxis of inhalational anthrax (Figure 10). Some of these studies were performed to evaluate the efficacy of ETI-204 in combination with an antibacterial drug.

²⁹ FDA draft guidance for Industry (2009) Animal models –essential elements to address efficacy under the Animal Rule. (Replaced by 2015 Guidance document).

³⁰ FDA draft guidance for Industry (2014) Product Development Under the Animal Rule (Replaced by 2015 Guidance document).

³¹FDA guidance (2015) Product Development Under the Animal Rule
<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM399217.pdf>.

Figure 10: Overview of the Animal Efficacy Studies in ETI-204 Development Program



6.1. Natural History Studies (Animal model characterization)

The applicant did not conduct any natural history studies. However, the applicant does refer to three published studies as well as four studies sponsored by the National Institute of Allergy and Infectious Disease (NIAID, NIH) studies to support the choice of NZW rabbits (*Oryctolagus cuniculus*) and cynomolgus monkeys (*Macaca fascicularis*) challenged with *B. anthracis* spores of the Ames strain by aerosolization for measuring the efficacy of ETI-204. The NIAID studies were submitted to the DMF ^{(b) (4)}

6.1.1. New Zealand White rabbits

The applicant refers to two published studies (Zaucha *et al.*, 1998² and Yee *et al.*, 2010³²) and 3 NIAID studies (Study No. 589-G607604, 616-G607604, and 662-G607604) to support characterization of the NZW rabbit animal model for evaluating efficacy of ETI-204.

6.1.1.1. Zaucha *et al.*, 1998

All rabbits were infected with different inoculum doses of the Ames of *B. anthracis* spores with a 3-jet Collison nebulizer with a muzzle (nose) only exposure.² Briefly, the concentrations of spores in the aerosol were determined by culture of samples collected from an impinger which sampled the aerosol that actually reached the rabbits. The actual inhaled dose for each rabbit was calculated using plethysmography data and ranged from 8.34×10^4 to 1.03×10^7 cfu (Table 16). The aerosol LD₅₀ was determined to be 1.05×10^5 cfu and the 99% lethal dose (LD₉₉) was

³² Yee SB, Hatkin JM, Dyer DN, Orr SA and Pitt LM. Aerosolized *Bacillus anthracis* infection in New Zealand White rabbits: Natural history and intravenous levofloxacin treatment. *Comparative Medicine* (2010) 60: 461-468.

1.36×10^5 cfu. Clinical signs were not generally apparent until within 24 hours of death, at which time rabbits became progressively lethargic and weak. The mean time of death was 2.4 days; all rabbits died two to three days post-exposure (Table 16) and were necropsied.

Several rabbits, had brain or meningeal lesions, exhibited brief periods of excitation and hyperactivity within hours or minutes before death. Hemorrhage, mediastinitis, and edema were the most prevalent changes; the affected tissues include mandibular lymph nodes, spleens, and lungs.

Table 16: Inhalational anthrax in NZW Rabbits: Exposure doses, routes of infection, and day of death
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The aerosol 50% lethal dose (LD_{50}) was determined to be 1.05×10^5 cfu and the aerosol 99% lethal dose (LD_{99}) was 1.36×10^5 cfu.

† S = Subcutaneous; A = Aerosol.
Source: Zaucha *et al.*, 1998²

6.1.1.2. Yee *et al.*, 2010

In the study by Yee *et al.* (2010)³² NZW rabbits, *Bordetella*-free, obtained from Charles River were infected with 150X LD_{50} of the Ames strain of *B. anthracis* spores with a 3-jet Collison nebulizer with a muzzle only exposure for 10 minutes. Body temperature was recorded every 15 minutes by radiotelemetry. Pyrexia was defined as a body temperature above 40 °C for at least 3 sequential time points. Animals were followed from Day-1 prior to challenge until found dead or were euthanized for bacteremia, antigenemia (PA in whole blood by ECL immunoassay using three PA-specific monoclonal antibodies were used as a capture antibody mix³³), blood cell count, and clinical observations.

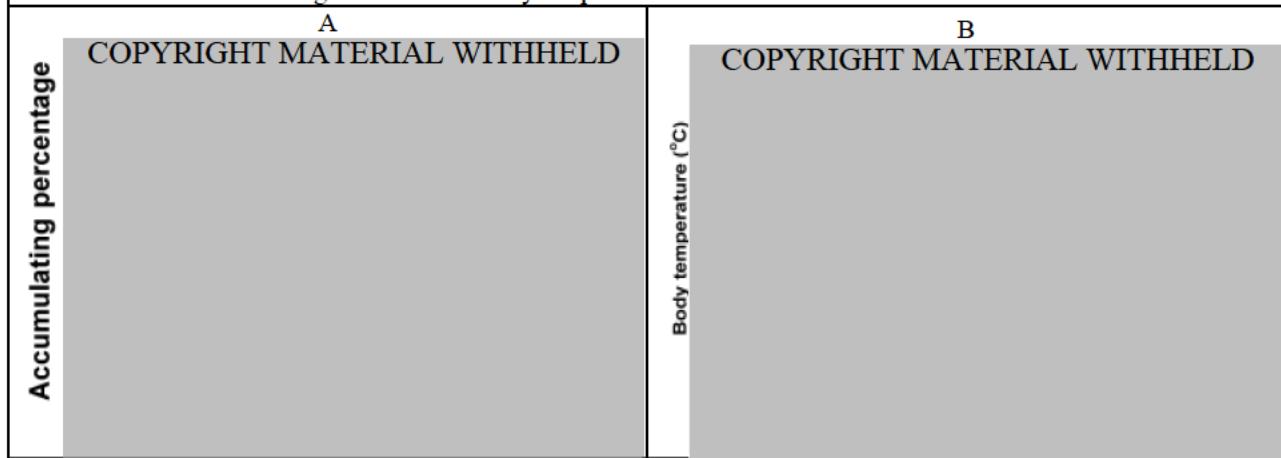
Reference ID: 3864561³³ The ECL limit of detection for PA-spiked whole blood was less than 2 ng/mL.

The rabbits were exposed to 155.6 ± 23.1 LD₅₀ and ranged from 51.1 to 313.8 LD₅₀ (Table 17 and Figure 11). The mean survival time after exposure was 46.9 ± 3.3 hours (range, 29 to 61 hours). Both antigenemia [24.5±1.4 (range, 15 to 30) hours] and bacteremia [23.1±0.2 (range 15 and 30 hours)] developed before the occurrence of pyrexia [29.0±2.3 (range, 25.5-32.75) hours].

Table 17: Disease course for rabbits challenged with aerosolized *B. anthracis*

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Figure 11: The pattern for disease development – A: the appearance of antigenemia and bacteremia, as well as rabbits succumbing to disease B: Body temperature



The clinical observations were consistent with those reported by Zaucha *et al.*, 1998² and are summarized above.

Comments:

Overall, the study suggests that PA is a suitable early biomarker of infection and should serve as a trigger for treatment compared with bacteremia and pyrexia, allowing for early therapeutic intervention against inhalational anthrax.

6.1.1.3. Study 589-G607604

This was a non-GLP study in 14 specific pathogen free NZW rabbits (7 males and 7 females) purchased from [REDACTED] (b)(4) weighing between 2.50 - 3.13 kg conducted at the [REDACTED] (b)(4).
[REDACTED] (b)(4)³⁴ The objectives of the study were to

- determine the potential of NZW rabbits to serve as a therapeutic model for *B. anthracis* infections.
- examine time to onset of abnormal values in physiological and clinical signs to obtain an understanding of disease progression post-challenge.
- determine if a predictable overall response may provide an idea of an optimal window of time that can be used for therapeutic intervention.

Rabbits placed on study were in good health, free of malformations, with no clinical signs of disease. The details of health assessments of the rabbits were not included. Rabbits were surgically implanted with jugular vascular access ports (VAP) by the vendor ([REDACTED] (b)(4)) and shipped to the test facility [REDACTED] (b)(4) where they were quarantined for seven days prior to study initiation. Rabbits were observed for clinical signs of disease twice daily during the quarantine period. During this time, each rabbit was implanted with two transponder chips for measuring temperature (subcutaneous; one chip at shoulder blade area and one in the rump).

The Ames strain of *B. anthracis* used in the study was derived from a subculture of *B. anthracis* received from the United States Army Medical Research Institute for Infectious Diseases (USAMRIID). Of the 14 rabbits, 12 were challenged with *B. anthracis* spores (spore lot no. B23) of the Ames strain by aerosolization and two rabbits (one male and one female) were not challenged and served as controls. The aerosol exposure system consisted of four major components: the aerosol generation and delivery system (Collison nebulizer and mixing tube), the sampling system (glass impinger), the exposure chamber, and the exhaust. A modified three-jet Collison nebulizer was used to generate a controlled delivery of an aerosol of spores from a liquid suspension. These nebulizers generate aerosol droplets having an approximate mean diameter of 1-2 µm. The aerosol particle size was determined during each exposure using an aerosol particle sizer (APS) spectrometer. The mass median aerodynamic diameter (MMAD), determined by averaging the cumulative median size (50 percent mass) from the aerosol size distributions obtained from the APS for all aerosol exposures. The geometric standard deviation (GSD) represents one standard deviation for a normal distribution of particle sizes. The MMAD and GSD were determined for each exposure run and used to determine the variability in particle sizes for each aerosol exposure. Immediately prior to exposure to the infectious aerosol, plethysmography was used to determine the pulmonary function of individual animals, which was then used to calculate the length of exposure necessary to achieve the desired exposure concentration of *B. anthracis* spores. For each animal, the total inhaled dose was calculated from the concentration of viable organisms in the aerosol and the total inhaled volume of aerosol as follows:

$$\text{Inhaled Dose (in cfu)} = (C \times V)(S \times T)^{-1}(\text{TATV})$$

C = Impinger concentration (cfu/mL)
V = Impinger sampler volume (mL)
S = sampling rate (6 L/min)
T = Exposure time (min)

TATV Total accumulated tidal volume (L) (determined for each animal)³⁵

³⁴ [REDACTED] (b)(4) Study No. 589-G607604: Conducted under Contract No. NO1-AI-30061 Task Order No. 04. Potential of New Zealand White rabbits as a therapeutic model for *Bacillus anthracis* infection. Report dated March 12, 2007.
Reference ID: 3861581

Effluent air streams were collected directly from an animal exposure port by an in-line impinger to determine the number of cfu which actually reached the animal during exposures. Serial dilutions of impinger samples were enumerated by plating of the dilutions on TSA followed by incubation of the plates at 35 to 37 °C for 16-24 hours. Colonies were counted and the mean cfu/mL represented the concentration of *B. anthracis* in the aerosol. The actual inhaled dose of spores for each animal was then calculated using this concentration and the individual plethysmography data determined at the time of exposure. The LD₅₀ equivalents were calculated by dividing the total inhaled dose for each animal by the LD₅₀ dose reported by Zaucha *et al.* (1998)².

The animals were followed at different time intervals post-challenge for clinical signs and symptoms of disease that include temperature monitoring and laboratory parameters including microbiologic measurements (Table 18). To assess change in temperature for individual animals, the baseline body temperature for each individual rabbit was determined; a significant increase in body temperature (SIBT) was based upon each rabbit's inherent variation in baseline body temperature readings and defined as three consecutive readings (or two occurrences of two consecutive significant readings) that were greater than two times the standard deviation of that rabbit's baseline average temperature.

Table 18: Study 589-G607604-Blood collection and temperature monitoring

Group	Number of Rabbits	Temperature Monitoring (days)	Blood Collection for CBC and CRP (hours)	Blood Collection for Bacteremia (hours)*	Hourly Temperature Monitoring (hours post-challenge)
1	12	-3 to 14	-72, 0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72	0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72	12-72 [#]
2 (controls)	2	-3 to 14	-72, 0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72	0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72	12-72 [#]

*Aliquots taken from samples collected for hematology to perform bacteremia
[#] Reference Deviation Report #3476

Microbiologic measurements: Blood was collected at different time intervals (Table 18) for detection of bacteremia by culture and *B. anthracis* DNA by polymerase chain reaction (PCR). For blood cultures, 40 µL of blood was collected in EDTA tubes and cultured at 37°C for at least 48 hours. Plates having at least one colony consistent with *B. anthracis* morphology were documented as positive.

For detection of *B. anthracis* DNA by TaqMan® Real-time quantitative (q) PCR assay, bacterial DNA was isolated from 100 µL whole blood (with EDTA) using the Qiagen DNeasy® Tissue Kit. The primer/probe selection was based on published sequence data available in GenBank; oligonucleotides against *B. anthracis* DNA-directed RNA polymerase. Beta subunit (*rpoB*) gene was selected. The *rpoB* gene was selected because it is a highly conserved housekeeping gene (*Bacillus* genus). Also, due to its essential role in cellular metabolism, at least one copy is expected to be present in all bacteria. All reactions were performed in triplicate, and each plate contained known negative (genomic DNA isolation procedure using dH₂O) and positive (genomic DNA isolated from a *B. anthracis* vegetative culture) controls.

Results

The MMAD for challenge material aerosols was 1.19 µm which is within the expected range for the size distribution of the aerosol. The inhaled dose of *B. anthracis* ranged from 168-274 LD₅₀

³⁵ After the aerosol concentration was estimated, the volume (total accumulated tidal volume [TATV]) of

Reference ID: 3861561 atmosphere that an animal needs to inhale to reach the target LD₅₀ was determined.

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(Table 19). Both challenged and unchallenged animals had similar baseline temperature values. A trend towards an increase in body temperature was observed between 13 and 33 hours post-challenge; there was no change in the 2 unchallenged animals.

All animals were bacteremic by culture and qPCR methods within 29 and 59 hours post-challenge, became lethargic, and died between Day 2 and 5 of challenge (Table 19 and Figure 12). Bacteremia by culture was detected around the same time as qPCR except for one animal (K87707) which was culture positive at 30 hours and qPCR positive at 60 hours. Three rabbits were not bacteremic when their body temperatures were significantly elevated; each of these animals exhibited a positive bacteremia within six hours of their increased temperature. The two unchallenged control rabbits did not exhibit clinical or physiological changes throughout the course of this study.

Table 19: Study 589-G607604 - Characterization of NZW rabbit model of inhalational anthrax. Summary of events

Animal ID (n=12)	Sex	Inhaled dose		Time to Positive Test (hours)		Time to 2° F Temperature Increase** (hours)	Time to Death (Day)
		LD ₅₀ Dose	cfux10 ⁷	Culture	qPCR*		
K87704	M	273	(b) (4)	31	31	28	5
K87701	M	274		25	25	29	2
K87977	F	168		19	19	38	2
K87976	M	216		31	37	14	4
K87974	F	225		24	24	26	3
K87979	F	221		24	24	28	2
K87978	F	247		24	24	26	4
K87703	M	223		24	24	13	3
K87702	M	189		23	23	29	4
K87707	M	202		29	59	33	3
K87973	F	266		29	41	33	4
K87705	M	194		23	29	29	2
Mean ± SD	NA	224.8±34.4		25.5±3.7	30±11.1	27.2±7.2	3.2±1.0

M= Male; F = Female;

NA = Not Applicable

*qPCR +ve based on Mean C_t values >38.0

**To assess change in temperature trends, the average and standard deviation (SD) for individual baseline body temperature for each individual rabbit were determined. A significant increase in body temperature (SIBT) was dependent upon each rabbit's inherent variation in baseline body temperature readings and was defined as three consecutive readings (or two occurrences of two consecutive significant readings) that were greater than two times the SD of that rabbit's baseline average temperature.

Note:

The uninfected control animals: There does not appear to be any change in body temperature in the unchallenged animals. The applicant states that the temperature transponder of one animal (K87707, challenge group) malfunctioned at the rump and monitoring was performed from the shoulder. Both animals were culture negative; one positive result was obtained by qPCR and was thought to be attributed to potential contamination of the sample — but this was not confirmed.

Both uninfected animals survived until Day 14.

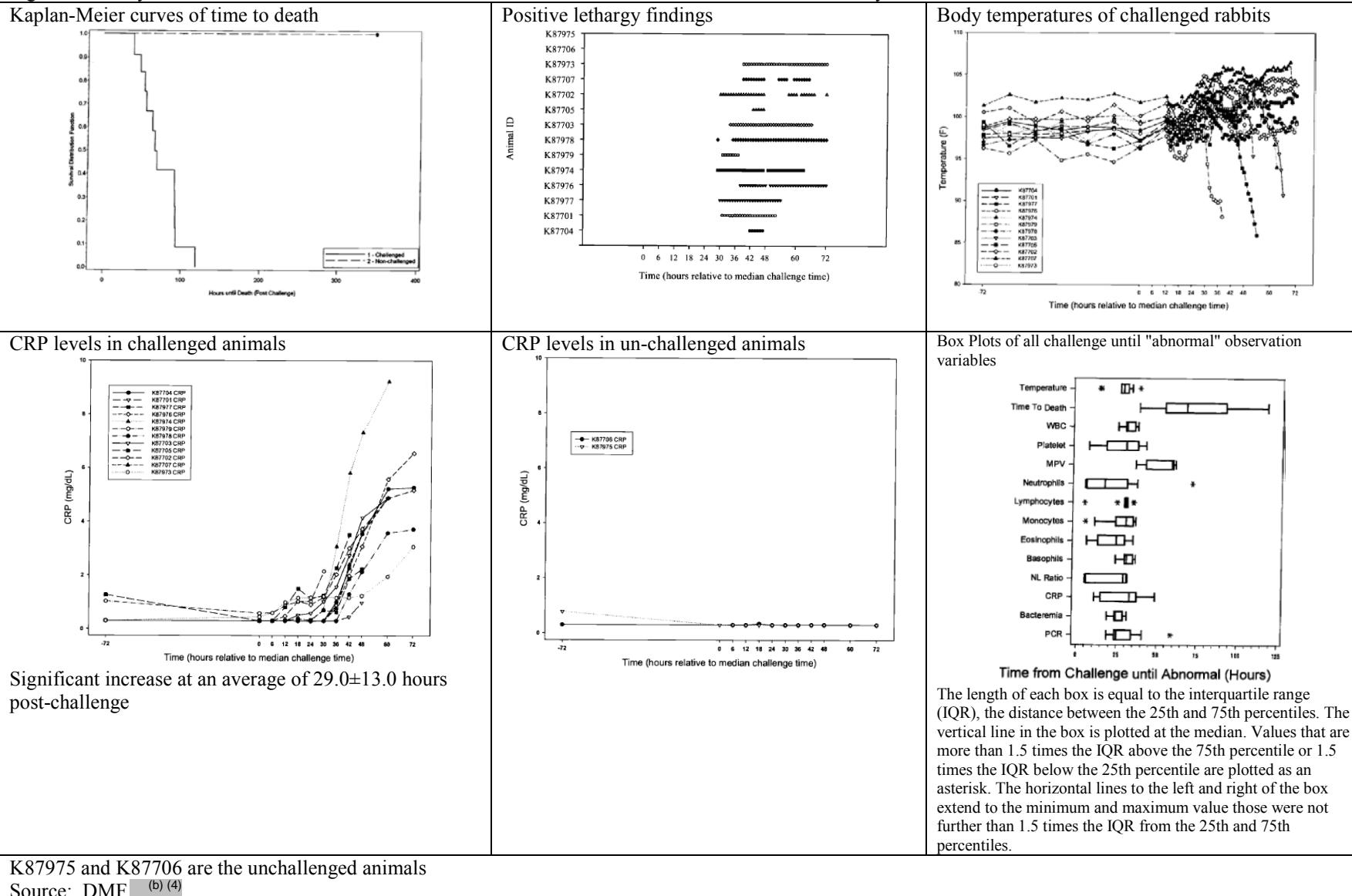
All challenged rabbits exhibited a significant increase in body temperature, increase in neutrophil/lymphocyte (N/L) ratio, decrease in white blood cells (WBC), increase in C-reactive protein (CRP), and positive bacteremia (Figure 12). All rabbits succumbed to disease with an average time to death of approximately 3.2 days (73 hours) following challenge (Figure 12).

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Figure 12: Study 589-G607604 - Characterization of NZW rabbit model of inhalational anthrax. Summary of events



Comments

All challenged rabbits exhibited a significant increase in body temperature, decrease in white blood cells (WBC), increase in neutrophil/lymphocyte (N/L) ratio, increase in C-reactive protein (CRP), positive bacteremia, and succumbed to disease with an average time to death of approximately 3.2 days (73 hours) following challenge. Lethargy, inappetence, respiratory, and stool abnormalities were common abnormal clinical observations documented. The two unchallenged control rabbits did not exhibit clinical or physiological changes during the period of observation.

Abnormal clinical observations occurred relatively late in comparison to other parameters such as temperature, hematology, bacteremia, and CRP. Detection of bacteremia by culture appears to be the most sensitive method. However, cultures results can take approximately 24 to 48 hours. As rabbits die rapidly (the average time to death was approximately 3 days), culture may not be an appropriate screening assay for detecting bacteremia for the purpose of treatment intervention. Detection of bacteremia by qPCR may be more appropriate as a screening assay as it corresponded closely with culture results. However, in this study qPCR was less sensitive than culture and treatment intervention could be delayed by approximately 10 hours.

The applicant did not provide information regarding PA detection. However, in the study report for the Study 662-G607604, it was stated that “circulating protective antigen (PA) and lethal factor (LF) levels were assessed and confirmed negative at every blood collection time point for each of the unchallenged control rabbits on [REDACTED] ^{(b)(4)} Study 589-G607604 (data not shown).” It appears that specimens from challenged animals were not tested for the presence of PA.

The study suggests that NZW rabbits provide a useful model for evaluating treatment and prophylaxis and treatment against inhalational anthrax in humans. Presence of bacteremia appears to be an appropriate trigger for intervention.

6.1.1.4. Study 616-G607604

The study was performed in 9 rabbits weighing between 2.53-3.23 kg challenged with the 200 LD₅₀ spores (spore lot no. B23) of the Ames strain of *B. anthracis*.³⁶ The study design was similar to that of Study No. 589-G607604 (summarized above in section 6.1.1.3) except that the study included two groups; animals (10 rabbits) in one of the group were treated with an anti-PA antibody. Additionally, bacteremia was measured by both quantitative (qPCR; same as for Study 589-G607604) and qualitative PCR (+/- PCR; using *pagA* primer/probe set). Although qPCR was stated to be quantitative, the results were expressed as positive or negative.

The findings in the control group of animals are summarized below.

Results

The MMAD was for challenge material aerosols was 1.14 µm. The average challenge dose varied from 245 to 450 (225±34) LD₅₀. The time to increase in SIBT (17 to 69 hours; average

³⁶ [REDACTED] ^{(b)(4)} Study No. 616-G607604: Conducted under Contract No. N01-AI-30061 Task Order No.04. Determining the robustness of New Zealand White rabbits as a therapeutic model for *Bacillus anthracis* infection. Report dated July18, 2007.

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30.6 hours) was consistent with the observations for animals in Study 589-G607604 summarized above (Table 20). The average time to death from the documentation of an SIBT was 49.81 hours (range 29.23-70.39 hours).

All animals were bacteremic by culture and PCR (quantitative and qualitative) within 23 and 75 hours post-challenge, became lethargic, and died between Day 2 and 5 of challenge (Table 20 and Figure 13); bacteremia by culture as well as qualitative and quantitative PCR was detected at the same time in all animals.

Table 20: Study 616-G607604 - Characterization of NZW rabbit model of inhalational anthrax. Summary of events								
Animal ID (n=9)	Sex	Inhaled dose		Time to Positive Test (hours)			Time to Increase in Temperature** (hours)	Time to Death (Day)
		LD ₅₀ Dose	cfux10 ⁷	Culture	+/-PCR*	qPCR		
K87708	M	252	(b) (4)	75	75	75	69	5
K87716	M	341		23	23	23	25	3
K87720	M	245		23	23	23	25	2
K87721	M	395		26	26	26	29	4
K87722	M	245		24	24	24	25	3
K87724	M	365		25	25	25	28	2
K87988	F	319		27	27	27	17	2
K87990	F	490		27	27	38	30	5
K87994	F	298		61	61	61	27	5
Mean ± SD		328±81		34.6±19.4	34.6±19.4	35.8±19.2	30.6±14.9	3.4±1.3

M= Male; F = Female;
*qPCR and +/-PCR +ve results based on Mean C_t values >38.0
**To assess change in temperature trends, the average and standard deviation (SD) for individual baseline body temperature for each individual rabbit were determined. A significant increase in body temperature (SIBT) was dependent upon each rabbit's inherent variation in baseline body temperature readings and was defined as three consecutive readings (or two occurrences of two consecutive significant readings) that were greater than two times the SD of that rabbit's baseline average temperature.

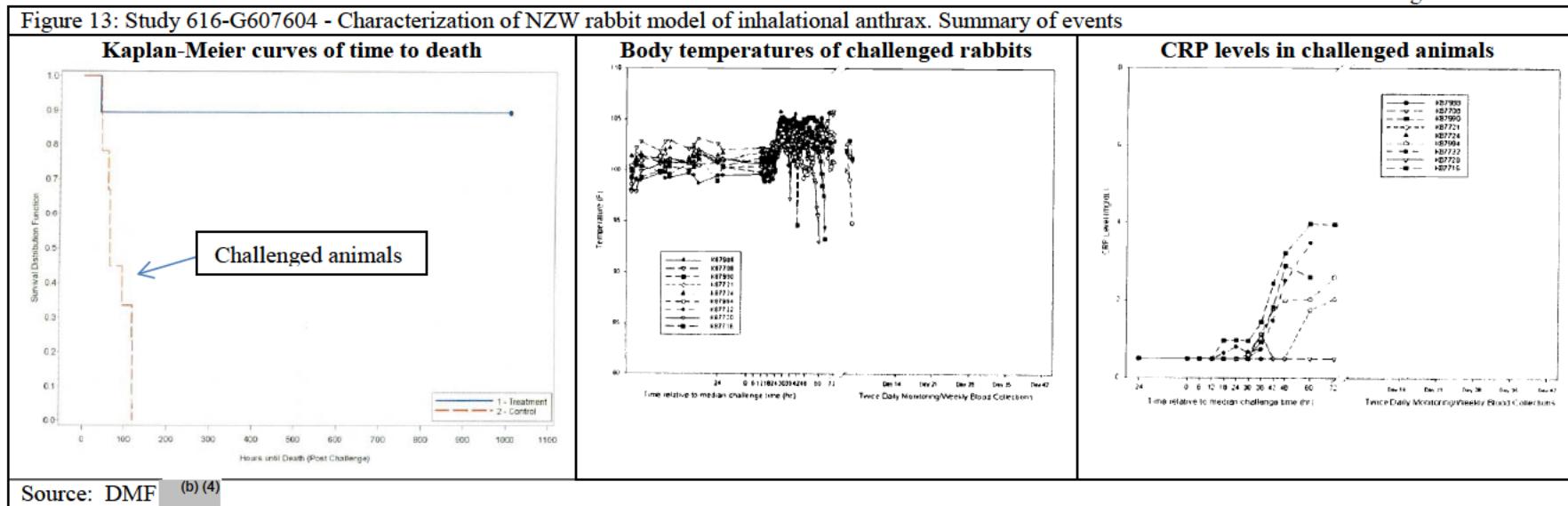
Alterations in hematological parameters measured showed a similar trend as observed for the animals in Study 589-G607604 summarized above.

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Figure 13: Study 616-G607604 - Characterization of NZW rabbit model of inhalational anthrax. Summary of events



Comments:

All challenged rabbits exhibited a significant increase in body temperature, decrease in white blood cells (WBC), increase in neutrophil/lymphocyte (N/L) ratio, increase in C-reactive protein (CRP), positive bacteremia, and succumbed to disease with an average time to death of approximately 3.4 days (82 hours) following challenge. Lethargy, inappetence, respiratory, and stool abnormalities were common abnormal clinical observations documented. The detection of bacteremia by culture or any of the PCR methods was at the same time. PA was not measured in this study.

The study suggests that NZW rabbits provide a useful model for evaluating treatment and prophylaxis against inhalational anthrax in humans. Presence of bacteremia appears to be an appropriate trigger for intervention.

6.1.1.5. Study 662-G607604

The study was performed in 40 rabbits weighing between 2.84-3.46 kg challenged with the 200 LD₅₀ spores (spore lot no. B30) of the Ames strain of *B. anthracis* and included 4 groups one of which (Group 4) was an infected control group (n=10).³⁷ The study design was similar to that of Study No. 589-G607604 (summarized above in section 6.1.1.3) except that qPCR was not performed and PA concentrations were measured in plasma by sandwich ELISA (modified version of [REDACTED]^{(b)(4)} SOP Number [REDACTED]^{(b)(4)} X-101) using rabbit anti-PA IgG antibody purified at [REDACTED]^{(b)(4)}. Additionally, gross necropsies and histopathology were performed on all rabbits found dead or euthanized due to illness to confirm death or illness due to *B. anthracis* infection.

The objectives of the study were to:

- determine the protective efficacy of a monoclonal antibody or antibiotic when administered alone or in combination following a SIBT (onset of illness).
- continue to build a database of information pertaining to the clinical profile of rabbits following a *B. anthracis* (Ames strain spores) aerosol challenge.
- provide further evidence supporting the use of body temperature, bacteremia, and white blood cell (WBC) counts as predictors of illness in the NZW rabbit and their usefulness as time to treat indicators.

The findings in the control group of animals are summarized below.

Results

The MMAD was for challenge material aerosols was 1.13 µm. The average challenge dose varied from 245 to 450 (432 ± 62) LD₅₀. The time to increase in body temperature (30.1 hours; range 13.4-43.9 hours) was consistent with the observations for animals in the two studies (589-G607604 and 616-G607604) summarized above.

All animals were bacteremic by culture and PCR within 48 and 60 hours post-challenge, respectively; bacteremia by culture and PCR was detected at the same time in all animals except 2 (Table 21). All animals became lethargic and died between Days 2 and 4 post-challenge (Table 21 and Figure 14). The average time to death was 2.5 days (60 hours). Eighty percent of

³⁷ [REDACTED]^{(b)(4)} Study No. 662-G607604: Determining the therapeutic efficacy of a monoclonal anti-PA antibody given in combination with levofloxacin to New Zealand White rabbits following a *Bacillus anthracis* inhalation challenge. Report dated December 7, 2009.

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the animals were culture positive for *B. anthracis* within 30 hours of challenge, with the remaining animals becoming positive ~18 hours later. PA was detected in all the rabbits by 48 hours post challenge; the PA levels ranged from 10 to 2,083 ng/mL.

Table 21: Study No. 662-G607604 -Characterization of NZW rabbit model of inhalational anthrax. Summary of events

Animal ID (n=10)	Sex	Inhaled dose		Time to Positive Test (hours)		PA by ELISA		Time to Death (Day)
		LD ₅₀ Dose	cfux10 ⁷	Culture	+/-PCR*	Time to positive (hours)	Concentration (ng/mL) [†]	
L06032	M	462	(b) (4)	24	24	24	14	2
L06045	F	376		24	24	24	420	2
L06026	M	462		24	24	30	154	2
L06043	F	446		36	30	36	25	4
L06039	F	503		30	30	30	16	3
L06014	M	380		30	30	36	2083	2
L06024	M	417		48	60	48	14	3
L06046	F	435		24	24	24	123	2
L06053	F	316		24	24	30	451	2
L06047	F	519		30	30	30	10	3
Mean ± SD		432±62		29.4±7.7	30.0±11	31.0±7.5	331±638	2.5±0.7

M= Male; F = Female;
* +/-PCR +ve results based on Mean C_t values >38.0
**To assess change in temperature trends, the average and standard deviation (SD) for individual baseline body temperature for each individual rabbit were determined. A significant increase in body temperature (SIBT) was dependent upon each rabbit's inherent variation in baseline body temperature readings and was defined as three consecutive readings (or two occurrences of two consecutive significant readings) that were greater than two times the SD of that rabbit's baseline average temperature.
†Lower limit of detection 1 µg/mL (for details see Dr Lynette Berkeley's microbiology review)

Alterations in hematological parameters measured showed a similar trend as observed for the animals in Studies 589-G607604 and 616-G607604 summarized above.

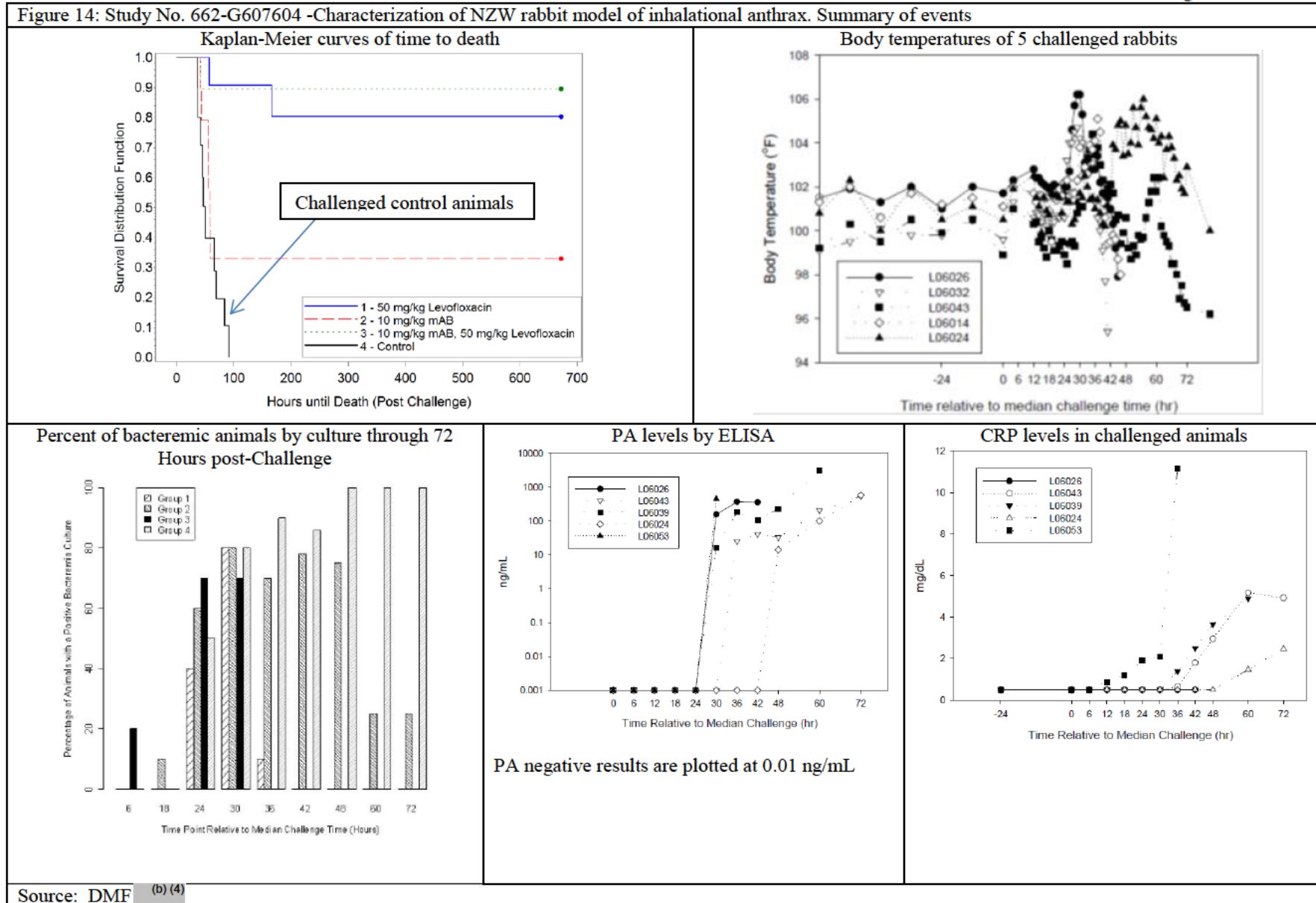
Gross lesions observed included red foci in the appendix, ovaries, and stomach; discoloration (red) of the lungs (representing hemorrhage); and enlargement of the spleen; these findings are consistent with those reported by Zaucha *et al.* (1998)². Microscopic findings were graded semi-quantitatively. Microscopic findings consistent with inhalation anthrax included presence of bacteria with appropriate morphology (large square ended bacilli) in vessels and extravascularly in tissues, with necrosis (particularly of lymphoid tissues), hemorrhage, and fibrin accumulation with or without a component of suppurative inflammation.

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Figure 14: Study No. 662-G607604 -Characterization of NZW rabbit model of inhalational anthrax. Summary of events



Source: DMF (b) (4)

Comments:

All of the untreated animals succumbed to disease. Assessment of body temperature, complete blood count (CBC) with differential, C-reactive Protein (CRP) levels, and onset of bacteremia was consistent with that for the two studies summarized above. All challenged rabbits exhibited a significant increase in body temperature, decrease in white blood cells (WBC), increase in neutrophil/lymphocyte (N/L) ratio, increase in C-reactive protein (CRP), positive bacteremia, and succumbed to disease with an average time to death of approximately 2.5 days (60 hours) following challenge. Lethargy, inappetence, respiratory, and stool abnormalities were common abnormal clinical observations documented. The detection of bacteremia by culture or PCR was around the same time. PA levels were also measured. Time to PA positive findings was consistent with culture positive findings. The applicant stated that the circulating PA and LF levels were assessed and confirmed negative at every blood collection time point for each of the unchallenged control rabbits on ^{(b)(4)} Study 589-G607604 (data not shown). Presence of bacteremia or PA appears to be an appropriate trigger for intervention.

The presence of either bacteremia or PA antigen could be considered a confirmation of active anthrax as the terminal phase of anthrax is associated with the proliferation of the bacteria in the blood stream and the production and distribution of large amounts of toxin components released into the circulation. The study suggests that NZW rabbits provide a useful model for evaluating treatment and prophylaxis and treatment against inhalational anthrax in humans.

Gross lesions observed included red foci in the appendix, ovaries, and stomach; discoloration (red) of the lungs (representing hemorrhage); and enlargement of the spleen; these findings are consistent with published pathology of rabbits with inhalational anthrax disease (Zaucha et al., 1998²)

6.1.2. Cynomolgus monkeys

The applicant refers to one published study (Vasconcelos et al. 2003³) and one NIAID study (Study No. 777-G607605) to support characterization of the animal model in cynomolgus monkeys (*Macaca fascicularis*).

6.1.2.1. Vasconcelos et al. 2003

In the study by Vasconcelos et al. 2003³, monkeys were exposed to aerosols containing 4.56×10^4 to 2.94×10^6 cfu of the Ames strain of *B. anthracis* spores in a head-only aerosol exposure chamber. The authors state that all animals were culture-positive for *B. anthracis* and died within 10 days (range 2 – 10 days) of aerosol exposure (Table 22), with a tendency for those receiving higher doses to succumb more quickly than those receiving lower doses (data not shown). All monkeys exposed to 2.0×10^5 cfus died within 4 days of exposure. The LD₅₀ determined was 6.1×10^4 (34,000 to 110,000) cfu. Additionally, animals generally showed few clinical signs until 1 to 2 hours prior to death, when they became progressively less responsive to external stimuli.

Table 22: Time to death and necropsy findings in 14 cynomolgus monkeys

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Gross Enlargements of Hematopoietic Tissues

COPYRIGHT MATERIAL WITHHELD

6.1.2.2. Study No. 777-G607605

A study to characterize the animal model was performed at [REDACTED] (b) (4) in 12 cynomolgus monkeys (*Macaca fascicularis*) five years of age and weighing between 2.7-7.3 kg; this was a part of the study that included another group of monkeys treated with an anti-PA monoclonal antibody (other than ETI-204).³⁸ A group of uninfected animals were also included to measure pharmacokinetics of the monoclonal antibody (Table 23).

³⁸ [REDACTED] (b) (4) Study No. 777-G607605: Evaluation of the therapeutic efficacy of an anti-PA monoclonal antibody in the cynomolgus macaque following inhalational challenge with *Bacillus anthracis*. March 24 2009 (submitted in the DMF [REDACTED] (b) (4) by NIAID, NIH).

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Table 23: Study 777-G607605-Study Groups

Group ID	Monkeys per Group	mAb Dose	Treatment Point Post-Challenge	Dose Route
1	12	10 mg/kg	Individual times were based on serum PA levels	IV (bolus injection)
2	12	N/A	N/A	N/A
3	6 (unchallenged)	10 mg/kg	Day 42 (relative to Day 0 of challenge day B animals)	IV (bolus injection)

The animals utilized in this study were transferred from a previous study; however, the study they were in was not specified. All monkeys were stated to be healthy prior to placement on study and had TA-D70 telemetry transmitters implanted prior to shipment to [REDACTED] (b) (4). Monkeys were tested and verified negative for tuberculosis; also, they were pre-screened within 30 days prior to receipt at [REDACTED] (b) (4) to confirm that they were seronegative for Simian Immunodeficiency Virus (SIV), Simian T-Lymphotropic Virus-1 (STLV-1), and *Cercopithecine herpesvirus* 1 (Herpes B virus) and negative for Simian Retrovirus (SRV1 & SRV2) by PCR.

Animals were challenged with the Ames strain of *B. anthracis*; the preparation of spores (spore lot no. B31) and aerosolization procedures were the same as for the NZW rabbit studies except that the monkeys were exposed in a head only chamber and were anesthetized prior to placement in the exposure chamber and throughout the exposure.

The applicant used a target dose of 200X LD₅₀ ([REDACTED] (b) (4)) spores that was based on the study by Vasconcelos *et al.* 2003³. The animals were challenged on 2 separate days; the MMAD of aerosolized anthrax for Challenge Days A and B were 1.18µm and 1.13µm, respectively, consistent with lower respiratory tract deposition. Aerosol concentrations of *B. anthracis* were quantified and results expressed as cfu. Effluent streams were collected directly from an animal exposure port by an in-line impinger model 7541 (Ace Glass, Inc). Serial dilutions of impinger samples were plated onto TSA plates and the number of cfu enumerated.

Microbiologic measurements: Blood samples were collected with and without EDTA prior to challenge and at different time intervals post-challenge for measuring bacteria by culture and PCR, PA by ECL and ELISA, CRP, and hematological parameters (Table 24).

Table 24: Study 777-G607605- Natural history study - blood collection and assay schedule

Group	Blood collections (Bacteremia culture, qPCR, Hematology, CRP, ECL, and PA-ELISA)
1	Day -7; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post median challenge time; Days 5*, 8*, 14*, 28*, PTT
2	Day -7; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post median challenge time; Days 5*, 8*, 14*, 28*
3	Day -7; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post median challenge time; Days 5*, 8*, 14*, 28*

* No ECL analysis was performed at these time points. Real time ECL testing was suspended after 72 hours as the purpose of such testing was to use test results as a trigger for intervention.

PTT a blood sample collected immediately prior to treatment.

Source: DMF [REDACTED] (b) (4)

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PA-ECL: The rapid protective antigen screening assay was performed in accordance with SOP (b) (4) V-061 at (b) (4) (for details see Dr Lynette Berkeley's microbiology review) using 25 µL serum specimens. The lower limit of detection was 4 ng/mL; however, a positive control of 2 ng/mL tested positive in the assay (for details see Dr Lynette Berkeley's review).

PA-ELISA: The ELISA assay was performed in accordance (b) (4) SOP (b) (4) X-180 (for details see Dr Lynette Berkeley's microbiology review) using purified polyclonal anti-PA IgG antibody.

Bacteremia (Culture): Approximately, 40 µL of whole blood was collected in EDTA tubes at different time intervals (Table 24) and streaked on blood agar plates; plates were incubated at 37°C ± 2°C for at least 48 hours. At least one colony consistent with *B. anthracis* morphology was documented as positive.

Bacteremia (qualitative PCR): Bacterial genomic DNA was isolated from 100 µL whole blood aliquots collected in EDTA tubes at different time intervals (Table 24) using the Qiagen DNeasy® Tissue Kit (Qiagen, Valencia, CA). Briefly, blood was mixed with bacterial enzymatic lysis buffer and incubated for 15 ± 5 minutes at 37°C followed by incubation with proteinase K for 30 minutes at 70°C. Absolute ethanol was added to each sample and added to a Qiagen spin column and washed sequentially. The purified genomic DNA was eluted from the spin column with 100 µL of buffer. The protective antigen (*pagA*) primer/probe was selected and the sequence was based on published data; oligonucleotides were designed to amplify a small fragment within the coding region of the *B. anthracis* PA (*pagA*) gene (Figure 15) as PA is a crucial part of three-part protein toxin complex secreted by *B. anthracis* and is the main protein component of most vaccine formulations. Oligonucleotide sequences were submitted to (b) (4) for the generation of a TaqMan® Gene Expression Assay (Figure 15).

Figure 15: Study 777-G607605-Targeted sequence within the *B. anthracis* protective antigen (*pagA*) gene and primer and probe sequence

Targeted sequence within the *B. anthracis* protective antigen (*pagA*) gene

144661	atggagaata	ttatttcttc	aaaaaatgag	gatcaatcca	cacagaatac	tgatagtcaa
144721	acgagaacaa	taagtaaaaa	tacttctaca	agttagcac	atactagtga	agtacatggaa
144781	aatgcagaag	TGCATGCGTC	GTTCTTGAT	AttGGTGGGA	GTGTATCTGC	Aggattttgt
144841	aattcqaatt	CAAGTACGGT	CGCAATTGAT	CATTcactat	ctcttagcagg	ggaaagaact
144901	tgggctgaaa	caatgggttt	aaataccgct	gatacagcaa	gattaaatgc	caatattttaga
144961	tatgttaaaa	ctgggacggc	tccaaatctac	aacgtgttac	caacgacttc	tttagtgttta

Sequence information was obtained from GenBank Accession Number AE017336. Underlined nucleotides indicate the sequence amplified in the qPCR assay. Uppercase and bolded nucleotides indicate the forward primer, reverse primer, and probe sequences.

TaqMan® Primer and Probe Sequences for the *Bacillus anthracis* *pagA* Gene

Gene	Primer/Probe	Oligonucleotide Sequence (5'-3')	Accession No. ¹
pagA	Forward	AATGATCAATTGCGACCGTACTT	AE017336
	Reverse	TGCATGCGTCGTCTTGATA	
	Probe	6FAM-TGCAGATACACTCCCACC-MGBNFQ	

¹ GenBank accession numbers are available online at <http://www.ncbi.nlm.nih.gov/>
Source: DMF (b) (4)

Qualitative real-time PCR was performed using TaqMan® Universal PCR Master Mix and ABI PRISM® Sequence Detection System; known negative (genomic DNA isolation procedure using dH2O) and positive (genomic DNA isolated from a *B. anthracis* vegetative culture) controls

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(b) (4)

were included in each run. Data were analyzed using [REDACTED]

A positive result report was based

on a median Ct value of <38.00.

Hematology parameters: Hematological measurements evaluated at different time intervals included complete blood count, differentials (white blood cell count, neutrophil/lymphocyte (N/L) ratio, and differential leukocyte count, hemoglobin, hematocrit, red blood cell count, platelet count) in EDTA containing blood. C-reactive protein was measured in plasma.

Toxin neutralizing antibodies: TNAs were measured in serum samples stored at < -70°C. However, no results were included.

Telemetry transmitters that collect body temperature and activity levels were surgically implanted in the animals for pre- and post-challenge assessments. Body temperature, weight, and activity were measured at regular intervals. The average body weight on Day 0 (prior to challenge) was 4.2 kg. Clinical observations included but not limited to anorexia, lethargy, respiratory distress, moribund, activity (recumbent, weak, or unresponsive), seizures, and other abnormal clinical observations (described by observer). In addition, appetite was monitored twice daily during the six hourly observation period. Observed signs of illness or live/found dead/euthanized animals were recorded at the time of observation on approved [REDACTED] (b) (4) forms. Animals judged to be moribund underwent a terminal blood draw prior to being euthanized according to [REDACTED] (b) (4) standard operating procedures.

Pathology: Gross necropsy was performed on all monkeys that were found dead or euthanized (SOP PATH.XI-001). Sections of target tissues including but not limited to brain/meninges, lungs, liver, spleen, kidney and mediastinal lymph nodes as well as all gross lesions were preserved in 10% neutral buffered formalin. Histopathology was performed, as deemed necessary by a board-certified Veterinary Pathologist, on two animals one of which (A03505) was in Group 2 (died on study with no specific gross lesions) to confirm death or illness due to anthrax.

Results:

Exposure: The mean aerosol exposure dose for the 12 infected animals was 433 (± 156) LD₅₀ equivalents (Table 25); the challenge Day A animals received a higher average challenge dose (537 LD₅₀) than the challenge Day B animals (329 LD₅₀).

Mortality: Two animals survived the period of observation i.e., 28 days (one from challenge day A and one challenge Day B); 10 (83.3%) animals died between Days 2 and 9 post-challenge (Figure 16). The reason for the two animals surviving is unclear. All six of the unchallenged control animals (Group 3) remained healthy throughout the course of the study until Day 28.

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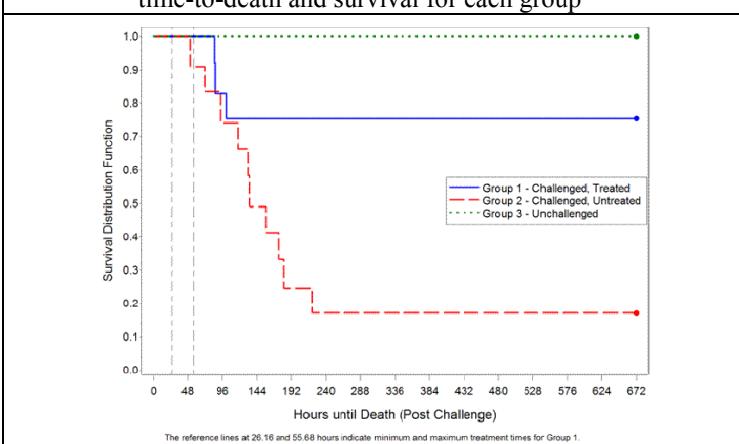
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Table 25: Study 777-G607605 - Characterization of cynomolgus monkey model of inhalation anthrax - Summary of events

Animal ID (n=12)	Sex	Body weight (kg)	Inhaled Dose		Time to Positive Test (hours)		PA titer by ELISA (ng/mL)	Time to Death hour (days)		
					Bacteremia					
			cfu x 10 ⁷	LD ₅₀ Dose	Culture	PCR				
Challenge Day A										
23393	M	5.2	(b) (4)	443	36	36	42 (48)	21.4	157 (6.54)	
A01249*	M	7.2		390	42	42	42 (48)	13.0	Survived	
A01180	F	4.3		612	36	36	42 (42)	3.7	174 (7.25)	
A01093	F	2.9		678	42	42	42 (42)	3.1	94 (3.92)	
A01218	F	3.1		601	24	24	30 (30)	8.7	118 (4.92)	
A03022	M	3.6		497	60	60	60 (72)	27.8	220 (9.17)	
Challenge Day B										
23379	M	7.3	(b) (4)	198	42	42	42 (48)	43.7	182 (7.58)	
A03526	M	4.0		364	36	36	36 (42)	37.0	133 (5.54)	
A01072	F	3.3		382	48	54	42 (48)	10.8	71 (2.96)	
A03580	F	2.7		402	30	30	30 (36)	6.1	131 (5.46)	
A03505	M	4.0		385	24	30	30 (36)	21.3	51 (2.13)	
A01176*	F	3.0		244	42	36	36 (42)	5.2	Survived	
Mean ± SD	NA	4.22 ± 1.6		433 ± 144	38.5±10.1	39 ± 10.1	39.5 ± 8.3 (44.5±10.3)	16.82±13.5	133± 52.2 (5.55± 2.2)	
M= Male; F = Female; NA = Not Applicable PCR values are for an undiluted sample The mass median aerodynamic diameter (MMAD) of aerosolized anthrax for Challenge Days A and B were 1.18µm and 1.13µm, respectively, consistent with lower respiratory tract deposition. *Survivors A01249 and A01176 had a positive culture at 42 hours and remained positive.										

Figure 16: Study 777-G607605-Kaplan-Meier curves representing time-to-death and survival for each group



Circulating PA (ECL and ELISA): Circulating PA was detected by **ECL** assay between 30 and 60 hours post challenge time (Table 25). For ECL assay, the threshold for a positive result was 2.0 ng/mL; this was based on the positive control included for testing; otherwise the LOD was determined to be 4 ng/mL (for details see Dr Lynette Berkeley's microbiology review); all challenged animals remained PA positive until 72 hours post-challenge (real-time ECL analysis was suspended after 72 hours-post median challenge as the results were used to indicate when treatment was to commence). One unchallenged animal (# 23375) in Group 3 was PA positive at 48 hours by ECL. The applicant states that this was a technical error as the specimen was stated to be PA negative on repeat testing.

PA levels in serum were quantitated by a sandwich **ELISA**. Based on data from initial development of the PA-ELISA, a preliminary LOD of 3.0 ng/mL was defined for this study. All

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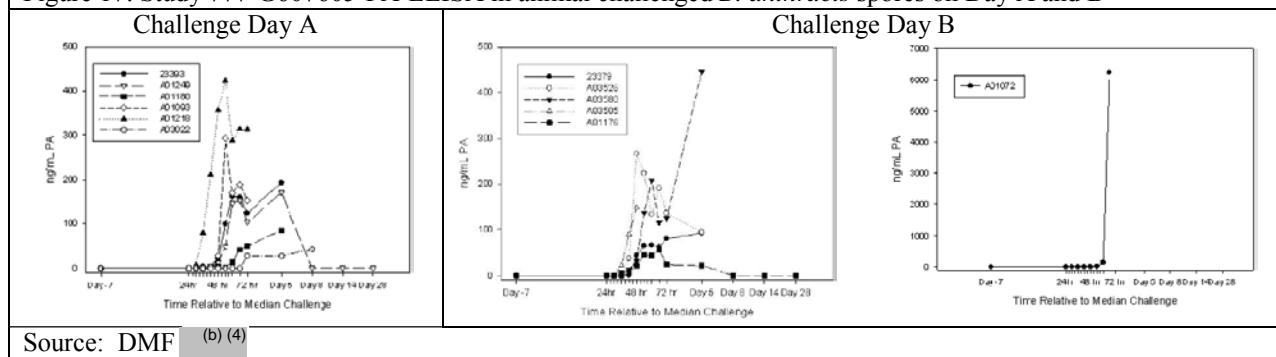
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the unchallenged animals exhibited results <3.0 ng/mL and challenged animals \geq 3.0 ng/mL (based on review of the validation report the LOD was determined to be \leq 4.2 ng/mL; for details see Dr Lynette Berkeley's review). The PA levels, time to peak, and the duration for which animal remained positive were highly variable among the animals (Figure 17). The animals which survived the period of observation became PA negative by Day 8 post-challenge and remained negative until Day 28 (Figure 17).

All the animals were PA positive by both ECL and ELISA (Table 25). Time to PA positivity was the same by either of the tests for 5 of the 12 animals; for the remaining 7 animals, PA positivity by ELISA, was delayed by 6 to 12 hours.

Figure 17: Study 777-G607605-PA-ELISA in animal challenged *B. anthracis* spores on Day A and B



Source: DMF (b) (4)

Bacteremia (culture): All the challenged animals were bacteremic; time to positive cultures varied from 24 hours to 60 hours post-challenge (Table 25). All animals that died (or were euthanized) following challenge exhibited positive terminal cultures. Eight of the 10 animals that survived through 72 hours post-challenge were culture positive at 72 hours. Of the two animals (A01249 and A01176) that survived until Day 28, one animal (A01249) was culture negative at 72 hours and positive on Day 5 and then stayed negative; the other animal remained culture negative at 72 hours and after (Tables 25 and 26). One of the unchallenged animal was stated to be culture positive with less than 5 colonies at Day 28 (Table 26); however, this animal was stated to be culture negative at Day 32; by qPCR the animal was stated to be negative at Day 28.

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Table 26: Study 777-G607605-Positive bacteremia results by culture

Animal ID	Group	Day -7	24h	30h	36h	42h	48h	54h	60h	66h	72h	PTT	Day 5	Day 8	Day 14	Day 28	Terminal
A01081	1			+	+	+	+					+					
22185	1					+	+	+	+	+	+	+					
A01015	1			+	+	+	+					+					+
23476	1					+	+	+				+					
A01012	1	+	+	+	+			+	+		+	+					
A00985	1		+	+	+							+	+				
23386	1		+	+	+	+	+	+	+	+	+	+					+
A03461	1			+	+							+					
A01092	1				+	+	+					+					+
A03210	1	+	+	+	+	+	+	+	+			+					+
A03184	1					+	+	+	+			+					
C16005	1			+	+		+		+			+					
23393	2				+	+	+	+	+	+	+		+				+
A01249	2					+	+	+	+	+			+				
A01180	2				+	+	+	+	+	+	+						+
A01093	2					+	+	+	+	+	+	+					+
A01218	2	+	+	+	+	+	+	+	+	+	+						+
A03022	2											+					+
23379	2					+	+	+	+	+	+	+					+
A03526	2				+	+	+	+	+	+	+	+					+
A01072	2					+	+	+	+								+
A03580	2		+	+	+	+	+	+	+	+	+	+					+
A03505	2	+	+	+	+	+	+	+	+	+	+	+					+
A01176	2				+	+	+	+	+	+							
A03575	3																
A01071	3																+
A01007	3																
A01215	3																
A01005	3																
23375	3																

■ Represent challenged Group 2 animals
■ Represent 2 challenged Group 2 animals that survived

Bacteremia (qPCR): Similar to culture results, positive bacteremia as assessed by qPCR was reported in all the challenged animals. All the animals that survived through Day 5 or 8 exhibited persistent bacteremia by qPCR. The two surviving animals at Day 28 were culture negative after Day 8 (Table 27).

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Table 27: Study 777-G607605 - Positive bacteremia results by qPCR

Animal ID	Croup	Day	Day -7	Hour 24	Hour 30	Hour 36	Hour 42	Hour 48	Hour 54	Hour 60	Hour 66	Hour 72	PTT	Day 5	Day 8	Day 14	Day 28	
22185	1	A	o	o	o	o	o	+	+	+	+	+	+	+	DEV	o	o	
23476	1	A	o	o	o	o	o	+	+	+	+	o	+	o	DEV	o	o	
A00985	1	A	o	o	+	+	+	+	+	+	+	+	+	o	o	o	o	
A01012	1	A	o	o	+	+	+	+	+	+	+	+	+	o	o	o	o	
A01015	1	A	o	o	o	+	+	+	+	+	+	+	+	o	DEV	o	o	
A01081	1	A	o	o	o	+	+	+	+	+	+	+	+	o	DEV	o	o	
23386	1	B	o	o	+	+	+	+	+	+	+	+	+	+	o	DEV	o	o
A01092	1	B	o	o	o	o	o	o	o	o	o	o	o	NI				
A03184	1	B	o	o	o	o	o	o	o	o	o	o	o	+	o	o	o	
A03210	1	B	o	o	+	+	+	+	+	+	+	+	+	+	o	o	o	
A03461	1	B	o	o	o	+	+	o	o	o	o	o	+	o	o	o	o	
C16005	1	B	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
22393	2	A	o	o	o	o	+	+	+	+	+	4	+	+				
A01093	2	A	o	o	o	o	o	+	+	+	+	+	+	+				
A01180	2	A	o	o	o	o	+	+	+	+	+	+	+					
A01128	2	A	o	o	+	+	+	+	+	+	+	+	+					
A01129	2	A	o	o	o	o	o	o	o	o	o	o	o					
A03012	2	A	o	o	o	o	o	o	o	o	o	o	o	+	DEV	o	o	
23379	2	B	o	o	o	o	o	o	o	o	o	o	o	+	+			
A01072	2	B	o	o	o	o	o	o	o	o	o	o	o	+	+			
A01176	2	B	o	o	o	o	o	o	o	o	o	o	o			o	o	
A03505	2	B	o	o	o	o	+	+	+	+	+	+	+	+	+			
A03576	2	B	o	o	o	o	o	o	o	o	o	o	o	+	+			
A03580	2	B	o	o	+	+	+	+	+	+	+	+	+	+	+			
A01062	3	A	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
A01071	3	A	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
A03575	3	A	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
23373	3	B	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
A01005	3	B	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
A01215	3	B	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	

DEV - Reference DR_5806

NI - No isolation was able to be performed due to low sample volume

 Represent challenged Group 2 animals

 Represent 2 challenged Group 2 animals that survived

Hematological parameters:

White Blood Cell Counts: The WBCs were elevated in challenged animals compared to pre-challenge levels; the two surviving animals showed peak WBC counts at Day 8 (> 40,000 cells/ μ L) that begin to resolve by Day 14 but did not decrease to baseline levels until Day 28. Unchallenged animals showed subtle elevations in WBC.

The unchallenged animals displayed a general diurnal rhythm (subtle increases in WBC counts (neutrophils, lymphocytes) during the night time hours with slight decreases during the day time hours). This is important when making comparisons between challenged and unchallenged animals, since changes of 1,000-2,000 cells/ μ L may be the normal variability throughout a 24 hours period for non-human primates. Additional consideration should be made regarding individual variability of normal values; each animal should be assessed by analyzing additional baseline blood samples collected prior to challenge.

Neutrophil Counts: Challenged animals showed an increase in neutrophil count (>15,000 cells/ μ L) beginning roughly 48 to 54 hours post-challenge; these levels peak prior to death (with the exception of the survivors). The change in neutrophil count appears to be later than WBC count in challenged animals; the average neutrophil count for the two surviving animals was also high. Normalization did not occur until Day 28.

Lymphocyte Counts: Lymphocytes counts decreased around 48 hours post-challenge followed by a rebound approximately 8 days following challenge, suggesting these cells may be recruited out of circulation after challenge. This could be due to the cell mediated immune response elicited by the bacteria that have infiltrated the lymphatic system. Animals that survive to Day 8 post-challenge showed a peak following the initial loss of lymphocytes that resolved between Days 14 and 28 post-challenge.

Neutrophil/Lymphocyte Ratio: A majority (11 of the 12) of the challenged animals showed significantly elevated N/L ratios; animal A03505 was the only challenged animal not to exhibit an elevated N/L ratio, and this animal died only 51 hours following challenge. By Day 8 post-challenge all surviving animal N/L ratios were within the normal range.

C-Reactive Protein: The CRP levels were increased in 11 of the 12 challenged animals between 22 and 66 hours post-challenge. The average time from abnormal CRP until death in challenged animals was 108.44 hours (4.5 days); one animal (A03505) that did not exhibit an increase in CRP was the first animal to die; the applicant states that it is possible that this animal died too soon to exhibit a CRP response or this animal didn't exhibit a CRP response and could have been more susceptible to disease. CRP levels were also increased in 3 of the 6 unchallenged animals. CRP is an acute phase reactant and could easily be affected by the frequent handling of the animals. From this study it appears that this non-specific indicator may not be beneficial in defining illness compared to other parameters such as bacteremia and PA.

Body temperature and activity: Ten of the twelve challenged animals exhibited SIBT-6 (6 consecutive significantly increased readings in temperature), with the two exceptions being A01072 and A01093; these two animals that did not show an elevated temperature following challenge (A01093 and A01072) did show slight disruptions in the diurnal pattern and were two of the first three animals to die. Animal A03580 did not show a persistent increase in temperature following challenge over normal day time temperature, but did lose the peaks typically seen during the night time hours, suggesting the animal may have been moderately febrile following challenge.

The challenged animals that exhibited body temperature increases also showed decreases in activity level during the same relative time frame. Prior to this decrease in activity many of the animals showed a short period of time of heightened activity. This increase in activity could be due to more frequent manipulation of the animals or may be a sign that the animals are becoming agitated as their disease progresses. Overall, there was a trend in an increase in body temperature and a decrease in activity level post-challenge; this is most likely due to anthrax disease progression. However, these observations should be interpreted with caution as temperature elevations were confounded by the diurnal temperature patterns typical of this species.

Clinical observations: Unchallenged animals were documented as having soft stool and hunched posture were documented in the unchallenged animals during the high frequency manipulation period and may likely be due to stress. Challenged animals showed clinical signs of disease including inappetence, stool abnormalities (e.g. soft stool, no stool, or diarrhea), posture changes (hunched or lying down in cage), inactivity or lethargy, signs of respiratory abnormalities (e.g. coughing, wheezing, and labored respirations), unresponsiveness, moribund, or found dead. The two challenged animals that survived through Day 28 were lethargic or had hunched posture through Day 10 (Animal A01176 – hunched posture) and Day 14 (Animal A01249 – lethargy).

When comparing the challenged animals to the unchallenged animals clinical signs were much more severe in the challenged animals. Persistent abnormal signs were typically observed beginning 48-60 hours post-challenge. The surviving challenged animals, except for inappetence, reverted back to normal approximately 7-9 days post-challenge.

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Pathology: Gross lesions at necropsy were consistent with anthrax, including body cavity effusions, red lung discoloration, liver foci, red or dark foci in the brain, enlargement and mottling of the ovary, and enlargement and/or dark color of multiple lymph nodes. Lesions of vascular damage (hemorrhage, edema and parenchymal necrosis) in multiple organ systems were typical of anthrax. Two of the ten animals in the challenged group showed lesions in the brain upon necropsy, whereas lesions were observed in many of the other tissues assessed in the untreated control animals (Table 28). In general, these observations were similar to those reported by Vasconcelos *et al.* (2003)³.

Histopathology was performed for only two animals (A03022 and A03505) that died on study with no specific gross lesions to confirm death due to anthrax; this decision was made by the study pathologist based on terminal bacteremia status. Microscopic findings considered consistent with anthrax (Twenhafel, 2010³⁹) were reported in both monkeys. Lesions typical of anthrax in this study included hepatic sinusoidal leukocytosis, pulmonary hemorrhage, mediastinal lymph node edema and lymphoid depletion, and splenic fibrin exudation and lymphoid depletion, as well as the presence of large rod-shaped bacteria consistent with *B. anthracis* in many of the organs evaluated histologically (Table 28B).

Table 28: Study 777-G607605 - Gross necropsy and histologic findings in animals found dead or euthanized in moribund condition

A: Gross necropsy findings		B: Microscopic Findings					
Group		1	2	3			
Number in group		12	12	6			
Mortality (%)		25%	83%	0%			
Sex	M	F	M	F	M	F	
ORGAN/lesion	# dead	2	1	5	5	0	0
BRAIN		2	1	2			
Accumulation/discoloration/foci (dark/red)							
CAVITY, ABDOMINAL							
Fluid							
CAVITY, PERICARDIAL							
Fluid							
CAVITY, THORACIC							
Fluid							
LIVER							
Foci							
LUNG							
Discoloration (red)							
LYMPH NODE, BRONCHIAL	1		2	5			
Enlarged/discoloration							
LYMPH NODE, INGUINAL							
Enlarged							
LYMPH NODE, MANDIBULAR							
Enlarged							
LYMPH NODE, MEDIASTINAL							
Enlarged							
LYMPH NODE, MESENTERIC/PANCREATIC							
Enlarged							
OVARY							
Enlarged/mottled							
SKIN							
Fluid (edema)							
Cells left blank have an incidence of 0							
Source: DMF		(b) (4)					
0 = lesion not present							

Comments:

Cynomolgus monkeys, challenged with the Ames strain of *B. anthracis* spores by aerosolization, were monitored for clinical and physiological changes following challenge; six uninfected animals were included as controls. All the monkeys included in the study were used previously in another study (the study was not specified). Two of the 12 challenged animals survived the

³⁹ Twenhafel N.A. 2010. Pathology of inhalational anthrax animal models. *Vet Pathol.* 47(5):819-30.

period of observation of 28 days; the reasons for their survival are unclear; these 2 animals were PA negative from Day 8 post-challenge. Immune status of the host was not measured.

Bacteremia by culture and PCR as well as circulating PA were detected relatively early in disease progression, whereas the occurrence of clinical parameters of disease (increase in body temperature, changes in hematologic parameters, and outward clinical signs of disease) were delayed. The challenged monkeys exhibited increased temperatures after spore challenge; however, temperature elevations may not be a reliable indicator of the onset of systemic disease as these measurements were confounded by the diurnal temperature patterns typical of this species.

Unchallenged control animals exhibited moderate changes in clinical parameters when manipulated at a frequency designed to monitor disease, beyond the normal monitoring of healthy study animals. There were distinct changes seen in challenged animals that were absent from unchallenged controls.

Assessment of body temperature, activity, blood cell count (CBC) with differential, C-reactive protein (CRP) levels, onset of bacteremia (culture and PCR), and presence of PA in circulation suggests a clinical profile similar to humans.

*The study suggests that cynomolgus monkeys exposed to *B. anthracis* via the inhalation exposure route can be utilized to assess the efficacy of a therapeutic agent. Detection of PA by ECL assay appears to be an appropriate trigger for intervention in cynomolgus monkeys. However, microbiological cultures should be performed to ensure that animals are bacteremic as one unchallenged animal (# 23375) in the uninfected group (Group 3) was PA positive at 48 hours by ECL assay; the applicant states that this was a technical error as on repeat testing the results were negative. White blood cells (WBC) counts and CRP have been used to help determine if a patient has an infection. Due to a general diurnal rhythm (subtle increases in WBC counts during the night time hours with slight decreases during the day time hours) observed in the unchallenged animals these changes may not be very useful as a trigger for intervention.*

6.2. Efficacy of ETI-204 (monotherapy) - treatment studies

The efficacy of intravenously administered ETI-204 was measured in NZW rabbits (2 studies) and cynomolgus monkeys (4 studies) to establish a dose of ETI-204 that

- protects animals from death due to inhalational anthrax.
- neutralizes serum PA when administered as a monotherapy.

Different products of ETI-204 (Baxter or Lonza) were used in different studies (see Appendix-4). ETI-204 was administered intravenously as a bolus after the onset of disease.

6.2.1. New Zealand White rabbits

The applicant conducted two studies (AR021 and AR033) to measure efficacy of ETI-204 in experimentally naïve NZW rabbits. All studies were conducted at [REDACTED] (b) (4)

6.2.1.1. Study AR021

This was a randomized, open-label, placebo-controlled, dose ranging, GLP study to evaluate the efficacy of ETI-204 (Baxter product) when administrated intravenously (IV), against lethality

due to inhalation exposure with the spores (spore lot no. B31) of the Ames strain of *B. anthracis* in NZW rabbits.⁴⁰ The objective was to identify a target ETI-204 dose that protected animals from lethality due to inhalational anthrax after exposure to 200 median LD₅₀s. Levofloxacin was included as a comparator.

Study design:

Sixty four rabbits purchased from [REDACTED] (b) (4) were quarantined at [REDACTED] (b) (4) and randomized to five dose groups (Table 29). The applicant states that rabbits that were in good health, free of malformations, and exhibited no signs of clinical disease were placed on study; however, the medical records were not included in the submission. Rabbits were challenged on three separate days. The study design was similar to that for natural history studies except that treatment was initiated based on a positive PA in serum by the ECL assay or when they had exhibited a significant increase in body temperature (SIBT); SIBT was achieved when an animal had exhibited three consecutive critical temperature readings or when an animal had exhibited two consecutive critical temperature readings twice. Body temperatures were measured at regular intervals (twice daily except from ~18 to ~72 hours post-challenge, when temperatures were measured once every hour). PA levels by ELISA or quantitative bacteremia were not measured.

Animals were treated with a single IV dose of 1, 4, or 16 mg/kg ETI-204; levofloxacin was administered by gastric intubation for 3 days (Table 29). Animals were monitored for abnormal clinical signs (such as respiratory distress, inactivity, seizures and moribundity) for 28 days post-challenge. Blood samples were collected for pharmacokinetics, hematological parameters, and microbiologic measurements that include bacterial cultures of EDTA containing blood [except at PTT i.e., prior to treatment - blood was collected in sodium polyanethol sulfonate (SPS tubes)] up to Day 28 and measurement of PA by ECL assay until initiation of treatment (Table 29). Complete gross necropsies were conducted on all rabbits found dead or euthanized due to illness to confirm *B. anthracis* infection. At the time of necropsy, spleen and if possible mediastinal or bronchial lymph nodes were collected and processed for bacterial cultures.

⁴⁰ [REDACTED] (b) (4) Study Number 832-G924202: AR021: Evaluating the efficacy of ETI-204 when administered therapeutically in the New Zealand White rabbit inhalational anthrax (October, 2009).

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Table 29: Study AR021 - Study design and blood collection schedule

Study design:

Group	Number of Animals	ETI-204 Dose mg/kg	Levofloxacin Dose ^x
1	10	Saline [^]	Control*
2	10	1.0	Control*
3	17	4.0	Control*
4	17	16.0	Control*
5	10	Saline [^]	50 mg/kg

* Water For Injection (WFI) was administered as a control (at 2 ml/kg) for levofloxacin.

[^] Saline was administered as a control (at 0.5ml/kg) for ETI-204 (See Appendix B, DR-7243)

^x Levofloxacin or control material was administered in three doses: upon meeting treatment intervention criteria and at 24 (± 1) and 48 (± 3) hours after the initial treatment.

^{*} Trigger for treatment intervention was either first positive PA result (via ECL assay) or three consecutive critical temperature readings or when an animal had exhibited two consecutive critical temperature readings twice.

A single IV bolus of ETI-204 or saline was administered, via the VAP, immediately (within 10 minutes) after administration of the 1st dose of levofloxacin or vehicle.

Blood draw schedule:

Approximate Time Point	Blood Tube type/ Approximate Blood volume	Bacteremia: (Culture)	CBC/ CRP	Serum PA level (via ECL assay)	Serum for ETI-204 assay	Plasma for Levofloxacin Assay
Day -7 *EDTA ~1.5 ml *EDTA ~1.0ml SST ~2.0ml		X	X	X	X	X
^18hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
^24hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
^30hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
^36hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
^42hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
^48hr PC EDTA ~1.5 ml SST ~1.0 ml		X	X	X		
PTT EDTA ~1.5 ml SST ~1.0ml *EDTA ~1.0ml SFS ~1.0ml		X [#]	X	X ^f		X
1hr PT *EDTA ~1.0ml						X
24hr PT (prior to Trt2) EDTA ~1.5 ml *EDTA ~1.0ml SST ~1.0ml		X	X		X	X
49hr PT (1hr PTrt3) *EDTA ~1.0ml						X
72hr PT (24hr PTrt3) *EDTA ~1.0ml		X	X			X
7 days PC ^b EDTA ~2.0 ml		X	X			
14 days PC ^b EDTA ~2.0 ml		X	X			
21 days PC ^b EDTA ~2.0 ml		X	X			
28 days PC ^b EDTA ~2.0 ml		X	X			
Terminal ^{c,d} *EDTA ~1.0ml		X	CRP only			X ^e

PC = Post-Challenge

Trt2 = Treatment 2

PTT = Prior to Treatment

Trt3 = Treatment 3

PT = Post-Treatment

^a Post-challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort. Post-treatment bleed times were calculated from the time each animal's IV treatment ended. Blood samples occurred within ± 60 minutes of the calculated time, except for the 1hr PT and 49hr PT samples which occurred within 15 minutes of the calculated time. The Day -7, Day 7, Day 14, Day 21 and Day 28 post-challenge bleed times were relative to the day of challenge.

^b Blood samples 7, 14, 21 and 28 days post-challenge were not collected from the VAP.

^c The terminal sample, if occurring >7 days post-challenge, was not collected from the VAP.

^d If collection was possible.

^e Plasma for Levofloxacin analysis was only collected from terminal samples occurring ≤ 48 hours post treatment #3.

^f This sample for Serum PA level (via ECL assay) was not be run immediately onsite (relative to collection time).

* Chilled EDTA for Levofloxacin Analysis

[#] Post-Challenge, pre-treatment sampling stopped once decision to treat was made.

[#] PTT Bacteremia performed on sample collected in SPS tube

ETI-204 or control material (saline) was administered post-challenge within 0.2 to 3.9 hours of determining a positive serum PA-ECL screening assay or an increase in temperature.

Results:

Baseline characteristics: Prior to challenge, all animals were culture negative as well as PA negative by the ECL assay. Age, gender, body weight, and challenge dose were comparable among three groups. The mean \pm standard deviation (SD) LD₅₀ was 180.4 \pm 55.9; the LD₅₀ was \geq 200 in approximately 36% of the animals. The average MMAD was 1.12, 1.10 and 1.09 μm for challenge Days A, B and C respectively. The MMAD of the aerosol particles generated for this study were consistent with lower respiratory tract deposition.

All animals were PA negative by the ECL assay and culture negative on Day-7 (prior to challenge). Animals were either PA positive by ECL assay or showed increased temperature between 24 and 44 hours post-challenge (Table 30 and Figure 18). The applicant states that the variability in the shoulder temperatures was significantly less than the rump temperatures (p-value=0.0044) for challenge Day A. Thus, the shoulder temperatures were used for all animals in the analyses with the exception for Animal K99423, which lost functionality in the shoulder implant causing measurements to be taken from the rump. About 84% of the animals were bacteremic at the time of treatment (Table 30).

The mean time from trigger to treatment was similar (less than 4 hours) in all the five groups (Table 30).

The baseline characteristics (disease stage) of animals were similar in animals in all the groups.

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Table 30: Study AR021 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	ETI-204				Levofloxacin 50 mg/kg (Group 5) N=10
	Placebo (Group 1) N=10	1 mg/kg (Group 2) N=10	4 mg/kg (Group 3) N=17	16 mg/kg (Group 4) N=17	
Baseline characteristics					
Age (months) estimated range	7-8	7-8	7-8	7-8	7-8
Body weight (kg) Mean ± SD	3.2±0.1	3.2±0.1	3.2±0.1	3.2±0.1	3.2±0.1
Inhaled Dose					
Total Inhaled Dose (cfu x 10 ⁷) Mean ± SD (Range)	(b) (4)				
LD ₅₀ dose	184.6±71.8* (85-343)	167.7±41.3* (99-217)	200.0±51.8 (89-309)	174.9±61.2 (86-300)	183.0±54.9 (79.0-343.0)
<200 LD ₅₀ dose n (%)	7 (70.0)	8 (80.0)	9 (52.9)	10 (58.8)	8 (80.0)
≥200 LD ₅₀ dose n (%)	3 (33.3)	2 (22.0)	8 (47.1)	7 (41.2)	2 (20.0)
Trigger for treatment^a					
Positive screening PA/ECL Assay n (%)	5 (55.0)	6 (66.0)	6 (35.3)	8 (47.1)	6 (60.0)
SIBT n (%)	4/9 (44.4) [‡]	3/9 (33.3) [‡]	11 (64.7)	9 (52.9)	4 (40)
Bacteremia prior to treatment					
Enriched (qualitative) bacteremia n (%)	10 (100.0)	9 (90.0)	15 (88.2)	14 (82.4)	9 (90.0)
Time (hours) between challenge, trigger, and treatment					
Time to trigger post-challenge^a n Mean±SD (Range)	9 [‡] 30.7±7.7 (20.9-43.8)	9 [‡] 26.9±4.8 (21.8-35.6)	17 27.7±4.1 (22.2-35.6)	17 28.8±5.3 (21.6-40.3)	10 24.9±3.4 (18.5-30.4)
Time to bacteremia n Mean±SD (Range)	9 [‡] 37.7±21.8 (23.8-94.1)	9 [‡] 43.3±25.5 (23.7-104.3)	15 38.2±15.2 (23.6-60.7)	14 27.5±3.7 (23.8-35.7)	7 25.0±2.3 (23.7-30.1)
Time from trigger to treatment n Mean±SD (Range)	9 [‡] 1.7±1.2 (0.3-3.5)	9 [‡] 1.7±12 (0.3-3.5)	17 1.4±1.2 (0.2-3.5)	17 1.6±1.3 (0.2-3.5)	10 2.1±1.4 (0.2-3.9)
Survivors at the end of study (Day 28)					
n (%)*	1 (10)	4 (40)	13 (76.5)**	16 (94.1)**	9 (90.0)**
n (%) based on bacteremic (enriched culture) animals at some time prior to treatment [*]	0/9 (0)	3/8 (38)**	11/15 (73)**	13/14 (93)**	8/9 (89)**

SD Standard deviation

PTT Prior to treatment; PA LOD=4 ng/mL.

^aThe trigger for treatment was defined as the time from challenge to a positive PA-ECL or SIBT post-challenge.

*Results based on exclusion of 2 animals (Animal K99373 in the placebo group and animal K99383 treated with ETI-204 1 mg/kg) that were inadvertently dosed with levofloxacin.

[‡]K99373 and K99383 were negative for *B. anthracis* in the LB data set

**Statistically significant between the ET-204 treated group and the control group

Detection of Bacteremia and PA: The variability among the results of two bacterial culture methods and the PA by ECL assay is shown in Table 31. The samples used for these three assays were collected at the same times post-challenge. Of the 62 rabbits in the study, 55 and 42 were positive by enriched and qualitative culture methods, respectively, and 51 were PA positive by

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ECL assay; 42 samples were positive by all the three assays. Qualitative enriched bacteremia was most sensitive [55/62 (88.9%)] for diagnosis.

Table 31: Study AR021 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Qualitative Enriched Culture*	Detection Method			Treatment Group									
		Quantitative Culture	Screening PA (ECL Assay)*	Quantitative		1 mg/kg ETI-204			4 mg/kg ETI-204		16 mg/kg ETI-204		50 mg/kg Levo (n=10)	Total (n=62)
				PA (ELISA Assay)	Placebo (n=9)	n=9	n=17	n=17	n=10	n=10	n=10	n=10		
+	+	ND	+	ND	8	5	8	14	7	42				
-	+	ND	+	ND	0	2	6	0	1	9				
-	+	ND	-	ND	1	1	1	0	1	4				
-	-	ND	-	ND	0	1	2	3	1	7				

n = Number of treated animals. Results determined on a per animal basis, not for individual tests. Animal with a positive test from any time prior to treatment is considered as positive. ND = Not Done Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

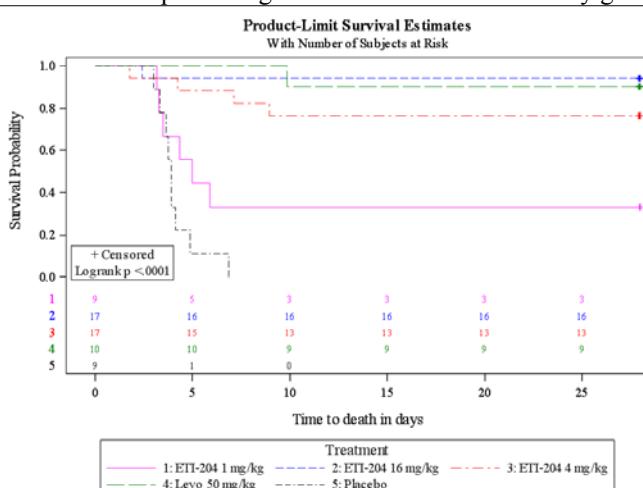
*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (1 ng/mL)- the LOD was 4 µg/mL

Effect of treatment on survival and microbial burden: The results show that ETI-204, at all the doses tested, was effective in improving survival compared to the control group; such an effect was dose-dependent; 16 mg/kg dose was most effective in improving survival (Figure 18 and Table 30). Levofloxacin, at a dose of 50 mg/kg, improved survival in 90% of the animals. Of the rabbits that died, 100% (20/20) had a positive terminal bacteremia sample (does not include animal K99426 for which a terminal sample could not be collected). By seven days post-challenge, all rabbits which survived (to the end of the study) were culture negative and remained culture negative for the duration of the study.

One rabbit (K99373) in the control group that survived was treated based on SIBT as a trigger; this animal was PA negative by ECL and culture positive. However, at 24 hours post-treatment, the rabbit was culture negative and remained negative for the duration of the study. No bacteria were observed by culture in any of the tissues. The reason for survival of one rabbit in the control group is unclear.

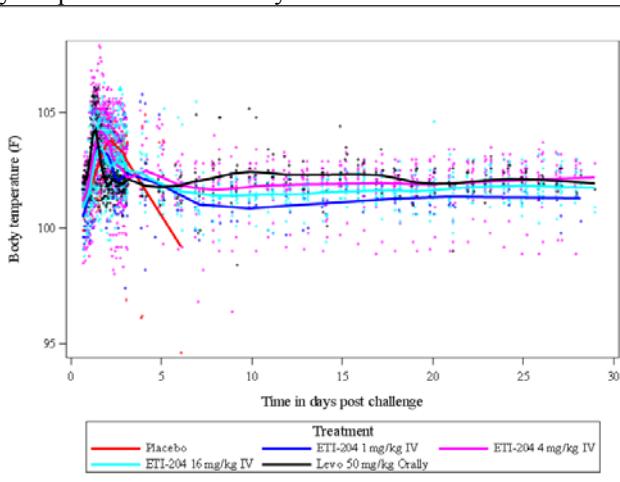
Figure 18: Study AR021 - Survival and change in body temperature

A: Kaplan-Meier curves representing time-to-death and survival by group



Not including animals inadvertently doses with levofloxacin (K99373 and K99383)

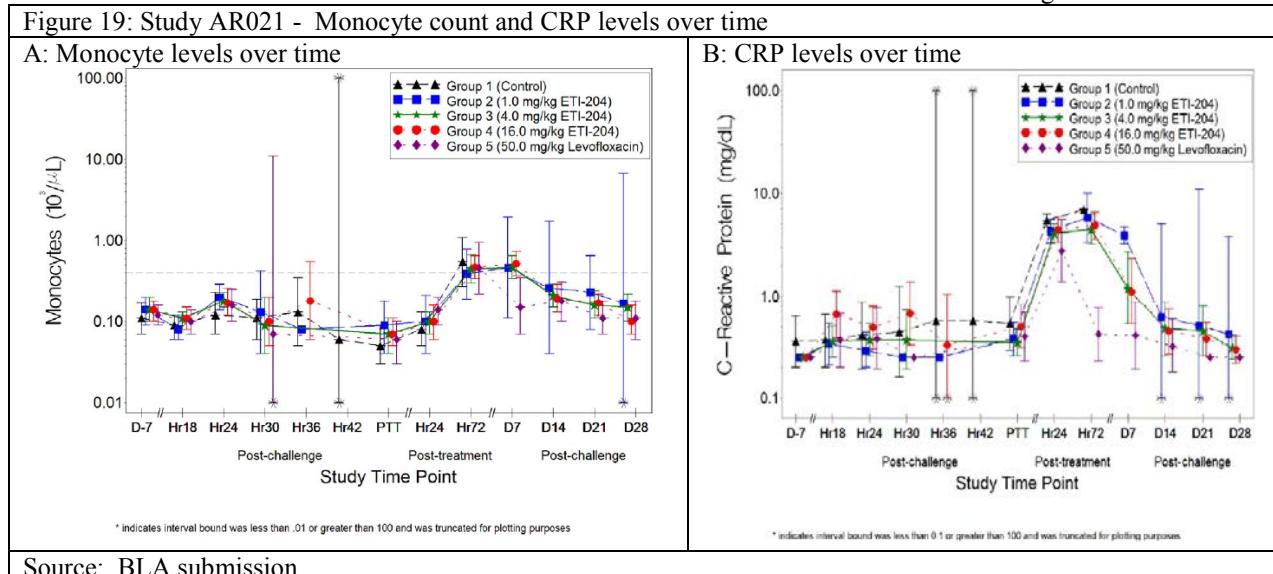
B: Mean body temperature over time by treatment



Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Hematological parameters: Several red blood cell parameters were relatively unaffected following challenge with *B. anthracis*. The white blood cell types were assessed for both total number and percentage of the whole population. Trends were observed when assessing the neutrophil, lymphocyte, basophil, and monocyte cell populations which were similar to those summarized for the natural history studies summarized above. Neutrophils increased post-challenge and began to return to normal levels around 72 hours post-treatment. Lymphocytes decreased until the prior to treatment time point and subsequently increased post treatment until 7 days post-challenge. Basophils generally decreased post-challenge until between 24 and 72 hours post-treatment when the levels began to return to normal. Monocytes were elevated generally at the 72 hour post-treatment and 7 days post-challenge time points and then subsequently began to return to normal levels (Figure 19A). Overall, the hematology data suggests that the animals demonstrate non-specific indicators of illness post-challenge. CRP levels were relatively consistent prior to challenge. CRP levels increased and peaked between 24-72 hours post-treatment. A majority of the animals that survived showed low CRP levels at 14 days post-challenge (Figure 19B).

Figure 19: Study AR021 - Monocyte count and CRP levels over time



Source: BLA submission

Clinical observations: The clinical observations post-challenge were consistent with those observed in the natural history studies summarized above and include decrease in food consumption, lethargy, no stool, soft stool and respiratory abnormalities. Except for occasional loss of appetite, the surviving animals returned to normal between Days 5-8 post-challenge.

Necropsy and Histopathology: Gross lesions in rabbits dying post-challenge included discolorations and/or foci in the appendix, brain, lung and large intestine; enlargement of multiple lymph nodes; and fluid in multiple body cavities (effusion) and the ventral skin (edema). These gross lesions are typical of anthrax in rabbits and correlated histologically with hemorrhage, necrosis, edema and acute inflammation. No gross lesions were reported for the surviving animals that were terminated at study completion for gross and microscopic evaluation (Table 32). These observations are consistent with those observed in the natural history studies summarized above.

Tissues from all rabbits that were found dead following challenge as well as two animals (one male and one female) from each of the five groups, which survived to the end of the study, were examined microscopically for evidence of anthrax; the exception included few minor tissues not successfully processed to slides. Microscopic findings were graded semi-quantitatively according to the following scale, with the associated numerical score used to calculate average severity grades for each lesion by group and sex as follows:

Minimal (Grade 1) represented the least detectable lesion.

Mild (Grade 2) represented an easily discernible lesion unlikely to have any biological relevance.

Moderate (Grade 3) represented a change affecting a large area of the represented tissue that had the potential to be of some relevance.

Marked (Grade 4) represented a lesion that approached maximal.

Microscopic findings considered consistent with anthrax were present in all rabbits that died or became moribund during the study. Lesions included acute fibrinous to heterophilic inflammation, necrosis, hemorrhage, edema, and the presence of large rod-shaped bacteria in the brain (meninges), heart, kidney, liver, lung, spleen, cecum/appendix and multiple lymph nodes (Table 32). Slightly more bacteria were evident in organs from the control group animals as compared to other groups. There were no other qualitative differences in lesions of anthrax.

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among the control and experimental groups. No significant microscopic lesions were present in the six rabbits that survived until Day 28.

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Table 32: Study AR021 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)	50 mg/kg Levo n/N (*)
Brain					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	9/ 9 (NA)	5/ 8 (NA)	2/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	9/ 9 (2.22)	4/ 7 (2.75)	1/ 6 (2.00)	0/ 3	0/ 1
Hemorrhage	5/ 9 (2.40)	3/ 7 (3.00)	2/ 6 (2.00)	0/ 3	0/ 1
Inflammation, acute	3/ 9 (2.33)	4/ 7 (2.25)	1/ 6 (3.00)	0/ 3	0/ 1
Necrosis	2/ 9 (1.50)	3/ 7 (2.00)	1/ 6 (1.00)	0/ 3	0/ 1
Kidney					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	9/ 9 (NA)	2/ 8 (NA)	3/15 (NA)	1/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	7/ 9 (2.00)	1/ 7 (2.00)	2/ 6 (1.50)	0/ 3	1/ 1 (2.00)
Hemorrhage	1/ 9 (1.00)	0/ 7	0/ 6	0/ 3	0/ 1
Inflammation, acute	0/ 9	1/ 7 (2.00)	2/ 6 (1.50)	0/ 3	0/ 1
Necrosis, tubular epithelium	1/ 9 (2.00)	1/ 7 (4.00)	0/ 6	0/ 3	1/ 1 (1.00)
Nephropathy	3/ 9 (2.00)	0/ 7	2/ 6 (1.00)	1/ 3 (1.00)	0/ 1
Liver					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	8/ 9 (NA)	3/ 8 (NA)	2/15 (NA)	1/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	7/ 9 (1.86)	1/ 7 (2.00)	0/ 6	0/ 3	1/ 1 (1.00)
Necrosis, hepatocytes	0/ 9	1/ 7 (3.00)	0/ 6	0/ 3	0/ 1
Sinusoidal leukocytosis	8/ 9 (1.25)	3/ 7 (1.67)	1/ 6 (1.00)	1/ 3 (1.00)	0/ 1
Lymph Node, Bronchial					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	9/ 9 (NA)	5/ 8 (NA)	4/15 (NA)	1/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	8/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	7/ 8 (2.57)	2/ 7 (1.50)	1/ 6 (1.00)	1/ 3 (2.00)	1/ 1 (2.00)
Depletion/necrosis, lymphocytes	8/ 8 (2.38)	5/ 7 (1.00)	2/ 6 (2.00)	1/ 3 (3.00)	1/ 1 (1.00)
Edema	2/ 8 (2.00)	0/ 7	0/ 6	1/ 3 (1.00)	0/ 1
Hemorrhage	7/ 8 (2.29)	4/ 7 (1.75)	2/ 6 (2.50)	1/ 3 (2.00)	1/ 1 (1.00)
Inflammation, acute	6/ 8 (2.33)	4/ 7 (1.75)	2/ 6 (2.00)	1/ 3 (1.00)	1/ 1 (1.00)
Lymph Node, Mandibular					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	3/ 9 (NA)	1/ 8 (NA)	0/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	8/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	2/ 8 (3.00)	0/ 7	0/ 6	0/ 3	0/ 1
Depletion/necrosis, lymphocytes	2/ 8 (3.50)	1/ 7 (1.00)	0/ 6	0/ 3	0/ 1
Edema	1/ 8 (3.00)	0/ 7	0/ 6	0/ 3	0/ 1
Hemorrhage	2/ 8 (2.00)	1/ 7 (2.00)	0/ 6	0/ 3	0/ 1
Inflammation, acute	1/ 8 (3.00)	0/ 7	0/ 6	0/ 3	0/ 1
Lymph Node, Mediastinal					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	9/ 9 (NA)	5/ 8 (NA)	4/15 (NA)	1/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	9/ 9 (2.78)	2/ 7 (2.00)	3/ 6 (1.00)	1/ 3 (2.00)	1/ 1 (2.00)
Depletion/necrosis, lymphocytes	9/ 9 (3.44)	4/ 7 (2.00)	4/ 6 (3.00)	1/ 3 (4.00)	1/ 1 (1.00)
Edema	1/ 9 (1.00)	0/ 7	1/ 6 (1.00)	1/ 3 (1.00)	0/ 1
Hemorrhage	7/ 9 (2.14)	3/ 7 (1.00)	2/ 6 (3.50)	1/ 3 (2.00)	0/ 1
Inflammation, acute	7/ 9 (2.43)	4/ 7 (2.25)	4/ 6 (2.50)	1/ 3 (2.00)	1/ 1 (3.00)

NA, not applicable.

*Mean severity of lesion Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 32 (continued): Study AR021 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)	50 mg/kg Levo n/N (*)
Lymph Node, Mesenteric					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	0/ 9 (NA)	1/ 8 (NA)	0/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	0/ 9	1/ 7 (3.00)	0/ 6	0/ 3	0/ 1
Depletion/necrosis, lymphocytes	0/ 9	1/ 7 (4.00)	0/ 6	0/ 3	0/ 1
Hemorrhage	0/ 9	1/ 7 (4.00)	0/ 6	0/ 3	0/ 1
Inflammation, acute	0/ 9	1/ 7 (1.00)	0/ 6	0/ 3	0/ 1
Lung					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	8/ 9 (NA)	4/ 8 (NA)	4/15 (NA)	2/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	6/ 9 (2.50)	1/ 7 (4.00)	0/ 6	0/ 3	1/ 1 (3.00)
Edema	1/ 9 (3.00)	1/ 7 (1.00)	1/ 6 (1.00)	0/ 3	0/ 1
Hemorrhage	1/ 9 (2.00)	2/ 7 (1.00)	0/ 6	0/ 3	1/ 1 (3.00)
Inflammation, acute	5/ 9 (1.60)	2/ 7 (1.00)	4/ 6 (1.75)	0/ 3	1/ 1 (3.00)
Inflammation, chronic	0/ 9	0/ 7	0/ 6	1/ 3 (1.00)	0/ 1
Necrosis, BALT	1/ 9 (2.00)	0/ 7	0/ 6	1/ 3 (1.00)	1/ 1 (4.00)
Spleen					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	8/ 9 (NA)	5/ 8 (NA)	4/15 (NA)	1/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	7/ 9 (3.86)	1/ 7 (3.00)	1/ 6 (1.00)	0/ 3	1/ 1 (1.00)
Depletion/necrosis, lymphocytes	8/ 9 (2.25)	4/ 7 (1.25)	1/ 6 (4.00)	1/ 3 (3.00)	1/ 1 (1.00)
Hemorrhage	0/ 9	0/ 7	1/ 6 (1.00)	0/ 3	1/ 1 (1.00)
Inflammation, acute	3/ 9 (2.00)	3/ 7 (2.00)	3/ 6 (1.67)	1/ 3 (3.00)	1/ 1 (1.00)
Heart					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	8/ 9 (NA)	1/ 8 (NA)	1/15 (NA)	0/14 (NA)	1/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	8/ 9 (2.25)	1/ 7 (3.00)	1/ 6 (2.00)	0/ 3	1/ 1 (3.00)
Inflammation, acute	0/ 9	0/ 7	1/ 6 (2.00)	0/ 3	0/ 1
Cavity, Abdominal					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	1/ 9 (NA)	1/ 8 (NA)	0/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2,3}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Cavity, Pericardial					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	1/ 9 (NA)	0/ 8 (NA)	1/15 (NA)	1/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2,3}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Cavity, Thoracic					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	4/ 9 (NA)	0/ 8 (NA)	1/15 (NA)	1/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1,2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9

NA, not applicable.

*Mean severity of lesion Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 32 (continued): Study AR021 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)	50 mg/kg Levo n/N (*)
Appendix					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	2/ 9 (NA)	0/ 8 (NA)	1/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1, 2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	2/ 9 (3.50)	0/ 7	1/ 6 (2.00)	0/ 3	0/ 1
Depletion/necrosis, GALT	2/ 9 (3.00)	0/ 7	0/ 6	0/ 3	0/ 1
Edema	2/ 9 (2.50)	0/ 7	0/ 6	0/ 3	0/ 1
Hemorrhage	2/ 9 (3.00)	0/ 7	1/ 6 (2.00)	0/ 3	0/ 1
Cecum					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	2/ 9 (NA)	1/ 8 (NA)	0/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1, 2}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9
Bacteria	2/ 9 (2.00)	1/ 7 (3.00)	0/ 6	0/ 3	0/ 1
Depletion/necrosis, GALT	2/ 9 (1.50)	1/ 7 (4.00)	0/ 6	0/ 3	0/ 1
Hemorrhage	2/ 9 (2.00)	1/ 7 (4.00)	0/ 6	0/ 3	0/ 1
Inflammation, acute	1/ 9 (2.00)	1/ 7 (4.00)	0/ 6	0/ 3	0/ 1
Skin					
Macroscopic Finding ¹					
# Necropsied/Total Infected	9/ 9	8/ 8	15/15	14/14	9/ 9
Gross Lesions	3/ 9 (NA)	0/ 8 (NA)	1/15 (NA)	0/14 (NA)	0/ 9 (NA)
Microscopic Finding ^{1, 2, 3}					
# Necropsied/Total Infected	9/ 9	7/ 8	6/15	3/14	1/ 9

NA, not applicable.

*Mean severity of lesion Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the surviving treated rabbits irrespective of the ETI-204 dose (Table 33). A majority of the tissues [brain, kidney, liver, lymph nodes (mandibular and mesenteric), lung, spleen] from one animal treated with 16 mg/kg dose that died were negative for bacteria by histological examination. However, bacteria were observed in a majority of the tissues from non-surviving animals treated with the lower doses of ETI-204 (Table 33).

Culture: A majority of the spleens (20/21) and lymph nodes (19/21) from non-surviving animals, were culture positive (Table 33). All these animals were bacteremic at the time found dead or necropsied. Culture of tissues from surviving animals was not performed. It appears that culture is more sensitive than histological examination for detection of bacteria in tissues.

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Table 33: Study – AR021-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 1mg/kg		ETI-204 4mg/kg		ETI-204 16mg/kg		Levo 50mg/kg	
	Non Survivors (N=0)	Non Survivors (N=9)	Non Survivors (N=3)	Non Survivors (N=6)	Non Survivors (N=13)	Non Survivors (N=4)	Non Survivors (N=16)	Non Survivors (N=1)	Non Survivors (N=9)	Non Survivors (N=1)
Presence of bacteria by microscopy [1, 3, 4]										
Brain	0/0	9/9	0/2	4/6	0/2	1/4	0/2	0/1	0/0	0/1
Kidney	0/0	7/9	0/2	2/6	0/2	2/4	0/2	0/1	0/0	1/1
Liver	0/0	7/9	0/2	1/6	0/2	0/4	0/2	0/1	0/0	1/1
Lymph Node, Bronchial	0/0	7/8	0/2	2/6	0/2	1/4	0/2	1/1	0/0	1/1
Lymph Node, Mandibular	0/0	2/8	0/2	0/6	0/2	0/4	0/2	0/1	0/0	0/1
Lymph Node, Mediastinal	0/0	9/9	0/2	2/6	0/2	3/4	0/2	1/1	0/0	1/1
Lymph Node, Mesenteric	0/0	0/9	0/2	1/6	0/2	0/4	0/2	0/1	0/0	0/1
Lung	0/0	6/9	0/2	2/6	0/2	0/4	0/2	0/1	0/0	1/1
Spleen	0/0	7/9	0/2	2/6	0/2	1/4	0/2	0/1	0/0	1/1
Heart	0/0	8/9	0/2	1/6	0/2	1/4	0/2	0/1	0/0	1/1
Cavity, Abdominal	0/0	0/9	0/2	0/6	0/2	0/4	0/2	0/1	0/0	0/1
Cavity, Pericardial	0/0	0/9	0/2	0/6	0/2	0/4	0/2	0/1	0/0	0/1
Cavity, Thoracic	0/0	0/9	0/2	0/6	0/2	0/4	0/2	0/1	0/0	0/1
Appendix	0/0	2/9	0/2	0/6	0/2	1/4	0/2	0/1	0/0	0/1
Cecum	0/0	2/9	0/2	1/6	0/2	0/4	0/2	0/1	0/0	0/1
Skin	0/0	0/9	0/2	0/6	0/2	0/4	0/2	0/1	0/0	0/1
Presence of bacteria by culture [1, 2]										
Lymph Node, Bronchial	ND	9/9	ND	5/6	ND	3/4	ND	1/1	ND	1/1
Spleen	ND	9/9	ND	5/6	ND	4/4	ND	1/1	ND	1/1

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate. ND=Not Done

[3] Histopathology performed at (b) (4)

[4] Not all animal were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ECL) and bacteremia (qualitative, and qualitative enriched) at any point prior to treatment: K99372, K99386, K99387, K99404, K99409, K99419, K99423

Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: K99381, K99388, K99399, K99426

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

Comments:

- The baseline characteristics of animals in the different groups were similar. A majority of the animals were bacteremic, PA positive, or showed an increase in temperature at the time of initiation of treatment.
- The study showed that ETI-204 at all the doses (1, 4 and 16 mg/kg) tested was effective in improving survival up to Day 28 compared to the untreated control group. Such an effect was dose-dependent. The survival rate at the ETI-204 dose of 16 mg/kg was similar to that of levofloxacin. By seven days post-challenge, all rabbits which survived (to the end of the study) were culture negative and remained negative for the duration of the study.
- All of the non-surviving animals died within 10 days of challenge. Of the rabbits that died, 100% (20/20) had a positive terminal bacteremia sample and spleen and lymph nodes were culture positive. Gross lesions and microscopic findings consistent with anthrax were present in all rabbits that died or became moribund during the study. No significant lesions or microscopic findings were noted in animals surviving to the end of study.
- Slightly more bacteria were evident in organs from control animals as compared to other groups. No bacteria were observed in any of the surviving treated rabbits irrespective of the ETI-204 dose. A majority of the tissues [brain, kidney, liver, lymph nodes (mandibular and mesenteric), lung, and spleen] from one animal treated with 16 mg/kg dose that died were negative for bacteria by histological examination. However, bacteria were observed in a

majority of the tissues from non-surviving animals treated with the lower doses of ETI-204 (Table 33). It appears that culture is more sensitive than histological examination for detection of bacteria in tissues.

- *Overall, the study suggests that ETI-204 at a dose of 16 mg/kg is effective in protecting against death due to anthrax when administered intravenously after the onset of disease.*

6.2.1.2. Study AR033

This was a randomized, blinded, placebo-controlled, dose ranging, GLP study conducted at [redacted] (b) (4) to evaluate the efficacy of ETI-204 (Baxter product) when administrated, IV, against lethality due to inhalational exposure with the spores (spore lot no. B37) of the Ames strain of *B. anthracis* in 70 NZW rabbits.⁴¹ The objective was to identify a target dose for ETI-204.

Study design:

The study design was similar to that of study AR021 summarized above except that the doses of ETI-204 tested were 1, 4, 8, and 16 mg/kg; levofloxacin was not included as a comparator (Table 34). Additionally, blood samples were collected at different time points for quantitating bacteremia by culture and free PA levels by ELISA (Table 34). The capture antibody used in the PA assay is ETI-204 and therefore PA bound to ETI-204 will not be detected as ETI-204 is known to bind to the PAD4 (for more details see Dr Berkeley's microbiology review).

It should be noted that the frequency at which treatment triggers were collected varied for temperature (every hour) vs PA by ECL assay (every 6 hours). Once an animal met the treatment criteria for SIBT, treatment was administered as soon as possible whereas animals treated based on a positive PA-ECL result were not treated for several hours (time between actual sample collection and the result of the PA-ECL assay were not specified).

When possible, cerebral spinal fluid (CSF) was collected and processed for microbiological cultures. Selected left brain hemispheres from some of the rabbits (survivors and non-survivors) were processed for neuropathological examination.

⁴¹ [redacted] (b) (4) Study Number 1185-100003006: AR033: Evaluating the efficacy of ETI-204 when administered therapeutically in the New Zealand White rabbits (February, 2013).

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Table 34: Study AR033 - Study groups and schedule of blood collection for different assays

Study groups

Animal Numbers	Treatment	Blinded Group Assignments
14	0 (Saline)	4
14	1 mg/kg ETI-204	3
14	4 mg/kg ETI-204	1
14	8 mg/kg ETI-204	5
14	16 mg/kg ETI-204	2

Schedule of blood collection for different assays

Time-point	Collection Window	Collection Tube and Approximate Volume	Bacteremia (Qualitative & Quantitative)	CBC/CRP	Serum PA level (via ECL assay)	Serum for PA ELISAs	Serum for ETI-204 Levels	Serum for Anti-drug antibodies	CSF for bacterial assessment
Day -7	-	EDTA ~1.5 mL SST ~2.5 mL	X (Qual. only)	X	X	X	X	X	
^24hr PC	± 60 min	EDTA ~0.7 mL SST ~2.0 mL	X		X	X			
^30hr PC	± 60 min	EDTA ~0.7 mL SST ~2.0 mL	X		X	X			
^36hr PC	± 60 min	EDTA ~0.7 mL SST ~2.0 mL	X		X	X			
^42hr PC	± 60 min	EDTA ~0.7 mL SST ~2.0 mL	X		X	X			
# PTT	-	EDTA ~1.5 mL SST ~2.0 mL SPS ~1.0 mL	X	X	X	X			
15 min PT	± 5 min	SST ~2.0 mL				X	X		
4hr PT	± 15 min	EDTA ~0.7 mL SST ~2.0 mL	X			X	X		
8 hr PT	± 15 min	SST ~2.0 mL				X	X		
24hr PT	± 60 min	EDTA ~1.5 mL SST ~2.0 mL	X	X		X	X		
48 hr PT	± 60 min	EDTA ~0.7 mL SST ~2.0 mL	X			X	X		
72 hr PT	± 60 min	EDTA ~1.5 mL	X	X					
*8 days PC	-	EDTA ~1.5 mL SST ~2.0 mL	X	X		X	X		
*12 days PC	-	SST ~2.0 mL				X	X		
*16 days PC	-	EDTA ~2.0 mL SST ~4.0 mL	X	X		X	X		
*20 days PC	-	EDTA ~2.0 mL SST ~4.0 mL	X	X		X	X		
*28 days PC	-	EDTA ~1.5 mL SST ~4.0 mL	X	X		X	X	X	X
Terminal ^b	-	EDTA ~1.5 mL SST ~4.0 mL	X	CRP only		X	X		X

PC = Post-Challenge, PTT = Prior to Treatment, PT = Post-Treatment, - = not applicable

^a Post-challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort.

Post-treatment bleed times were calculated from the time each animal's IV treatment ends.

^b If collection was possible (performed by cardiac stick if required).

* Collected via ear.

^ Post-Challenge, pre-treatment sampling stopped once decision to treat has been made.

PTT Bacteremia enrichment performed on sample collected in SPS tube (see section 4.10)

Results:

Baseline characteristics: Prior to challenge, all animals were culture negative as well as PA negative by the ECL assay and ELISA. Age, gender, body weight, and challenge dose were comparable among three groups. The average MMAD was between 1.23 and 1.27 µm for the three challenge days; the MMAD of the aerosol particles generated for this study was consistent with lower respiratory tract deposition and Study AR021 summarized above. The mean±SD LD₅₀ was 200.7±35.4; the LD₅₀ was ≥200 in approximately 54% of the animals (Table 35). Overall, the baseline characteristics (disease stage) of animals were similar among animals in all the groups.

All animals were PA negative by the ECL assay and culture negative on Day-7 i.e., prior to challenge. Animals were either PA positive by ECL assay or showed SIBT between 24 and 44 hours post-challenge (Table 35 and Figure 20). Approximately, 24% (17/70) of the animals were

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treated based on a positive PA-ECL result; the remaining animals were treated based on temperature. About 89% of the animals were bacteremic between 24 and 104 hours post-challenge (Table 35 and Figure 20). The mean time to challenge until a positive PA-ECL result among different groups varied between 26.77 and 28.90 hours. All animals were treated within 4 hours of trigger.

The mean time from trigger to treatment was similar (approximately 1 to 4 hours) in all the five groups (Table 35).

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Table 35: Study AR033 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Placebo (Group 1) N=14	ETI-204			
		1 mg/kg (Group 2) N=14	4 mg/kg (Group 3) N=14	8 mg/kg (Group 4) N=14	16 mg/kg (Group 5) N=14
Baseline characteristics					
Age (months) estimated range	8.9±1.2 (7.0-12.0)	9.3±2.0 (7.0-12.0)	9.8±2.2 (7.0-15.0)	8.9±2.2 (7.0-13.0)	9.9±3.5 (7.0-19.0)
Body weight (kg) Mean ±SD	3.6±0.2	3.5±0.2	3.6±0.2	3.5±0.1	3.6±0.2
Inhaled Dose					
Total inhaled dose (cfu x 10 ⁷) GM ± SD (Range)	(b) (4)				
LD ₅₀ dose Mean±SD	201.6±33.8 (132-263)	208.7±27.8 (155-255)	208.5±45.4 (102-278)	188.6±38.0 (137-290)	196.1±30.2 (129-238)
<200 LD ₅₀ dose n(%)	6 (42.9)	3 (21.4)	6 (42.9)	12 (85.7)	5 (35.7)
≥200 LD ₅₀ dose n(%)	8 (57.1)	11 (78.6)	8 (57.1)	2 (14.3)	9 (64.3)
Trigger for treatment^a					
PA ^{+ve} (screening) by ECL assay ^b n (%)	2 (14.3)	3 (21.4)	5 (35.7)	5 (35.7)	2 (14.3)
SIBT n (%)	12 (85.7)	11 (78.6)	9 (64.3)	9 (64.3)	12 (85.7)
PA levels (ng/mL) by ELISA prior to treatment					
N (%)	1	1	1	2	1
Log ₁₀ Mean±SD (Range)	0.7±0.2 (0.7-1.3)	0.7±0.2 (0.7-1.4)	0.8±0.3 (0.7-1.7)	0.8±0.2 (0.7-1.2)	0.8±0.3 (0.7-1.8)
Geometric mean	5.3	5.5	5.7	5.7	5.8
Bacteremia prior to treatment					
Enriched (qualitative) bacteremia n (%)	13 (92.9)	12 (85.7)	11 (78.6)	13 (92.9)	13 (92.9)
Log ₁₀ (cfu/mL) Mean±SD (Range)	2.8±1.6 (0.3-4.7)	3.1±1.7 (0.3-5.1)	3.3±1.5 (0.3-0.5)	3.3±1.5 (0.3-5.3)	3.1±1.5 (0.3-4.8)
GM±SD (cfu/mL)	705.9	1310.1	1937.1	2050.0	1362.2
Time between challenge, trigger, and treatment					
Time to trigger (PA ^{+ve} or SIBT) post challenge (hours) ^a Mean±SD (Range)	25.78±5.3 (18.4-36.9)	26.83±3.61 (20.4-33.4)	27.40±5.87 (19.8-42.8)	25.94±4.75 (19.9-36.3)	27.73±5.34 (17.8-37.1)
Time to quantitative bacteremia (hours) n Mean±SD (Range)	14 36.7±20.8 (22.1-103.7)	12 31.3±13.6 (22.9-73.7)	13 28.2±6.5 (23.0-44.8)	14 30.1±12.2 (22.4-69.0)	12 34.8±18.9 (23.7-92.6)
Time from trigger to treatment (hours)	0.95±1.23 (0.30-4.48)	0.95±1.05 (0.28-3.22)	1.60±1.35 (0.37-4.25)	1.45±1.55 (0.23-4.22)	0.71±0.75 (0.27-2.82)
Survivors at the end of study (Day 28)					
n (%)	0/14 (0)	4/14 (29)**	6/14 (43)**	10/14 (71)**	9/14 (64)**
N (%) Based on bacteremic animals (enriched bacteremia) at some time prior to treatment	0/13 (0%)	2/12 (16.7)	3/11 (27.3)**	9/13 (69)**	8/13 (62)**
SD Standard deviation PTT Prior to treatment; Quantitative bacteremia Limit of detection (LOD) =100 cfu/mL; PA ELISA Lower limit of quantification (LLOQ) =9.68 ng/mL.					
^a The trigger for treatment was defined as the time from challenge to a positive PA-ECL post-challenge.					
^b The time to treatment was defined as the time from challenge to treatment.					
**Statistically significant between the ETI-204 treated group and the control group					
^number positive >LLOQ					

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Detection of Bacteremia and PA: The variability among the results of three bacterial culture methods as well as PA detection by ELISA and ECL assays is shown in Table 36. The samples used for all these assays were collected at the same times post-challenge. Of the 70 rabbits in the study, 62, 55 and 60 were positive by enriched, qualitative, and quantitative culture methods, respectively; 53 and 6 were PA positive by ECL and ELISA, respectively. Only 6 samples were positive by all the 5 methods. Qualitative enriched bacteremia was the most sensitive [62/70 (88.6%)]. Detection of PA by the ECL method was more sensitive than ELISA; this may be due to approximately 10-fold higher sensitivity of the ECL assay (LOD 4 ng/mL; however, the positive control used for testing was 1 ng/mL and showed positive results) compared to ELISA (LOD 9.68 ng/mL); for details see microbiology review by Dr Berkeley.

Table 36: Study AR033 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Detection Method				Treatment Group							
	Qualitative		Quantitative Culture‡	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)‡		Placebo (n=14)	1 mg/kg ETI-204 (n=14)	4 mg/kg ETI-204 (n=14)	8 mg/kg ETI-204 (n=14)	16 mg/kg ETI-204 (n=14)	Total (n=70)
	Enriched Culture*	Quantitative Culture‡			PA (ELISA Assay)‡	PA (ELISA Assay)‡		ETI-204 (n=14)	ETI-204 (n=14)	ETI-204 (n=14)	ETI-204 (n=14)	
+	+	+	+	+	+	+	1	1	1	2	1	6
+	+	+	+	+	-	-	8	10	9	9	10	46
+	+	+	-	-	-	-	0	0	1	1	1	3
-	+	+	+	+	-	-	1	0	0	0	0	1
-	+	+	-	-	-	-	1	1	0	1	0	3
-	+	-	-	-	-	-	2	0	0	0	1	3
-	-	+	-	-	-	-	0	0	1	0	0	1
-	-	-	-	-	-	-	1	2	2	1	1	7

n = Number of treated animals. Results determined on a per animal basis, not for individual tests. Animal with a positive test from any time prior to treatment is considered as positive.

*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (1 ng/mL); LOD was 4 ng/mL

‡LLOQ by quantitative culture 100 cfu/mL; LLOQ by free PA ELISA 9.68 ng/mL

There was a positive correlation between bacterial burden and PA levels (0.33056; P-value 0.0055).

Effect of treatment on survival and microbial burden: A dose dependent increase in survival was observed up to 8 mg/kg dose of ETI-204; survival at 8 mg and 16 mg/kg doses was similar (Figure 20 and Table 35). All the control group of animals died within Day 6 of challenge.

All surviving rabbits were culture negative by Day 8 post-challenge and remained negative (below the LOD) for the duration of the study (Figures 20 and 21).

Between 8 and 48 hours post-treatment, the PA levels were detectable and higher in the control group animals whereas in animals treated with ETI-204, there were very few animals (6/69; 9%) with PA levels above the lower limit of quantitation (LLOQ); therefore, comparison of PA levels with survival could not be made (Figures 20 and 22).

Effect of microbial burden on survival: There appears to be a trend towards a decrease in survival in animals with higher microbial burden.

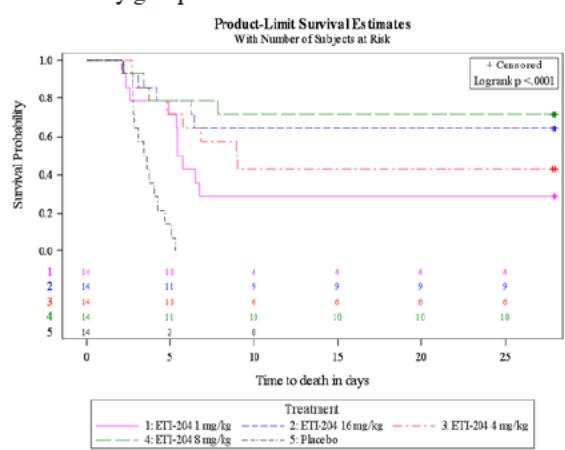
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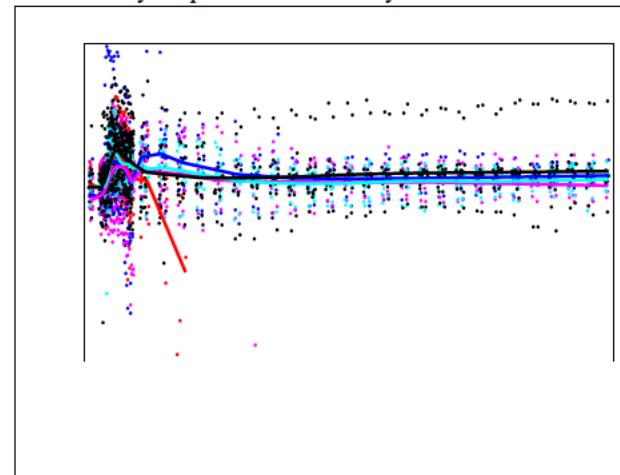
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Figure 20: Study AR033 – Survival, changes in body temperature over time, and relationship between microbial burden and time to death

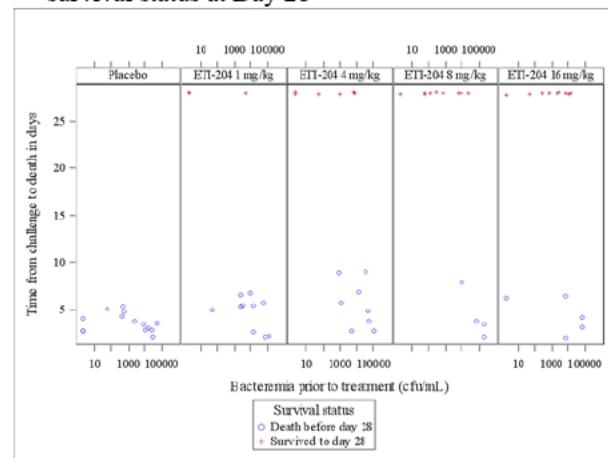
A: Kaplan-Meier curves representing time-to-death and survival by group



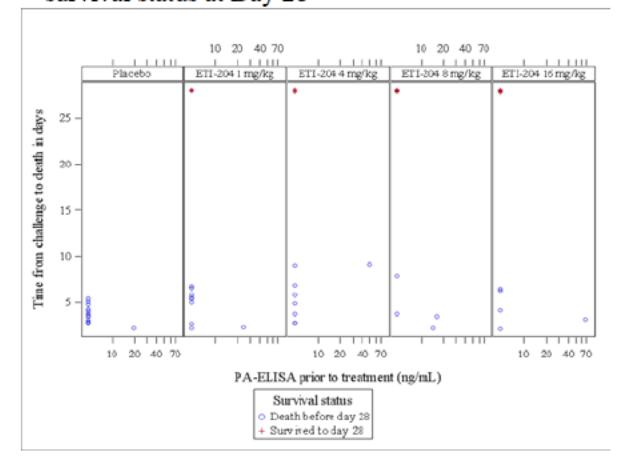
B: Mean body temperature over time by animal and treatment



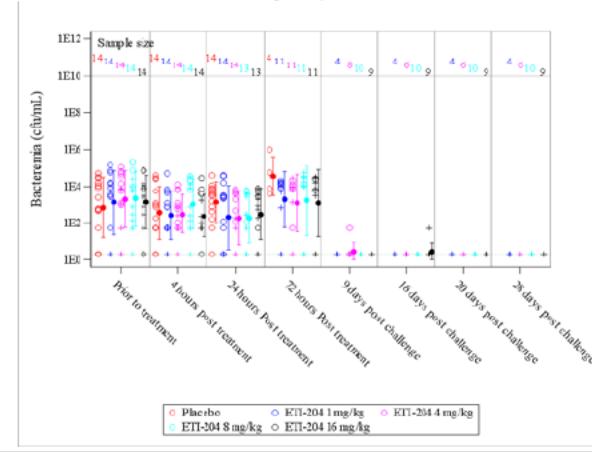
C: Time to death versus bacteremia prior to treatment by survival status at Day 28



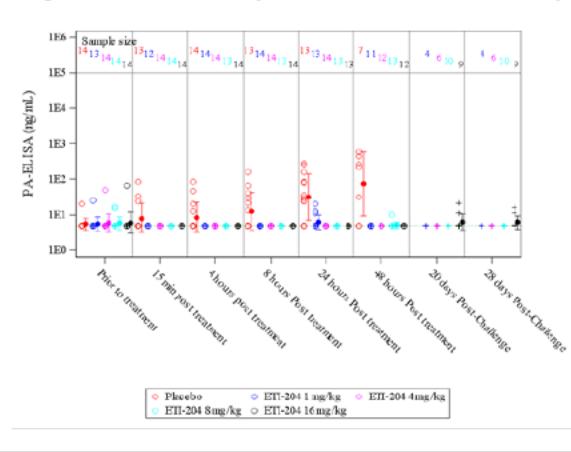
D: Time to death versus PA-ELISA prior to treatment by survival status at Day 28



E: Bacteremia with geometric mean and standard deviation (by survival status: plus=survived to day 28; circle=death before day 28)



F: PA-ELISA prior to treatment with geometric mean and standard deviation (by survival status: plus=survived to day 28; circle=death before day 28)



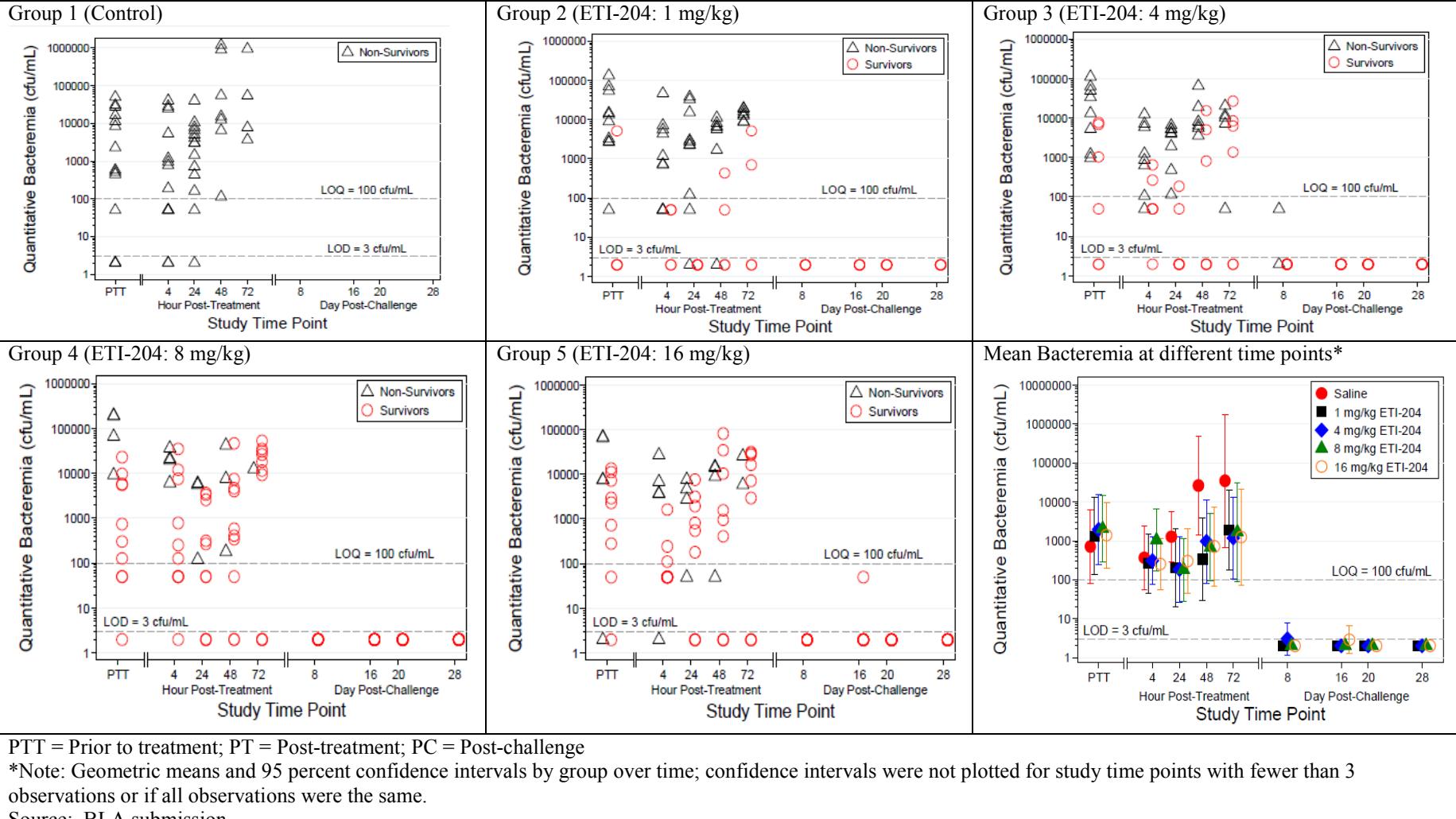
Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

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Figure 21: Study AR033 - Observed bacteremia levels over time in survivors and non-survivors

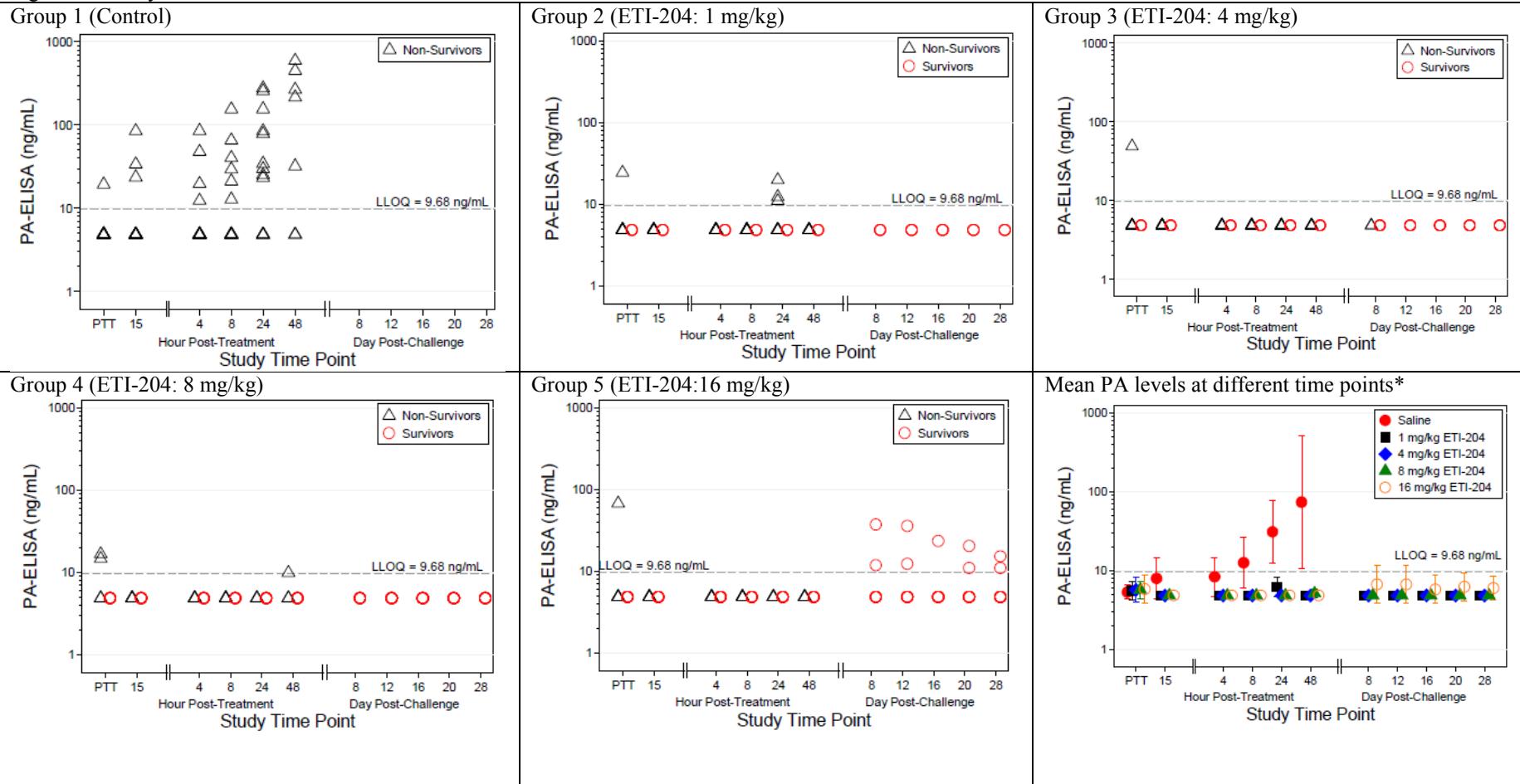


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Figure 22: Study AR033 - Observed PA levels over time in survivors and non-survivors



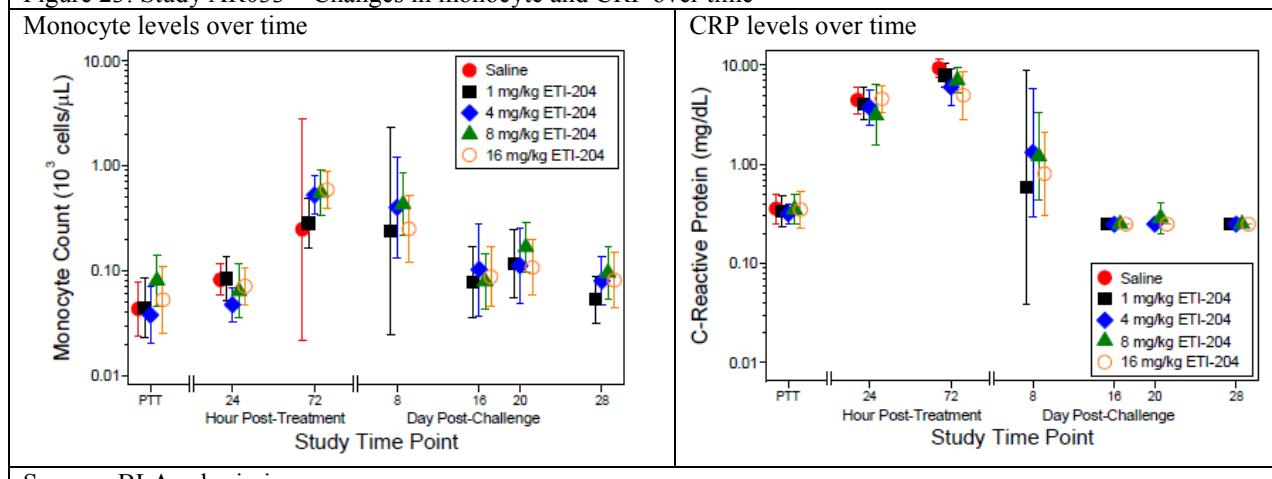
PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge

*Note: Geometric means and 95 percent confidence intervals by group over time; confidence intervals were not plotted for study time points with fewer than 3 observations or if all observations were the same.

Source: BLA submission

Hematological parameters: The changes in hematological parameters were similar to that for the Study AR021 summarized above. For example, the most apparent trend was observed with monocytes which were elevated generally between 72 hour post-treatment and 7 days post-challenge time points and then subsequently began to return to normal levels (Figure 23). Overall, the data suggests that the animals demonstrated non-specific indicators of illness post-challenge. CRP levels were relatively consistent prior to challenge. CRP levels increased and peaked between 24-72 hours post-treatment followed by returning to baseline (Figure 23). These results should be interpreted with caution as assessments were infrequent during this study.

Figure 23: Study AR033 – Changes in monocyte and CRP over time



Source: BLA submission

Clinical Observations: Clinical observations following challenge were similar to those summarized above for Study AR021 and the natural history studies. Animals that succumbed to disease showed a characteristic progression of signs that followed from unremarkable to observations of lethargy, respiratory abnormalities, and finally moribundity. Animals that survived until scheduled sacrifice were documented as lethargic with signs of respiratory abnormalities and reduced food consumption within the first few days post-challenge. The animals returned to normal observations generally between Day 7 and 11 post-challenge with occasional stool abnormalities or reduced food consumption.

Necropsy and Histopathology: Gross lesions and microscopic findings in rabbits dying post-anthrax challenge were similar to those summarized above for the Study AR021 and natural history studies. These lesions included discolorations and/or foci in the brain, and large intestines (appendix); enlargement of mediastinal and bronchial lymph nodes; and fluid effusion in the pericardial cavity, thymus, and thoracic cavity (fluid in body cavities was not examined microscopically). These gross lesions correlated histologically with hemorrhage, necrosis, edema and acute inflammation. No gross lesions were evident among the surviving animals that were terminated at study completion for gross and microscopic evaluation (Table 37).

Microscopic findings included acute fibrinous to heterophilic inflammation, necrosis, hemorrhage, edema, and the presence of large rod-shaped bacteria in the brain (meninges), kidney, liver, lungs, mediastinal or bronchial lymph nodes, spleen, spinal cord, and any gross lesions. Slightly more bacteria were evident in organs from saline control Group 1 animals compared to other groups. There were no other qualitative differences in lesions of anthrax

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among the control and treated animals. No significant microscopic lesions were present in the six rabbits terminated on Study Day 28.

Neuropathological observations: The brains of 12 surviving animals (3 survivors per group) and 15 animals that were found dead (between Study Days 2 and 8; 3 non-survivors per group) were microscopically evaluated. The applicant stated that of the 15 animals that died, nine died with either no microscopic changes or with just scattered hemorrhage and/or areas of intravascular bacteria suggesting that these animals died prior to the bacteria leaving the blood stream due to septicemia without any notable morphologic response. Six of the non-surviving animals had notable microscopic changes. These changes included observations of an inflammatory response in four animals and extravascular bacteria/notable hemorrhage in two animals without a visible inflammatory response; however, there was a lack of a dose response. All three animals at the 8 mg/kg dose that died were without detectable microscopic changes, whereas 3/3 of the 4 mg/kg animals and 2/3 of the 16 mg/kg animals died with extravascular bacteria, extensive hemorrhage and/or evidence of an inflammatory response (Table 37). The lack of a dose response may be due to biologic variation. However, these findings should be interpreted with caution due to a small number of animals examined.

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Table 37: Study AR033 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Brain					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	1/13 (NA)	1/12 (NA)	0/12 (NA)	1/13 (NA)	2/13 (NA)
Microscopic Finding ^{2,3}					
# Necropsied/Total Infected	3/13	5/12	6/12	6/13	6/13
Brain, basal nuclei: Bacteria, extravascular	0/3	0/5	2/6 (4.00)	0/6	1/6 (4.00)
Brain, basal nuclei: Hemorrhage	0/3	0/5	2/6 (3.00)	0/6	1/6 (4.00)
Brain, basal nuclei: Inflammation	0/3	0/5	1/6 (4.00)	0/6	0/6
Brain, basal nuclei: Inflammation, 100%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, basal nuclei: Vasculitis	0/3	0/5	2/6 (3.50)	0/6	0/6
Brain, cerebellum: Bacteria, extravascular	0/3	0/5	1/6 (3.00)	0/6	1/6 (3.00)
Brain, cerebellum: Hemorrhage	0/3	1/5 (1.00)	1/6 (3.00)	0/6	1/6 (3.00)
Brain, cerebral cortex: Bacteria, extravascular	0/3	0/5	3/6 (3.00)	0/6	2/6 (3.00)
Brain, cerebral cortex: Bacteria, intravascular	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, cerebral cortex: Hemorrhage	1/3 (2.00)	0/5	3/6 (2.00)	0/6	1/6 (4.00)
Brain, cerebral cortex: Inflammation	0/3	0/5	2/6 (1.50)	0/6	1/6 (2.00)
Brain, cerebral cortex: Inflammation, 100%granulocytes	0/3	0/5	1/6 (NA)	0/6	1/6 (NA)
Brain, cerebral cortex: Inflammation, 20%mononuclear cells	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, cerebral cortex: Inflammation, 80%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, cerebral cortex: Parenchymal, necrosis	0/3	0/5	2/6 (2.00)	0/6	0/6
Brain, cerebral cortex: Vasculitis	0/3	0/5	1/6 (3.00)	0/6	1/6 (2.00)
Brain, hippocampus: Bacteria, extravascular	0/3	0/5	3/6 (3.33)	0/6	1/6 (2.00)
Brain, hippocampus: Hemorrhage	0/3	0/5	2/6 (3.00)	0/6	1/6 (4.00)
Brain, hippocampus: Inflammation	0/3	0/5	2/6 (2.50)	0/6	1/6 (2.00)
Brain, hippocampus: Inflammation, 10%mononuclear cells	0/3	0/5	0/6	0/6	1/6 (NA)
Brain, hippocampus: Inflammation, 100%granulocytes	0/3	0/5	2/6 (NA)	0/6	0/6
Brain, hippocampus: Inflammation, 90%granulocytes	0/3	0/5	0/6	0/6	1/6 (NA)
Brain, hippocampus: Neuronal necrosis/neuron loss	0/3	0/5	0/6	1/6 (1.00)	0/6
Brain, hippocampus: Parenchymal, necrosis	0/3	0/5	1/6 (3.00)	0/6	1/6 (4.00)
Brain, hippocampus: Pigment, melanin (variation of normal)	0/3	0/5	0/6	1/6 (1.00)	0/6
Brain, hippocampus: Vasculitis	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, medulla oblongata: Bacteria, extravascular	0/3	0/5	2/6 (3.00)	0/6	1/6 (3.00)
Brain, medulla oblongata: Hemorrhage	2/3 (1.00)	0/5	1/6 (3.00)	0/6	1/6 (3.00)
Brain, meninges: Bacteria, extravascular	0/3	0/5	1/6 (2.00)	0/6	1/6 (3.00)
Brain, meninges: Bacteria, intravascular	0/3	1/5 (1.00)	0/6	0/6	0/6
Brain, meninges: Hemorrhage	0/3	0/5	1/6 (2.00)	0/6	0/6
Brain, meninges: Inflammation	0/3	1/5 (1.00)	1/6 (3.00)	0/6	1/6 (4.00)
Brain, meninges: Inflammation, 10%mononuclear cells	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, meninges: Inflammation, 100%mononuclear cells	0/3	1/5 (NA)	0/6	0/6	0/6
Brain, meninges: Inflammation, 20%mononuclear cells	0/3	0/5	0/6	0/6	1/6 (NA)
Brain, meninges: Inflammation, 80%granulocytes	0/3	0/5	0/6	0/6	1/6 (NA)
Brain, meninges: Inflammation, 90%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, meninges: Vasculitis	0/3	0/5	0/6	0/6	1/6 (2.00)
Brain, midbrain: Bacteria, extravascular	0/3	0/5	2/6 (3.00)	0/6	0/6
Brain, midbrain: Hemorrhage	1/3 (1.00)	0/5	2/6 (2.50)	0/6	0/6
Brain, midbrain: Inflammation	0/3	0/5	2/6 (2.00)	0/6	0/6
Brain, midbrain: Inflammation, 100%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, midbrain: Inflammation, 50%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, midbrain: Inflammation, 50%mononuclear cells	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, midbrain: Vasculitis	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, pons region/pontine nuclei: Hemorrhage	1/3 (2.00)	0/5	1/6 (3.00)	0/6	0/6
Brain, thalamus/hypothalamus: Bacteria, extravascular	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, thalamus/hypothalamus: Inflammation	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, thalamus/hypothalamus: Inflammation, 100%granulocytes	0/3	0/5	1/6 (NA)	0/6	0/6
Brain, thalamus/hypothalamus: Vasculitis	0/3	0/5	1/6 (3.00)	0/6	0/6
Brain, ventricular system: Choroid plexus, extravascular, bacteria	0/3	0/5	0/6	0/6	1/6 (2.00)
Brain, ventricular system: Inflammation	0/3	0/5	0/6	0/6	1/6 (4.00)
Brain, ventricular system: Inflammation, 20%mononuclear cells	0/3	0/5	0/6	0/6	1/6 (NA)
Brain, ventricular system: Inflammation, 80%granulocytes	0/3	0/5	0/6	0/6	1/6 (NA)

NA, not applicable.

*Mean severity of lesion.

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 37 (continued): Study AR033 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Kidney					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	0/12 (NA)	0/12 (NA)	1/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Bacteria	12/13 (2.25)	5/12 (1.60)	3/12 (1.33)	1/13 (1.00)	0/13
Dilatation, Pelvis	0/13	0/12	0/12	1/13 (3.00)	0/13
Inflammation	0/13	0/12	2/12 (1.50)	0/13	1/13 (2.00)
Infract, Multifocal, Unilateral, Chronic	0/13	0/12	0/12	1/13 (3.00)	0/13
Nephropathy	0/13	0/12	0/12	1/13 (2.00)	1/13 (2.00)
Liver					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	0/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	11/12	12/12	13/13	13/13
Bacteria	12/13 (2.50)	6/11 (1.50)	0/12	1/13 (1.00)	0/13
Inflammation	0/13	0/11	0/12	0/13	1/13 (1.00)
Necrosis	2/13 (1.50)	3/11 (2.33)	3/12 (1.67)	2/13 (3.00)	1/13 (2.00)
Necrosis, Hepatocyte	0/13	0/11	1/12 (1.00)	0/13	0/13
Sinusoidal Leukocytosis	12/13 (1.08)	7/11 (1.29)	3/12 (1.00)	2/13 (1.50)	2/13 (1.00)
Lymph Node, Bronchial					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	1/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	12/13	13/13
Bacteria	12/13 (3.33)	7/12 (2.43)	0/12	2/12 (1.50)	1/13 (1.00)
Depletion/Necrosis, Lymphoid	12/13 (3.50)	8/12 (2.88)	5/12 (3.00)	4/12 (3.00)	4/13 (2.75)
Fibrin	11/13 (2.27)	7/12 (1.71)	6/12 (2.67)	4/12 (2.50)	3/13 (2.33)
Hemorrhage	11/13 (2.36)	6/12 (2.17)	5/12 (2.20)	2/12 (2.50)	3/13 (1.67)
Inflammation	12/13 (2.33)	8/12 (1.50)	4/12 (2.25)	3/12 (2.00)	3/13 (1.33)
Lymph Node, Mediastinal					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	1/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	12/13	12/12	12/12	13/13	13/13
Bacteria	12/12 (3.50)	8/12 (1.88)	1/12 (1.00)	2/13 (1.00)	0/13
Depletion/Necrosis, Lymphocytes	12/12 (3.08)	8/12 (2.50)	4/12 (2.25)	3/13 (3.00)	4/13 (2.00)
Depletion/Necrosis, Lymphoid	0/12	0/12	0/12	1/13 (3.00)	0/13
Fibrin	10/12 (2.60)	9/12 (1.56)	5/12 (1.80)	4/13 (2.50)	3/13 (1.67)
Hemorrhage	9/12 (1.89)	6/12 (2.17)	3/12 (2.00)	2/13 (2.00)	1/13 (2.00)
Inflammation	11/12 (2.45)	7/12 (1.71)	3/12 (2.00)	3/13 (2.33)	3/13 (2.00)
Lung					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	0/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Alveolar Histiocytosis	0/13	0/12	0/12	0/13	1/13 (2.00)
Bacteria	12/13 (3.08)	8/12 (2.13)	0/12	3/13 (1.00)	0/13
Edema	1/13 (2.00)	4/12 (1.75)	0/12	1/13 (1.00)	0/13
Fibrin	2/13 (2.00)	7/12 (1.71)	3/12 (1.33)	1/13 (1.00)	1/13 (1.00)
Fibrin, Alveolus	0/13	0/12	0/12	0/13	1/13 (1.00)
Hemorrhage	1/13 (2.00)	4/12 (1.25)	2/12 (1.00)	0/13	0/13
Inflammation	2/13 (2.00)	7/12 (1.57)	2/12 (1.00)	1/13 (1.00)	2/13 (1.50)
Necrosis, BALT	2/13 (4.00)	3/12 (1.33)	0/12	0/13	0/13

NA, not applicable.

*Mean severity of lesion Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 37 (continued): Study AR033 - Incidence summary of gross, microscopic, and severity of lesions in rabbits bacteremic at the time of treatment.

Organ/Lesion	Treatment Group				
	Placebo n/N (*)	1 mg/kg ETI-204 n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Spleen					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	0/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Bacteria	12/13 (3.42)	3/12 (2.67)	0/12	0/13	0/13
Depletion/Necrosis, Lymphocytes	13/13 (2.92)	10/12 (2.90)	3/12 (3.67)	2/13 (3.00)	1/13 (4.00)
Depletion/Necrosis, Lymphoid	0/13	0/12	0/12	1/13 (4.00)	0/13
Fibrin	12/13 (1.58)	9/12 (2.11)	4/12 (2.50)	4/13 (2.75)	3/13 (2.00)
Inflammation	5/13 (1.00)	7/12 (1.43)	2/12 (1.00)	2/13 (1.00)	1/13 (3.00)
Cavity, Pericardial					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	1/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4,5}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Cavity, Thoracic					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	1/13 (NA)	1/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected ⁵	13/13	12/12	12/12	13/13	13/13
Intestine, Large					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	2/13 (NA)	2/12 (NA)	0/12 (NA)	1/13 (NA)	1/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Bacteria, Appendix	2/13 (1.00)	2/12 (2.00)	0/12	1/13 (2.00)	0/13
Hemorrhage, Appendix	2/13 (1.00)	2/12 (1.50)	0/12	1/13 (2.00)	1/13 (2.00)
Necrosis/Depletion, Lymphoid, Appendix	2/13 (1.00)	2/12 (2.00)	0/12	1/13 (2.00)	1/13 (2.00)
Spinal Cord					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	0/13 (NA)	0/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Bacteria	8/13 (1.00)	3/12 (1.00)	0/12	0/13	0/13
Bacteria, Meninges	3/13 (1.00)	1/12 (1.00)	1/12 (1.00)	0/13	2/13 (2.00)
Hemorrhage	1/13 (1.00)	1/12 (2.00)	2/12 (2.50)	0/13	0/13
Inflammation, Meninges	0/13	0/12	0/12	0/13	1/13 (3.00)
Thymus					
Macroscopic Finding ¹					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Gross Lesions	1/13 (NA)	0/12 (NA)	0/12 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,4}					
# Necropsied/Total Infected	13/13	12/12	12/12	13/13	13/13
Atrophy	1/13 (4.00)	0/12	0/12	0/13	0/13
Bacteria	1/13 (1.00)	0/12	0/12	0/13	0/13
Edema	1/13 (2.00)	0/12	0/12	0/13	0/13
Fibrin	1/13 (1.00)	0/12	0/12	0/13	0/13
Hemorrhage	1/13 (1.00)	0/12	0/12	0/13	0/13
Inflammation	1/13 (1.00)	0/12	0/12	0/13	0/13

NA, not applicable.

*Mean severity of lesion Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

Animals K99373 and K99383 were excluded from statistical analysis, because they were inadvertently dosed with levofloxacin.

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

Tissue bacterial assessments:

Histology: All the tissues [brain, kidney, liver, lymph nodes (mediastinal), lung, and spleen] from surviving animals irrespective of the ETI-204 dose were negative for bacteria by

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histological examination; however, bacteria were observed in several of the tissues from non-survivors from all the groups (Table 38).

Culture: Of the animals that survived treatment with ETI-204, one animal (treated with 16 mg/kg ETI-204) had a positive bronchial lymph node bacterial culture result, whereas all other tissues tested (brain, spleen, liver, lung, kidney, and lymph node) were negative (Table 38). All the rabbits that died had had a positive tissue bacterial result for at least two of the tissues tested (most of the animals that died had 5 or 6 positive tissue bacterial results).

Table 38: Study AR033 - Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 1mg/kg		ETI-204 4mg/kg		ETI-204 8mg/kg		ETI-204 16mg/kg	
	Non Survivors (N=0)	Non Survivors (N=14)	Non Survivors (N=4)	Non Survivors (N=10)	Non Survivors (N=6)	Non Survivors (N=8)	Non Survivors (N=10)	Non Survivors (N=4)	Non Survivors (N=9)	Non Survivors (N=5)
Presence of bacteria by microscopy [1, 5]										
Brain: Total [3, 4]	0/0	0/3	0/3	1/3	0/3	3/3	0/3	0/3	0/3	2/3
Brain, basal nuclei: extravascular	0/0	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	1/3
Brain, cerebellum: extravascular	0/0	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	1/3
Brain, cerebral cortex: extravascular	0/0	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	2/3
Brain, cerebral cortex: intravascular	0/0	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3
Brain, hippocampus: extravascular	0/0	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	1/3
Brain, medulla oblongata: extravascular	0/0	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	1/3
Brain, meninges: extravascular	0/0	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	1/3
Brain, meninges: intravascular	0/0	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Brain, midbrain: extravascular	0/0	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3
Brain, thalamus/hypothalamus: extravascular	0/0	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3
Brain, ventricular system: extravascular	0/0	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3
Kidney	0/0	13/14	0/4	5/10	0/6	3/8	0/10	1/4	0/9	0/5
Liver	0/0	13/14	0/4	6/9	0/6	0/8	0/10	1/4	0/9	0/5
Lymph Node, Bronchial	0/0	13/14	0/4	7/10	0/6	0/8	0/9	2/4	0/9	1/5
Lymph Node, Mediastinal	0/0	13/13	0/4	8/10	0/6	1/8	0/10	2/4	0/9	0/5
Lung	0/0	13/14	0/4	8/10	0/6	0/8	0/10	3/4	0/9	0/5
Spleen	0/0	13/14	0/4	3/10	0/6	0/8	0/10	0/4	0/9	0/5
Cavity, Pericardial	0/0	0/14	0/4	0/10	0/6	0/8	0/10	0/4	0/9	0/5
Cavity, Thoracic	0/0	0/14	0/4	0/10	0/6	0/8	0/10	0/4	0/9	0/5
Intestine, Large	0/0	2/14	0/4	2/10	0/6	0/8	0/10	1/4	0/9	0/5
Spinal Cord	0/0	12/14	0/4	4/10	0/6	1/8	0/10	0/4	0/9	2/5
Thymus	0/0	1/14	0/4	0/10	0/6	0/8	0/10	0/4	0/9	0/5
Presence of bacteria by culture [1, 2]										
Brain	0/0	14/14	0/4	10/10	0/6	7/8	0/10	4/4	0/9	3/5
Kidney	0/0	14/14	0/4	9/10	0/6	7/8	0/10	4/4	0/9	3/5
Liver	0/0	14/14	0/4	9/10	0/6	7/8	0/10	3/4	0/9	4/5
Lymph Node, Bronchial	0/0	14/14	0/4	10/10	0/6	5/8	0/10	3/4	1/9	3/5
Lung	0/0	14/14	0/4	9/10	0/6	7/8	0/10	4/4	0/9	2/5
Spleen	0/0	14/14	0/4	10/10	0/6	7/8	0/10	4/4	0/9	4/5
[1] All treated animals irrespective of bacteremia status prior to treatment										
[2] Animal was considered positive if at least 1-5 colonies were present on plate										
[3] Neuropathology performed at (b) (4)										
[4] Animals that were positive in at least one area for either extra- or intra-vascular bacteria										
[5] Histopathology performed at (b) (4) with exception of brain; Not all animals were assessed microscopically; numbers examined are shown										
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: L48728, L48754, L48759, L48769, L48779, L48793, L48794										
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None										
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: L48722, L48724, L48738, L48742, L48747, L48762, L48785, L48788, L48792, L48797										

Cerebral spinal fluid:

The CSF from a majority (22/26) of the non-survivors was culture positive; of these, 13 animals were in the control group, 3 treated with 1 mg/kg ETI 204, 2 treated with 4 mg/kg ETI-204, 2 treated with 8 mg/kg ETI-204, and 2 treated with 16 mg/kg ETI-204 (Table 39). The CSF from all the surviving rabbits was culture negative. These findings were consistent with the neuropathology results: non-survivors had evidence of an active *B. anthracis* infection in the brain whereas animals that survived to the end of the study did not.

Table 39: Study AR033 - Assessment of CSF culture results in non-survivors	
Group	CSF n/N (%)
Saline	13/13 (100)
ETI-204 treated	
1 mg/kg	3/5 (60)
4 mg/kg	2/3 (67)
8 mg/kg	2/3 (67)
16 mg/kg	2/2 (100)

Comments:

- The baseline characteristics of animals in the different groups were similar. All animals were bacteremic, PA positive, or showed an increase in temperature at the time of initiation of treatment. About 89% of the rabbits had a blood culture result positive for *B. anthracis* prior to treatment.
- The study showed that ETI-204 at all the doses (1, 4, 8 and 16 mg/kg) tested was effective in improving survival up to Day 28 compared to the untreated control group. The efficacy in the animals treated with either 8 or 16 mg/kg treated groups was similar. By eight days post-challenge, all rabbits which survived (to the end of the study) had negative bacteraemia results which remained negative for the duration of the study.
- All of the non-surviving animals died within 10 days of challenge. Of the rabbits that died, 100% all animals had a positive terminal bacteraemia sample; also, spleen, lymph nodes, and other organs were culture positive. Gross lesions and microscopic findings consistent with anthrax were present in all rabbits that died or became moribund during the study. No significant lesions or microscopic findings were noted in animals surviving to the end of study.
- High bacterial burden in the blood prior to treatment appears to be associated with decreased survival rate. PA levels above the LLOQ were detectable in more of the vehicle group of animals between 8 and 48 hours post-treatment, compared to the ETI-204-treated groups.
- Slightly more bacteria were evident in organs from control animals (Group 1) as compared to other groups. Of the animals that survived, one animal had a positive bronchial lymph node bacterial culture result, whereas all other tissues tested (brain, spleen, liver, lung, kidney, and lymph node) were negative by culture and histological examination. Most of the animals that died had 5 or 6 positive tissue bacterial results. The CSF from a majority (22/26) of the non-survivors was culture positive.
- Overall, the study suggests that ETI-204 at a dose of 16 mg/kg is effective in protecting against death due to anthrax when administered intravenously after the onset of disease.

6.2.2. Cynomolgus monkeys

The applicant measured the activity of different doses of ETI-204 (Baxter or Lonza products) in 4 studies (AP202, AP201, AP204, and AP203) in experimentally naïve cynomolgus monkeys. The primary efficacy endpoint was survival at the end of study compared to placebo. All the studies were conducted at [REDACTED] ^{(b)(4)}

6.2.2.1. Study AP202

This was a phase 3 randomized, blinded, 3-arm (placebo, Lonza and Baxter products of ETI-204), GLP, trigger-to-treat study, administrated IV, in experimentally naïve cynomolgus monkeys challenged with the spores of the Ames strain of *B. anthracis* by aerosolization with a head only chamber.⁴²

Primary objective: Evaluate the efficacy of a single, intravenous dose of ETI-204 manufactured at Lonza. Primary endpoint was survival to Day 28 post-challenge.

Secondary objective: Compare the efficacy of ETI-204 manufactured at Baxter with that manufactured at Lonza.

Study design:

Fifty-three juvenile (less than five years of age) animals weighing between 2.2 - 4.6 kg from [REDACTED] ^{(b)(4)} were included in the study. Of these, 51 monkeys were placed on study and the remaining two monkeys served as potential non-randomized replacements. Monkeys were tested and verified negative for tuberculosis; also, animals were prescreened within 30 days prior to receipt at [REDACTED] ^{(b)(4)} to confirm that the seronegative status for Simian Immunodeficiency Virus (SIV), Simian T-Lymphotropic Virus-1 (STLV-1), and *Cercopithecine herpesvirus* 1 (Herpes B virus) and negative for Simian Retrovirus (SRV1 and SRV2) by PCR. Only healthy monkeys free of malformations, clinical signs of disease, and negative for intestinal parasites were placed on study. About 28 of the animals were anti-measles antibody positive; majority received measles vaccine. Eight animals (C59119, C58856, C58858, C59381, C59383, C58888, C60817, and C58806) were positive for *Klebsiella*.

There were equal numbers of animals (n=17) randomized to each of the 3 arms i.e., placebo, Lonza (16 mg/kg) and Baxter (16 mg/kg) products of ETI-204 (Figure 24). Monkeys were transported into the ABSL-3 a minimum of 7 days prior to challenge to allow time for acclimation. Monkeys were quarantined for about a month at [REDACTED] ^{(b)(4)} and acclimated to pole/collar training and chair restraint during this period. Any animal that suffered from non-study-related illnesses between randomization and treatment was removed from the study at the discretion of the Study Director. Two of the animals were stated to be bacteremic (by enriched bacteremia) prior to challenge and were replaced. Any data associated with animals removed from the study before treatment trigger criteria were met and retained in the study file, but not reported. One animal (C58822) was positive (122 ng/mL) for anti-PA antibodies by ELISA performed at the [REDACTED] ^{(b)(4)} laboratory; this animal was not included in the study.

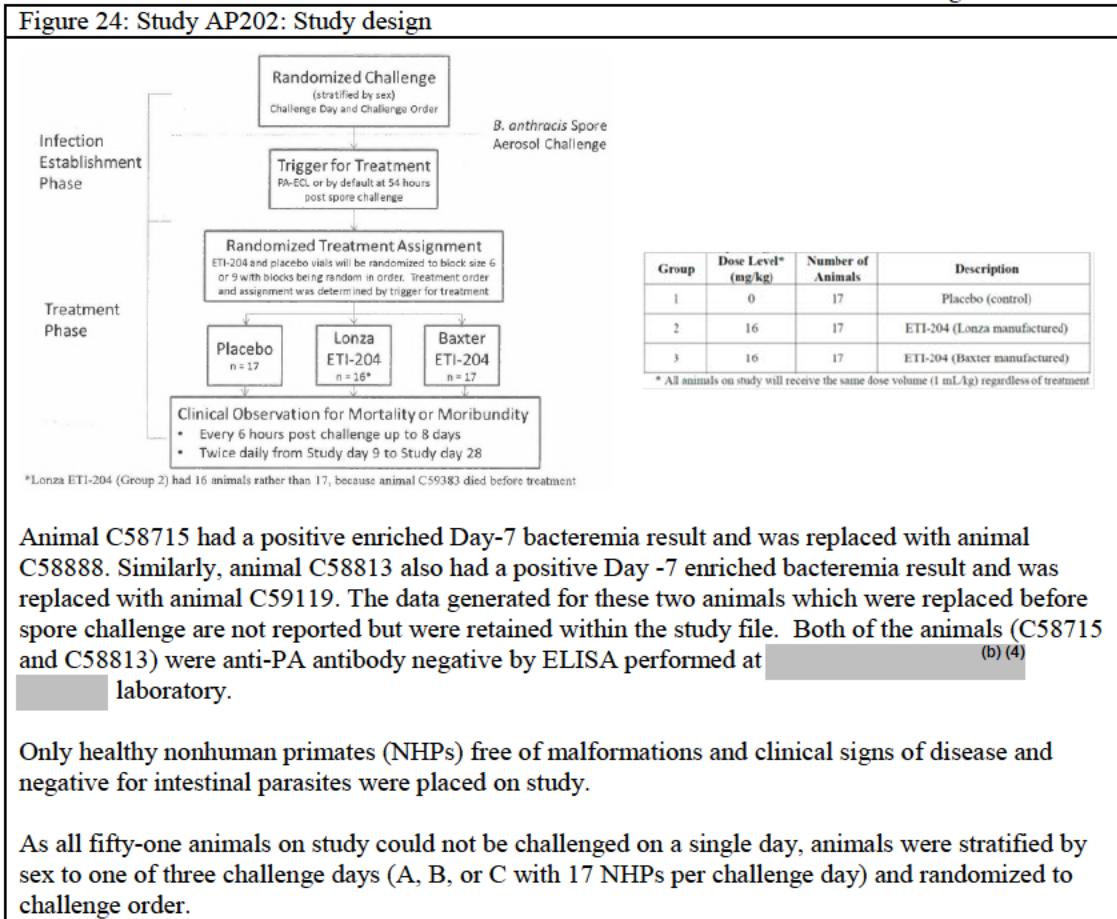
⁴² Elusys Study AP202: [REDACTED] ^{(b)(4)} Study Number 2826-100020847 - Three armed trigger-to-treat efficacy study of intravenously administered ETI-204 in cynomolgus monkeys with inhalational anthrax (January 23, 2015).

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Figure 24: Study AP202: Study design



- ***Establishment of inhalational anthrax and treatment***

- **Aerosol challenge**

Monkeys were transported into the Animal Biosafety Level-3 (ABSL-3) a minimum of 7 days prior to challenge to allow time for acclimation. On the day of challenge, monkeys were anesthetized with Telazol (1-6 mg/kg, IM) and placed into a plethysmography chamber and a Class III biosafety cabinet system as summarized above for the natural history studies at (b)(4). Monkeys were aerosol-challenged with a targeted 200X LD₅₀ [(b)(4) spores (spore lot no. B37)] based on the study by Vasconcelos *et al.* (2003)³.

- **Criteria for treatment trigger**

The criterion for treatment trigger was measurement of PA by the ECL assay (Table 40). For this, blood was collected in serum separator tubes (SST). The assay was run onsite, in real time (with the exception of the prior to challenge and prior to treatment time point samples) as a screening assay for the presence of circulating PA. Samples were considered negative when the mean ECL value of the test sample was less than the mean ECL value of the 2 ng/mL positive control; however the LLOD was 4 ng/mL (for details see Microbiology review by Dr Berkeley).

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Table 40: Study AP202 - Blood collection and assay schedule

Target Time Point	Blood Tube type/ Approximate Blood volume	Quantitative Bacteremia (Culture) ^a	Enriched Bacteremia (Culture) ^b	Serum for PA- ECL	Serum for PA- ELISA	Serum for ETI- 204 Concentration
Before Treatment ^c	Study Day -7	EDTA ~0.5 ml SPS ~1.0 ml SST ~4.0 ml	X	X	X	X
	~24 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	~30 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	~36 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	~42 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	~48 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	~54 hr PC	SPS ~1.0 ml SST ~1.0 ml	-	X	X	-
	PTT	EDTA ~0.5 ml SPS ~1.0 ml SST ~1.0 ml	X	X	-	X
Post Treatment	15 min PT	SPS ~1.0 ml SST ~2.5 ml	-	X	-	X
	96 hr PT	EDTA ~0.5 ml SPS ~1.0 ml SST ~1.5 ml	X	X	-	-
	7 days PT	EDTA ~0.5 ml SPS ~1.0 ml SST ~1.5 ml	X	X	-	-
	Day 28 PC (day of scheduled termination)	EDTA ~1.5 ml SPS ~1.0 ml SST ~4.0 ml	X	X	-	X
	Unscheduled Termination ^b	EDTA ~1.5 ml SPS ~1.0 ml SST ~2.0 ml	X	X	-	X

The day of spore challenge is Study Day 0, PC = Post Challenge, PTT = Prior to Treatment, PT = Post Treatment.

^a Post challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort. Post treatment bleed times were calculated from the time each animal's IV treatment ended. Blood samples occurred within ±60 minutes of the calculated time, except for the 15 min PT and 96 hr PT samples which occurred within 5 min and 3 hr of the their calculated times respectively. The Study Day -7 bloods were relative to the day of challenge and the 7 days PT blood samples were relative to the day of treatment.

^b If collection was possible | ^c Post Challenge, pre-treatment sampling stopped once decision to treat was made.

^a Bacteremia enrichment performed on sample collected in sodium polyanethol sulfonate (SPS) tube | ^b Quantitative Bacteremia performed on sample collected in EDTA tube

A single dose of either Baxter or Lonza products of ETI-204 (16 mg/kg) or placebo were administered intravenously after PA positive findings by the ECL assay. Animals that did not have a positive serum PA result up to 54 hours post-challenge time point were treated.

Study measurements included body weight, clinical observations, pharmacokinetics, and microbiological measurements at different time points prior to challenge, post-infection and post-treatment (Table 40). Microbiological parameters measured included PA measurement by ECL and ELISA and bacteremia by culture (enriched and quantitative). If blood could not be collected for all protocol-designated evaluations for any animal due to restrictions on the amount of blood that can be collected, the priority for blood collection post-challenge was PA (ECL) > enriched bacteremia > quantitative bacteremia > PA concentration (ELISA) prior to treatment. Post treatment the priority was ETI-204 concentration > quantitative bacteremia > PA (ELISA).

For **enriched bacterial cultures**, 1 mL of blood was collected in SPS tubes and cultured in brain heart infusion (BHI) broth or other appropriate culture broth at an approximate 1:10 dilution for a minimum of 24 hours and up to 64 hours at 37° C. A portion (~40 µL) of this broth culture was plated on blood agar plates to determine (qualitatively) the presence or absence of colony morphology consistent with *B. anthracis*.

Cultures for quantitating bacteremia were performed by plating of 100 µL of whole blood (collected in EDTA tubes) and a series (10^{-1} to 10^{-8}) of 1:10 serial dilutions in triplicate on TSA

plates. For the Day 7 post-treatment and Day 28 post-challenge quantitative bacteremia, only two (neat and 10^{-1}) samples were prepared and plated. Blood samples were stored at room temperature until diluted and plated; typically samples were plated within 6 hours of collection with the exception of terminal samples which may have been held longer. The results were expressed as cfu/mL. The majority of the cfu counts were based on 2 of the 3 replicates. The applicant states that typically, the acceptable countable cfu range was 25 to 250; average counts falling outside of this range were reported as being positive for bacteremia with no quantifiable number.

The LOD for quantitative bacteremia was 3 cfu/mL. Quantitative bacteremia levels less than the LOD or reported as "0" were replaced with one half of the LOD rounded to the nearest integer (2 cfu/mL) for the statistical analysis.

Free PA levels (quantitative) in serum by (ELISA) were measured at different time points pre-challenge, post-challenge, and post-treatment (Table 40). Blood was collected and serum samples stored at $\leq -70^{\circ}\text{C}$ until shipped on dry ice to [REDACTED] (b) (4) and [REDACTED] (b) (4). PA measurements were not conducted in a blinded fashion; however, the sample analysis results were not released to the Study Director or the applicant, until receiving a written memo of unblinding from the Study Director. In a case of sample discrepancy that could not be resolved within [REDACTED] (b) (4) and [REDACTED] (b) (4), the Contributing Scientist informed [REDACTED] (b) (4) and analysis was halted until the study was unblinded by the Study Director memo.

The LLOQ for PA-ELISA was 5 ng/mL. PA-ELISA values reported as less than the LLOQ were replaced with one half of the LLOQ (2.5 ng/mL) for the statistical analysis.

Clinical observations necropsy findings, and tissue collection was performed as summarized for the natural history studies. Gross necropsy was performed on all monkeys that were found dead or euthanized.

Bacterial culture of tissues: Spleen, brain (right cerebellar brain), kidney, liver, lung, and lymph nodes (mediastinal or bronchial) were collected at necropsy and cultures performed.

The applicant used modified intent-to-treat (mITT) population⁴³ as the primary analysis population. The ITT population⁴⁴ was used for sensitivity analysis.

Results:

Baseline characteristics: All animals were culture negative as well as PA negative by the ECL assay and ELISA on Day-7 i.e., prior to challenge. All animals included in the study were anti-PA IgG antibody negative; although one animal tested positive for antibodies but was not included in the study. Age, gender, body weight, and challenge dose (mean LD₅₀ 257; [REDACTED] cfu) were comparable among three groups; the LD₅₀ dose was ≥ 200 for approximately 84% of the animals (Table 41). The MMAD for each exposure day ranged from 1.13 - 1.18 μm which is consistent with the particle size range that would reach the alveoli.

⁴³ All animals assigned to a treatment vial excluding those animals that were not positive for bacteremia by enriched culture at the prior to treatment (PTT) sample collection time point.

⁴⁴ All animals assigned to a treatment vial regardless of bacteremia status at PTT.

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Table 41: Study AP202 - Baseline characteristics, inhaled dose of <i>B. anthracis</i> , time to treatment and survival			
Parameters	Placebo (Group 1) N=17	Lonza (Group 2) [#] ETI-204 16 mg/kg N=16	Baxter (Group 3) ETI-204 16 mg/kg N=17
Baseline characteristics			
Age (years) estimated range	2.7-5	2.7-5	2.7-5
Body weight (kg)	2.91±0.52	2.88±0.42	2.85±0.37
Inhaled dose			
Total Inhaled Dose: cfu x 10 ⁷	(b) (4)		
Mean ± SD (Range)			
LD ₅₀ dose	247.6±52.6	270.2±54.8	254.4±41.0
Mean ± SD (Range)	(172-318)	(166-402)	(182-323)
<200 LD ₅₀ dose n (%)	4 (23.5)	1 (6.3)	3 (16.7)
≥200 LD ₅₀ dose n (%)	13 (76.5)	15 (93.8)	14 (82.4)
Trigger for Treatment (PA by ECL)^a and microbial burden prior to treatment			
Positive Screening PA/ECL Assay n (%)	17/17 (100)	15/16 (93.8)*	17/17 (100)
Bacteremia (cfu/mL)			
Enriched (qualitative) bacteremia n (%)	17 (100)	16 (100)	17 (100)
Quantitative bacteremia			
n (%)	17 (100)	16 (100)	17 (100)
Log ₁₀ bacteremia (cfu/mL)	4.95±1.11	5.52±1.24	5.08±1.60
Geometric mean cfu/mL x 10 ⁴ /mL	8.9	32.7	12.1
PA levels by ELISA (ng/mL)			
Log ₁₀ ± SD (Geometric mean)	1.20±0.92 (15.9)	1.50±0.94 (31.9)	1.49±1.20 (30.7)
Time between challenge, trigger, and treatment			
Time (hours) to trigger - PA ^{+ve} post challenge ^a	34.5±5.5 (28.3-51.9)	34.1±4.6 (27.1-43.1)	35.1±4.5 (28.4-42.8)
Mean±SD (Range) n	17	15*	17
Time (hours) to bacteremia n	17	15*	17
Mean±SD (Range)	38.8±5.4 (28.3-51.9)	39.2±5.6 (31.0-53.1)	39.2±4.3 (32.3-46.0)
Time to treatment post challenge ^b (hours); Mean ± SD (Range)	38.9±5.4	39.3±5.6*	39.3±4.3
Time from trigger to treatment	4.3±0.8 (17)	4.2±0.8 (15)*	4.2±0.7 (17)
Survivors at the end of study (Day 28)			
Survivors	0 (0)	5 (31)**	6 (35)**
Survivors (bacteremic animals)			
#Group 2 only had 16 animals, because animal C59383 died before treatment and was excluded from the analysis			
*One animal (C60822) did not have a positive PA-ECL and was not included in the calculations. This animal was treated at 54 hours and was bacteremic and survived.			
^a The trigger for treatment was defined as the time from challenge to a positive PA-ECL post-challenge.			
^b The time to treatment was defined as the time from challenge to treatment.			
**Statistically significant compared to the control group by exact method and Boschloo's one-sided test; no difference between the animals treated with the Lonza or Baxter product			
SD=Standard deviation			
PTT Prior to treatment; Quantitative bacteremia LOD Limit of detection=3 cfu/mL; PA ELISA LLOQ Lower limit of quantification=5 ng/mL.			

The mean time to trigger ranged from 34 to 35 hours among the three groups. All the animals except one (C60822 in Group 2 treated with the Lonza product of ETI-204) became PA positive

post-challenge between 24 to 48 hours (Table 41). All animals became bacteremic within 53 hours. The mean time to treatment ranged from 38.9 to 39.3 hours among the groups. The time from trigger to treatment ranged from 4.2 to 4.3 hours among the groups (Table 41).

Detection of Bacteremia and PA: The variability among the results of two bacterial culture and the two assays (ECL and ELISA) used to detect PA is shown in Table 42. The samples used for these three assays were collected at the same time points post-challenge. All animals were positive for bacteremia by both the methods; both enriched and quantitative culture methods for measuring bacteremia were equally sensitive. Of the 50 monkeys in the study, 49 and 40 were PA positive by ECL or ELISA, respectively.

Table 42: Study AP202 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture	Detection Method				Treatment Group				Total (n=50)
	Qualitative Enriched Culture*	Quantitative Culture‡	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)‡	Placebo (n=17)	16 mg/kg ETI-204 (Lonza) (n=16)	16 mg/kg ETI-204 (Baxter) (n=17)	Total (n=50)	
ND	+	+	+	+	13	13	14	40	
ND	+	+	+	-	4	2	3	9	
ND	+	+	-	-	0	1	0	1	

n = Number of treated animals. Results determined on a per animal basis, not for individual tests.
Animal with a positive test from any time prior to treatment is considered as positive. ND = Not Done
*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative.
LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL).
‡LLOQ by quantitative culture 100 cfu/mL; LLOQ by free PA ELISA 5 ng/mL

Effect of treatment on survival and microbial burden: The results showed ETI-204 at a dose of 16 mg/kg was effective in improving survival compared to the untreated group; the efficacy of both Baxter and Lonza products of ETI-204 was similar (Figure 25 and Table 41). There was no significant difference in survival rate among the animals treated with the Lonza or Baxter products of ETI-204. All of the non-surviving animals died within 8 days of challenge.

The quantitative bacteremia levels at 7 days following treatment and 28 days post-challenge were less than the LOD for all animals surviving to the end of the study. Of the 11 animals treated with ETI-204 that survived to the end of the study, 10 (91%) were negative for enriched bacteremia by the Day 4 post-treatment; all of the 11 animals were culture negative by Day 7 post-treatment. All animals which were found dead or moribund and euthanized on study were bacteremic (positive enriched bacteremia) (Figures 26 and 27).

The decrease in the PA levels showed a similar trend as the decrease in bacteremia over time. At 15 minutes post-treatment and at unscheduled termination, there was a significant difference between the proportions of animals positive for PA when comparing each of the ETI-204 treated groups to the control group. PA was undetectable within 15 minutes of treatment in a majority of the animals treated with ETI-204. At Day 2 post-challenge, the PA levels in the treated animals were reduced (Figure 26 and 27).

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Figure 25: Study AP202 - Kaplan-Meier curves representing time-to-death and survival by group

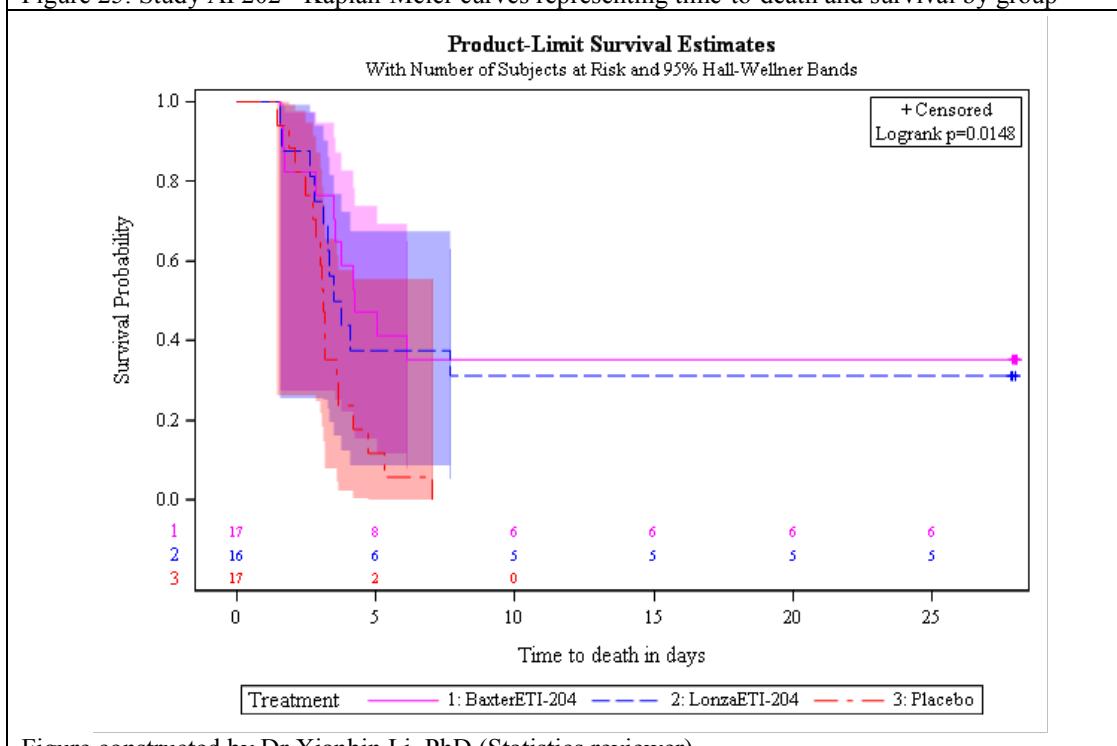


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Effect of microbial burden on survival: The quantitative bacteremia and PA levels, prior to treatment, though variable were comparable across the groups (Figures 26 and 27). There appears to be an inverse relationship between bacteremia or PA levels at the time of initiation of treatment and survival time; animals with higher bacteremia or PA levels, prior to treatment, had a lower probability of survival. The control group (Group 1), the Lonza ETI-204 treated group (Group 2) and the Baxter ETI-204 treated group (Group 3) had 8/17, 13/16 and 9/17 animals with quantitative bacteremia levels greater than the $1 \times 10^{4.74}$ threshold⁴⁵, respectively. No animals with a PA level over 1000 ng/mL survived.

⁴⁵A Cochran-Mantel-Haenszel test was performed by the applicant on the primary endpoint (survival) to determine its dependence on quantitative bacteremia levels greater than $1 \times 10^{4.74}$ cfu/mL at PTT. The quantitative bacteremia threshold of $1 \times 10^{4.74}$ cfu/mL at PTT was selected based on population pharmacokinetic and survival analysis modeling of ETI-204 (Mondick, J.T., Metrum Research Group LLC). Animals in each group were stratified based on whether the PTT quantitative bacteremia level was greater than $1 \times 10^{4.74}$ cfu/mL. This was used as a stratification variable in an analysis to determine if survival significantly differed among the groups after controlling for PTT quantitative bacteremia levels. Descriptive statistics for survival in each group were calculated at each level of the stratification variable.

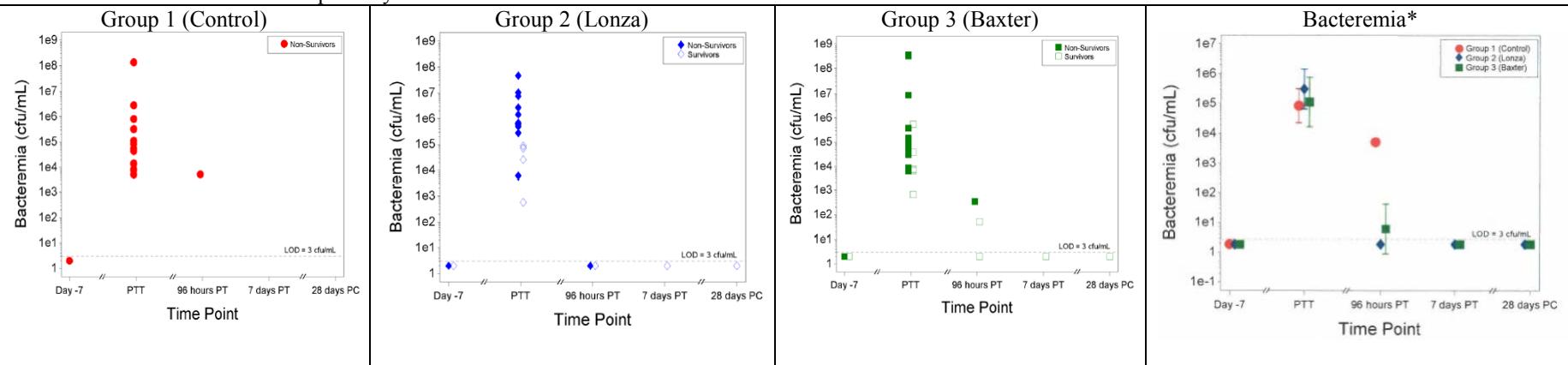
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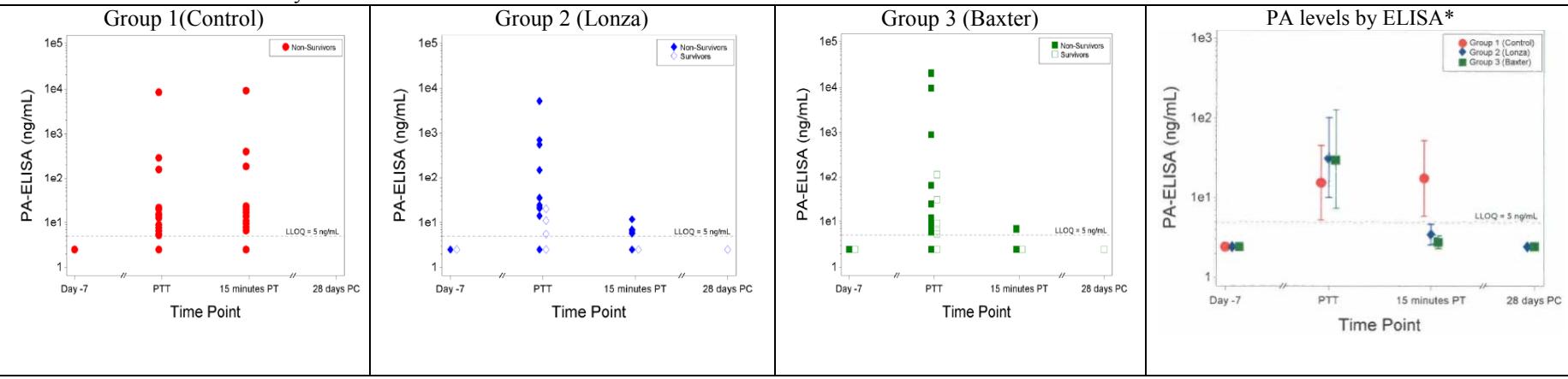
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Figure 26: Study AP202 - Observed bacteremia and PA levels over time in survivors and non-survivors at different time points

A: Bacteremia levels versus time point by survival status



B: Free PA-ELISA versus study time



PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge

*Plot of Group geometric means and 95 percent confidence intervals for bacteremia and PA levels versus study time

Source: BLA submission

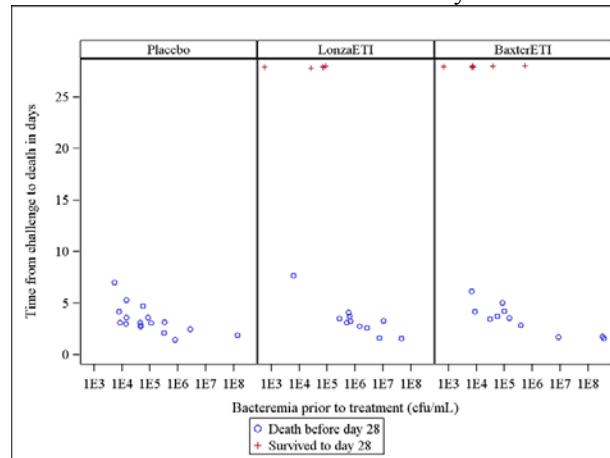
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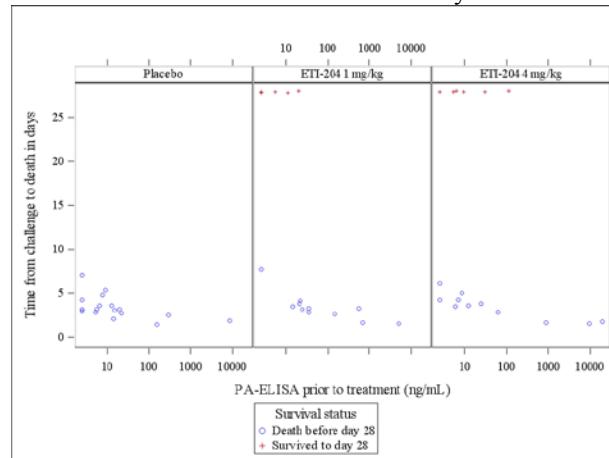
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Figure 27: Study AP202 - Survival and microbial burden (bacteremia and PA levels) at different time post-challenge

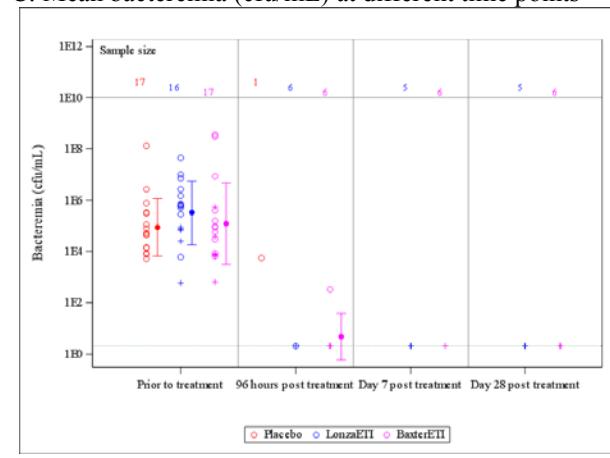
A: Time to death versus bacteremia prior to treatment by survival status for different animals at Day 28



B: Time to death versus PA levels prior to treatment by survival status for different animals at Day 28

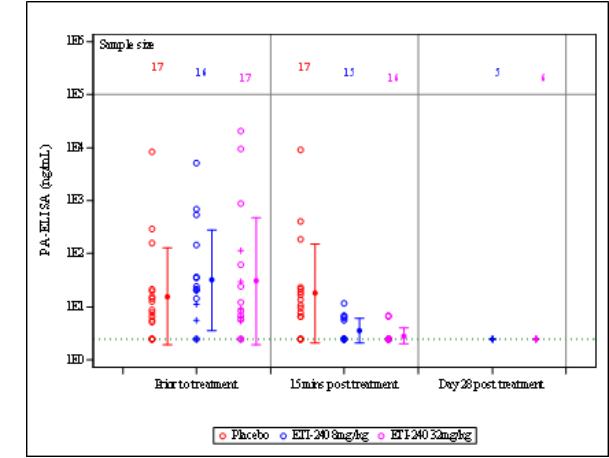


C: Mean bacteremia (cfu/mL) at different time points



The dotted green line is the reference line of 2 cfu/mL.

D: Mean PA levels (ng/mL) at different time points



The dotted green line is the reference line of 2.5 ng/mL.

PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge. LOD= lower limit of detection

+ = survivors

Figures constructed by Dr Xianbin Li PhD (Statistics reviewer)

Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these observations were consistent with those reported in the natural history studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed [other than sporadic stool abnormalities which is common with laboratory housed non-human primates (NHPs)] after Day 22 post-challenge (Figure 28).

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Figure 28: Study AP202 - Clinical observations

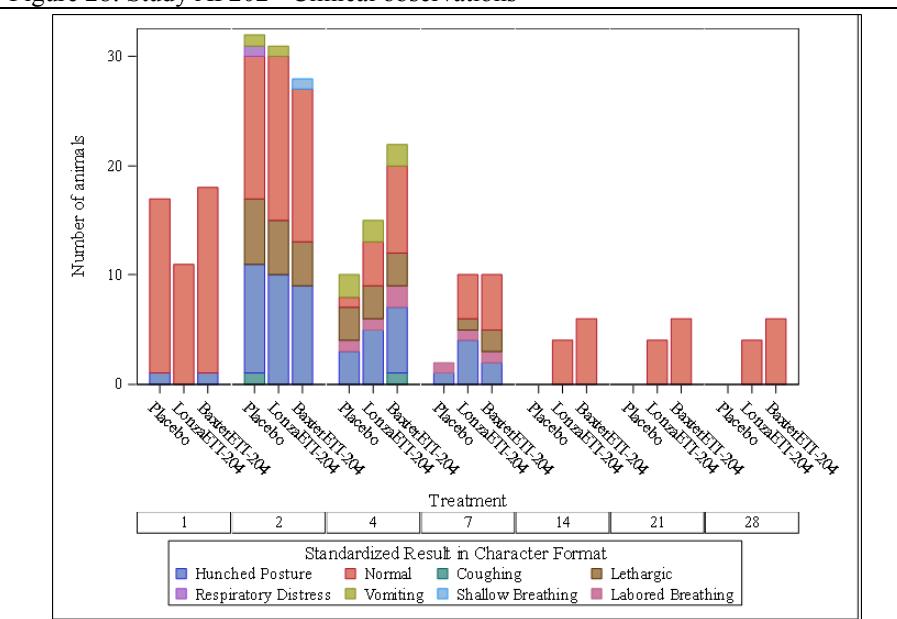


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

Hematological parameters: Not measured

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included discoloration of the brain and/or lung, enlarged lymph nodes (bronchial and/or renal), and/or fluid in the thoracic or abdominal cavities (Table 43). These lesions are typical of acute fulminant anthrax in NHPs and similar to those reported in the natural history studies summarized above. The applicant states that no gross lesions were found in animals that survived the period of observation. Histopathology was not performed.

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Table 43: Study AP202- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	16 mg/kg ETI-204 (Lonza) n/N (*)	16 mg/kg ETI-204 (Baxter) n/N (*)
Adrenal Glands			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Aorta			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Bone			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Bone Marrow			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Brain			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	1/17 (NA)	3/16 (NA)	3/17 (NA)
Cavity, Abdominal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	1/17 (NA)	0/16 (NA)	0/17 (NA)
Cavity, Thoracic			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	1/17 (NA)	0/16 (NA)	0/17 (NA)
Cecum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Colon			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Duodenum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Epididymis			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Esophagus			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Eyes			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Gallbladder			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)

NA, not applicable.

*Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

No histopathology performed

¹Gross necropsy pathology performed at (b) (4)

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Table 43 (continued): Study AP202- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	16 mg/kg ETI-204 (Lonza) n/N (*)	16 mg/kg ETI-204 (Baxter) n/N (*)
Heart			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Ileum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Injection Site			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Jejunum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Kidney			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Liver			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lung			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 1/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lymph Node			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 1/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lymph Node, Bronchial			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 2/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lymph Node, Mandibular			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lymph Node, Mediastinal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Lymph Node, Mesenteric			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			
Pancreas			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17 0/17 (NA)	16/16 0/16 (NA)	17/17 0/17 (NA)
Gross Lesions			

NA, not applicable.

*Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

No histopathology performed

¹Gross necropsy pathology performed at (b) (4)

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Table 43 (continued): Study AP202-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	16 mg/kg ETI-204 (Lonza) n/N (*)	16 mg/kg ETI-204 (Baxter) n/N (*)
Parathyroid Gland			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Pituitary Gland			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Rectum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Salivary Gland			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Sciatic Nerve			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Skeletal Muscle			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Skin			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Spinal Cord			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Spleen			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Stomach			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Thymus			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Thyroid Glands			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Tongue			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Trachea			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Ureter			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)
Urinary Bladder			
Macroscopic Finding ¹			
# Necropsied/Total Infected	17/17	16/16	17/17
Gross Lesions	0/17 (NA)	0/16 (NA)	0/17 (NA)

NA, not applicable.

*Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

No histopathology performed

¹Gross necropsy pathology performed at (b) (4)

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Tissue bacterial assessments: At least three of the six tissues, assessed from the animals that died or found moribund and euthanized on study, were positive for bacterial cultures consistent with *B. anthracis*. Almost all of the tissues assessed from ETI-204 treated animals that survived to the end of the study, were culture negative with the exception of the lung; the lung from all 11 survivors was culture positive (Table 44).

Table 44: Study AP202- Number of animals histologically and culture positive for <i>B. anthracis</i> in tissues						
Tissue	Placebo		ETI-204 Lonza 16 mg/kg		ETI-204 Baxter16 mg/kg	
	Survivors	Non-survivors	Survivors	Non-survivors	Survivors	Non-survivors
Presence of bacteria by microscopy: Not done						
Culture positive						
Brain	0	17/17	0/5	11/11	0/6	11/11
Bronchial lymph node	0	17/17	0/5	11/11	1/6	11/11
Kidney	0	17/17	0/5	8/11	0/6	10/11
Liver	0	17/17	0/5	10/11	0/6	10/11
Lung	0	17/17	5/5	11/11	6/6	11/11
Spleen	0	17/17	0/5	10/11	0/6	10/11
ND=Not Done						
[1] All treated animals irrespective of bacteremia status prior to treatment						
[2] Animal was considered positive if at least 1-5 colonies were present on plate						
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: None						
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None						
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: C60822						

Comments:

All animals included in the study were PA negative (by ECL). The study showed that both Lonza and Baxter products of ETI-204 at a dose of 16 mg/kg were effective in improving survival (31% and 35%, respectively) up to Day 28 compared to the untreated control group (all the untreated animals died by Day 7 post-challenge). All of the non-surviving animals died within 8 days of challenge.

All animals were bacteremic and PA positive at the time of initiation of treatment. For all animals surviving to the end of the study, the quantitative bacteremia levels at 7 days following treatment and 28 days post-challenge were below than the LOD. A higher bacteremia level and higher PA levels at the time of initiation of treatment appear to be associated with a lower survival rate. The study suggests that an animal with a high level of microbial burden prior to treatment is more likely to die after treatment with ETI-204.

Among monkeys that were found dead or euthanized, gross lesions were consistent with acute *B. anthracis* infection; additionally, the tissues from animals found dead or euthanized were culture positive for bacteria. No gross lesions were found in surviving animals; all tissues except lung from the animals that survived were negative; the lung was positive for bacterial cultures consistent with *B. anthracis*. The applicant states that this is consistent with the results from previous studies which have shown that spores can be found in the lung up to 56 days after challenge in surviving nonhuman primates ((b) (4) Study No. 1121- G924204; not a part of this BLA).

6.2.2.2. Study AP201

This was a randomized, blinded, placebo-controlled GLP study to evaluate the efficacy of ETI-204 (Baxter product) at a dose of 4 and 8 mg/kg administrated IV, against lethality due to inhalation exposure to the spores (spore lot no. B35) of the Ames strain of *B. anthracis* in 44 juvenile cynomolgus monkeys.⁴⁶ The study design was similar to that for Study AP202 except that the ELISA assay used for quantitation of free PA in serum was different from that used for Study AP202 and was performed at [REDACTED]^{(b)(4)} (Table 45). One animal (C36338) was PA positive (>30 ng/mL) by ELISA at Day -7; the anti-PA IgG antibodies were <LOD. It appears anti-PA antibodies were not measured for all animals. Additionally, hematological measurements, CRP levels and neuropathological evaluations were performed. Neuropathological evaluations were based on microscopic evaluation of brain and meninges from surviving and non-surviving monkeys.

The LOD and LLOQ for quantitative culture were 33 cfu/mL and 1,000 cfu/mL, respectively which are different from that for Study AP202. The reason for this is unclear as the same SOPs were used and testing was done in the same laboratory.

The mean age of the animals was 3.7 years (range 2.9-5.1 years) and mean body weight was 3.4 kg (range 2.5-5.3 kg) at the time of randomization. Monkeys were quarantined at [REDACTED]^{(b)(4)} and confirmed to be in good health, free of malformations, free of intestinal parasites, and exhibiting no signs of clinical disease by the study veterinarian. It is unclear if screening bacterial cultures were performed for all animals prior to challenge; two of the animals were stated to be culture negative for *Klebsiella*.

⁴⁶ [REDACTED] (b)(4) Study Number 834-G924202: AP201: Evaluating the efficacy of intravenously ETI-204 when administered therapeutically in the cynomolgus macaque with inhalational anthrax (August 8, 2011).

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Table 45: AP201 - Blood collection and assay schedule

Approximate Time Point	Blood Tube type/ Approximate Blood volume	Bacteremia (Culture)	CBC/ CRP	Serum PA levels (via ECL and PA-ELISA)	Serum for ETI-204 dose confirmation	* Retention Serum for Potential Future Use
Day -7	EDTA ~1.5ml SST ~2.0ml	X	X	X	X	X
^24hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
^30hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
^36hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
^42hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
^48hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
^54hr PC	EDTA ~0.5ml SST ~1.5ml	X		X		
PTT [#]	EDTA ~1.5 ml SST ~2.0ml SPS ~1.0 ml	X [#]	X	X		X
5 min PT	SST ~1.0ml					X
6hr PT	SST ~1.0ml					X
24hr PT	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c	X	X
96hr PT	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c		X
7 days PC	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c		X
14 days PC	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c		X
21 days PC	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c		X
30 days PC	EDTA ~1.5 ml SST ~2.0ml	X	X	X ^c		X
Terminal ^{b, d}	EDTA ~1.5 ml SST ~2.0ml	X	CRP only	X ^c		X

PC = Post-Challenge PTT = Prior to Treatment PT = Post-Treatment

^a Post-challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort. Post-treatment bleed times were calculated from the time each animal's IV treatment ended. Blood sample collection occurred within ±60 minutes of the calculated time, except for the 5 min PT, 6hr PT and 96hr PT samples which occurred within 2 min, 15 min and 3 hours of their calculated times, respectively. The Day -7, Day 7 PC, Day 14 PC, Day 21 PC and Day 30 PC bleeds were relative to the day of challenge.

^b If collection was possible

^c Samples tested via PA-ELISA only

^d Terminal samples were not collected on animals euthanized at the end of study as day 30 PC samples were already scheduled

^e If remaining serum was sufficient after aliquots for other analyses were made. Samples will be stored for potential future testing

[^]Post-Challenge, pre-treatment sampling stopped once decision to treat was made

[#] PTT Bacteremia enrichment performed on sample collected in SPS tube

Results:

Baseline characteristics: Age, gender, body weights, and challenge doses (mean LD₅₀ 199; (b) (4) cfu) were comparable among three groups; the LD₅₀ dose was ≥ 200 for approximately 40% of the animals (Table 46). The MMAD ranged from 1.11 - 1.12 µm which is consistent with the particle size range that would reach the alveoli.

All animals were culture negative at Day -7. All animals were PA negative by the ECL assay on Day-7 (prior to treatment); however, one animal (C36338 in the ETI-204 4 mg/kg treated group) was PA positive (>30 ng/mL) by ELISA on Day -7; the anti-PA IgG was below LOD of the assay. Although efforts were made to detect TNA in this animal prior to challenge and TNA was stated to be <LOD [effective dilution-50 (ED₅₀)⁴⁷ was ~45.3] it appears there were some

⁴⁷ Effective dilution-50 (ED₅₀) is defined as the reciprocal of the dilution of a serum sample that results in 50% neutralization of anthrax lethal toxin. This corresponds to the inflection point ('c' parameter) of a 4-parameter logistic log fit of the curve.

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technical issues; the applicant states that TNA measurement was performed with the remaining sera from the PA-ELISA assays “on study 834” and not the retention sera samples. However, the TNA assay could not be repeated due to logistic reasons. The anti-PA IgG for animal C36338 on Day 28 was 241.499 µg/mL. It is unclear if the PA positive finding, prior to challenge, in animal C36338 is due to cross-reactivity with another organism.

All animals except three [one animal (C38277) in the placebo group and two (C36383 and C37686) in the ETI-204 4 mg/kg group were triggered for treatment based on time] were treated within 6 hours of determining a positive serum PA by the ECL screening assay. Of the three animals that were PA negative by the ECL assay, two animals in the ETI-204 4 mg/kg treated group were PA positive by ELISA at the time of trigger and culture positive by qualitative enriched and quantitative culture methods. Animal C38277 in the control group was PA negative by ELISA at the time of trigger but culture positive by both enriched and quantitative culture methods; PA (52 ng/mL) was reported at 24 hours post-treatment that increased about 12-fold at 96 hours and was found dead by Day 7.

All animals were PA positive (by ECL or ELISA) and/or bacteremic between 25 and 55 hours post-challenge (Table 46). The mean time to trigger (about 39 hours) and treatment (43 hours) was similar in all the three groups (Table 46). The quantitative bacteremia and PA-ELISA levels though variable were comparable across the groups.

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Table 46: Study AP201 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Placebo (Group 1) N=14	ETI-204: 4 mg/kg (Group 2) N=14	ETI-204: 8 mg/kg (Group 3) N=15
Baseline characteristics			
Age (years) estimated range	3.6±0.6	3.6±0.6	3.7±0.6
Body weight (kg) Mean ±SD	3.4±0.8	3.3±0.6	3.3±0.5
Inhaled dose			
Total inhaled dose: cfu x 10 ⁷			(b) (4)
Mean ± SD (Range)			
LD ₅₀ dose	198.7±65.8	200.7±51.9	198.8±64.9
Mean ± SD (Range)	(96-305)	(140-280)	(109-356)
<200 LD ₅₀ dose n (%)	8 (57.1)	8 (57.1)	10 (66.7)
≥200 LD ₅₀ dose n (%)	6 (42.9)	6 (42.9)	5 (33.3)
Trigger for treatment (PA by ECL)^a and microbial burden prior to treatment			
Positive screening PA/ECL assay n (%)	13 (92.9)	12 (85.7)	15 (100)
Bacteremia			
Enriched (qualitative) bacteremia n (%)	14 (100)	13 (92.9)	15 (100)
Quantitative bacteremia n (%)	13 (92.9)	13* (92.9)	15 (100)
Log ₁₀ bacteremia (cfu/mL) Mean±SD	3.14±1.01 (1.23-5.85)	3.12±1.03 (1.23-5.52)	3.39±1.00 (1.23-6.38)
Geometric mean (Range) (cfu/mL x 10 ⁴)	0.14 (0.0017-70)	0.13 (0.0017-33.3)	0.25 (0.05-240)
PA levels (ng/mL) by ELISA			
n (%)	14 (100)	14 (100)	15 (100)
Log ₁₀ ±SD	1.00±0.73	1.08±0.86	1.07±0.89
Geometric mean	10.0	12.1	11.7
Time (hours) between challenge, trigger, and treatment			
Time to PA ^{+ve} (trigger) post challenge ^a n Mean±SD (Range)	13* 39.49±8.05 (28.6-52.6)	13* 37.96±10.12 (25.5-55.9)	15 38.65±8.00 (25.4-54.8)
Time to bacteremia Mean±SD (Range)	37.7±7.8 (28.6-55.4)	34.4 ±7.7 (25.5-52.1)	35.6±4.4 (25.4-41.8)
Time to treatment ^b Mean±SD (Range)	44.5±8.5 (31.8-58.7)	41.4 ±9.5 (29.1-59.1)	42.5±7.2 (29.4-58.0)
Time from trigger to treatment N Mean±SD (Range)	13* 3.9± 1.0 (2.87-5.62)	13* 3.14±1.47 (0.07-4.80)	15 3.89±1.41 (0.07-5.93)
Survivors at the end of study (Day 28)			
Survivors	2 (14.3)	11 (78.6)**	11 (73.3)**
Survivors (excluding one culture negative animal)	2 (14.3)	10/13 (76.9)**	11 (73.3)**

* One animal in the placebo group (C38277) and one in the ETI-204 4 mg/kg group (C37686) were triggered for treatment based on time and had missing values in trigger time so they were not included for this calculation.

Three animals from the 4 mg/kg ETI-204 group (C38261, C37686, and C36338) and 1 animal from the 8 mg/kg ETI-204 group (C37072) were treated as directed by the Study Director (per (b) (4) SOP (b) (4) V-061) as a result of failing ECL plates. In these instances, the Study Director reviewed the results on the failing ECL plates and compared the results (reported in ECL units) from the individual animals on the plates to the results obtained for the positive control on the respective plates. While the ECL plates failed, the ECL units for these individual animals were well above those obtained for the positive controls on the respective plates. One additional animal from the saline group (C38277) was treated after the 54-hour post-challenge time point despite a negative ECL result (per the protocol).

^aTime to trigger was based on PA positive findings by ECL assay.

^bThe time to treatment was defined as the time from challenge to treatment.

SD Standard deviation; PTT Prior to treatment; Quantitative bacteremia LOD Limit of detection=3 cfu/mL;

PA ELISA LLOQ Lower limit of quantification=5 ng/mL.

**Statistically significant between the ETI-204 8 mg/kg or 4 mg/kg treated groups and the control group by exact method and Boschloo's one-sided test.

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Detection of Bacteremia and PA: The variability among the results of three bacterial culture methods and the two assays (ECL and ELISA) used to detect PA is shown in Table 47. The samples used for these assays were collected at the same times post-challenge. Of the 43 animals, 39 (90.7%) were positive by all the 3 culture methods; 42 (98%) were positive by qualitative enriched culture and 43 (100%) by quantitative culture. PA was positive by both ECL and ELISA in 34 (79.1%) animals; PA was positive by ECL and ELISA in 41 (95%) and 35 (81%) animals, respectively. Enriched and quantitative culture methods for measuring bacteremia were most sensitive.

Table 47: Study AP201 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Qualitative Enriched Culture*	Detection Method				Placebo (n=14)	Treatment Group			Total (n=43)
		Quantitative Culture‡	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)‡	4 mg/kg ETI-204 (n=14)		8 mg/kg ETI-204 (n=15)	4 mg/kg ETI-204 (n=14)	8 mg/kg ETI-204 (n=15)	
+	+	+	+	+	11		10	11	11	32
+	+	+	+	-	1		1	4	0	6
+	+	+	-	+	0		1	0	0	1
+	+	+	-	-	1		0	0	0	1
+	ND	+	+	+	0		1	0	0	1
-	+	+	+	+	1		0	0	0	1
-	+	+	+	-	0		1	0	0	1

n = Number of treated animals. Results determined on a per animal basis, not for individual tests.

Animal with a positive test from any time prior to treatment is considered as positive.

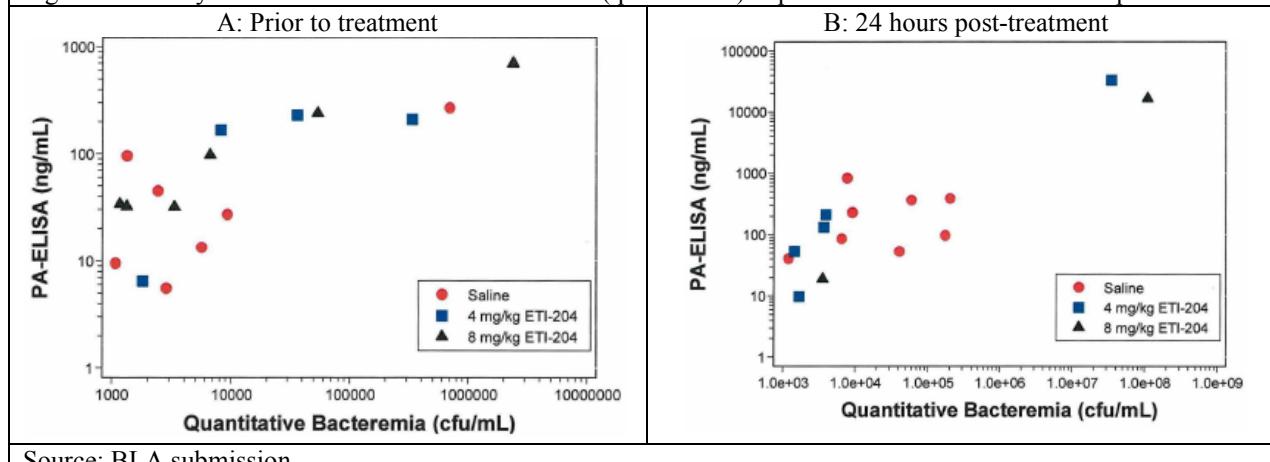
ND = Not Done

*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL); LOD 4 ng/mL

‡LLOQ by quantitative culture 1000 cfu/mL; LLOQ by PA ELISA 2.4 ng/mL

There was a positive correlation between quantitative bacteremia and PA levels at 36 hours post-challenge, prior to treatment (Figure 29), 24 hours post-treatment (Figure 29B) and 48 hours post-treatment suggesting a linear relationship between bacteremia and PA values at these study time points.

Figure 29: Study AP201 - PA-ELISA vs. Bacteremia (quantitative) at prior to treatment and 24 hours post-treatment



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Effect of treatment on survival and microbial burden: The results show that ETI-204 at a dose of 4 and 8 mg/kg was effective in improving survival compared to the control group (Figure 30 and Table 46). All of the non-surviving animals died within 10 days of challenge. All animals were bacteremic at the time of death; one animal (C37799) in the control group died on Day 9 and bacteremia at unscheduled terminal was not determined.

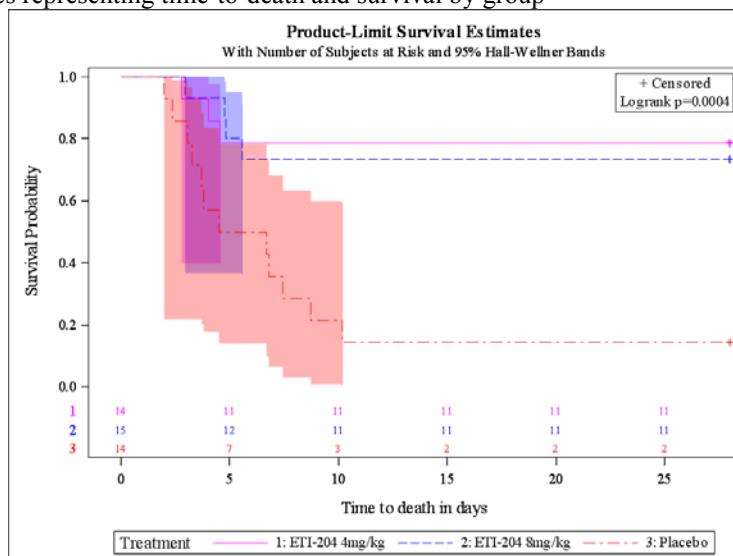
The bacteremia and PA levels decreased after initiation of treatment (Figures 30, 31 and 32). For all animals surviving to the end of the study, quantitative bacteremia levels at 7 days following treatment and 28 days post-challenge were below than the LOD. Complete resolution of bacteremia occurred by 96 hours post-treatment for ETI-204-treated monkeys. PA levels decreased by 96 hours post-challenge in survivors treated with ETI-204 4 mg/kg and 8 mg/kg.

Two animals (C37762 and C39097) in the control group survived the period of observation. Both animals were PA negative by Day 14; the PA levels at Day 7 post-challenge for survivors in the control group were 49 ng/mL (C37762) and 78 ng/mL (C39097). One animal was culture negative by Day 4 and another by Day 14.

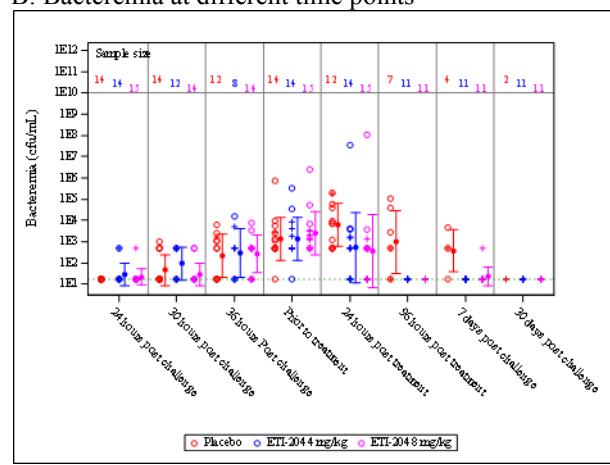
Overall, the results suggest ETI-204-treatment decreased PA and bacteremia levels.

Figure 30: Study AP201 - Survival and relationship between microbial burden at different time post-challenge

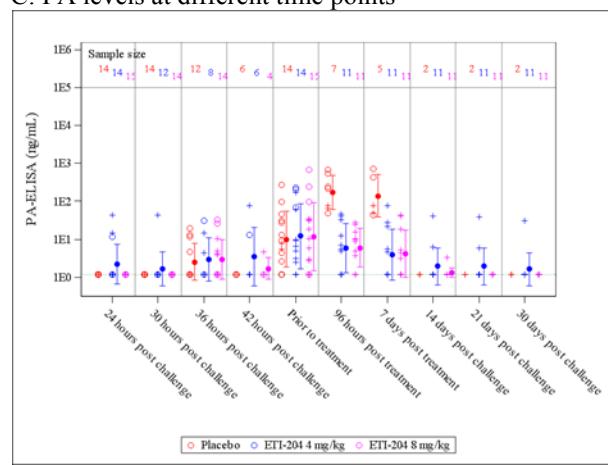
A: Kaplan-Meier curves representing time-to-death and survival by group



B: Bacteremia at different time points



C: PA levels at different time points



+=Survivors to Day 28; ○=Death before Day 28

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Effect of microbial burden on survival: Animals with higher bacteremia and PA levels at the time of initiation of therapy are less likely to survive after treatment with ETI-204 (Figures 31 and 32). The applicant states that the animals with PA levels <100 ng/mL, prior to treatment, are more likely to survive; however, this should be interpreted with caution as this may depend on the sensitivity of the assay.

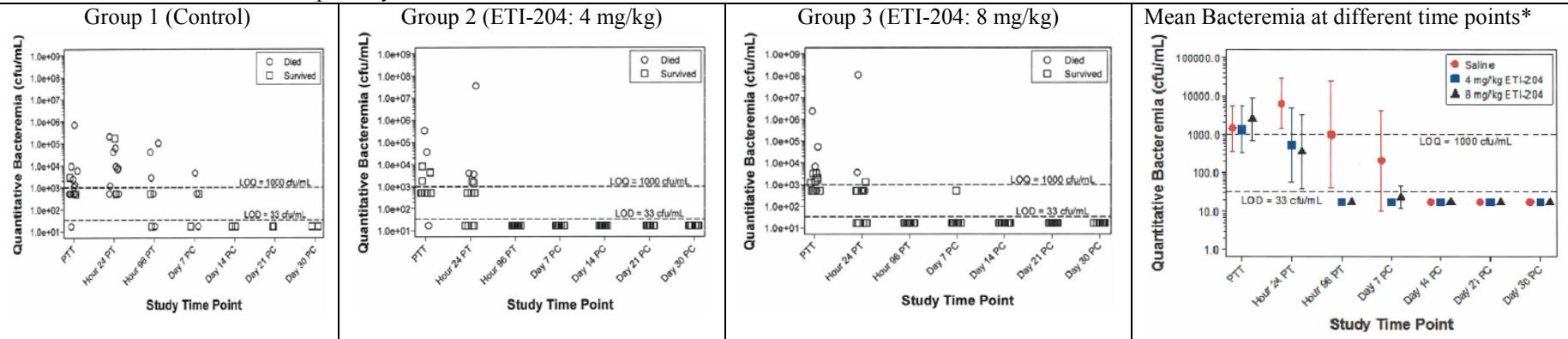
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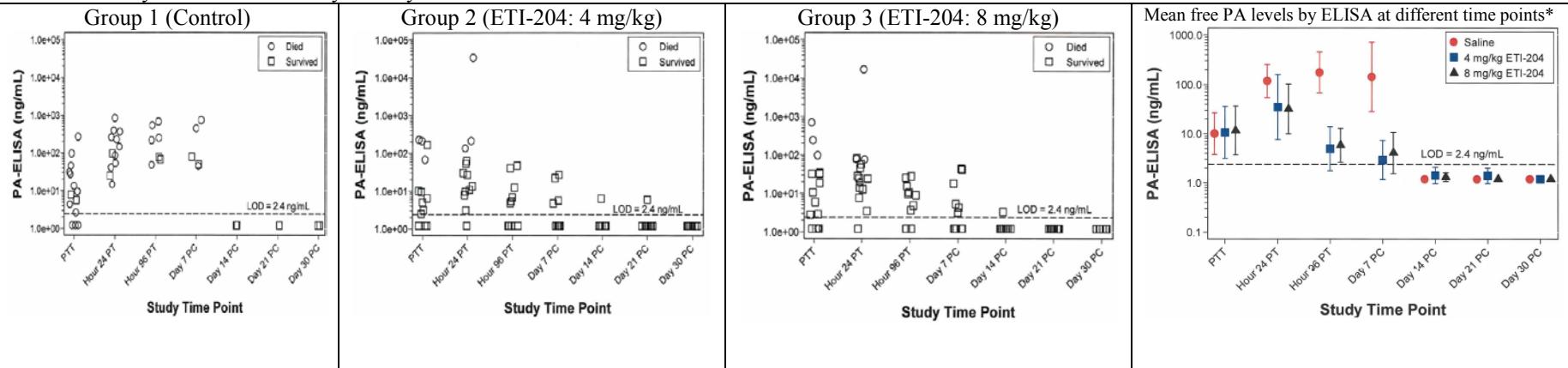
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Figure 31: Study AP201 - Observed bacteremia and PA levels over time in survivors and non-survivors

A: Bacteremia levels versus time point by survival status



B: PA levels by ELISA versus study time by survival status



PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge

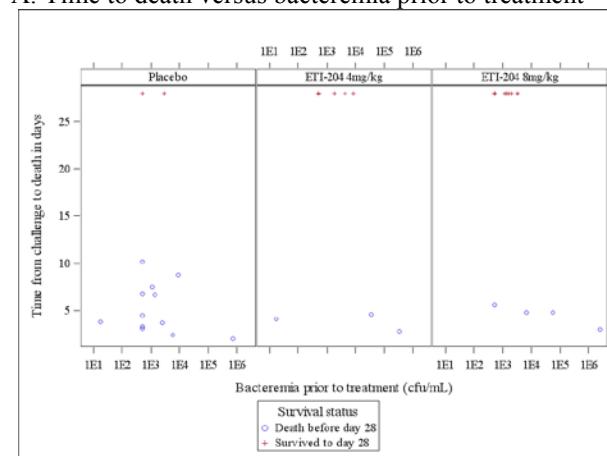
Animal C36338 had a positive PA-ELISA value prior to challenge. Since this animal was neither bacteremic nor positive for PA in the PA-ECL assay prior to challenge, this may indicate that the animal had a serum component(s) that cross-reacted with the PA-ELISA assay and, therefore, this animal was excluded from all analyses involving PA-ELISA

*Note: Confidence intervals not included for time points with two or less values (note: after Day 10 post-challenge there were only two surviving animals in the Saline group).

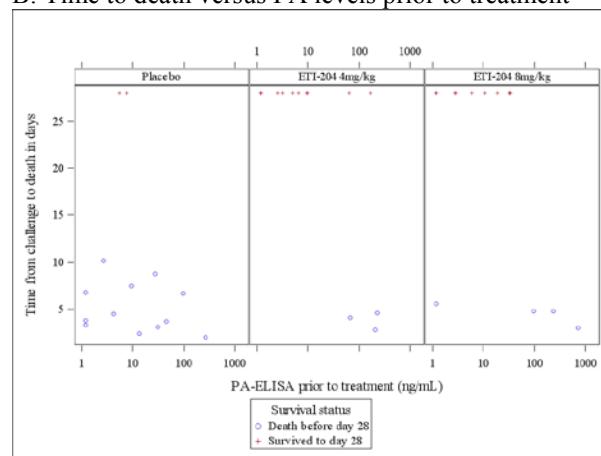
Source: BLA submission

Figure 32: Study AP201 - Relationship between microbial burden prior to treatment and time to death

A: Time to death versus bacteremia prior to treatment



B: Time to death versus PA levels prior to treatment

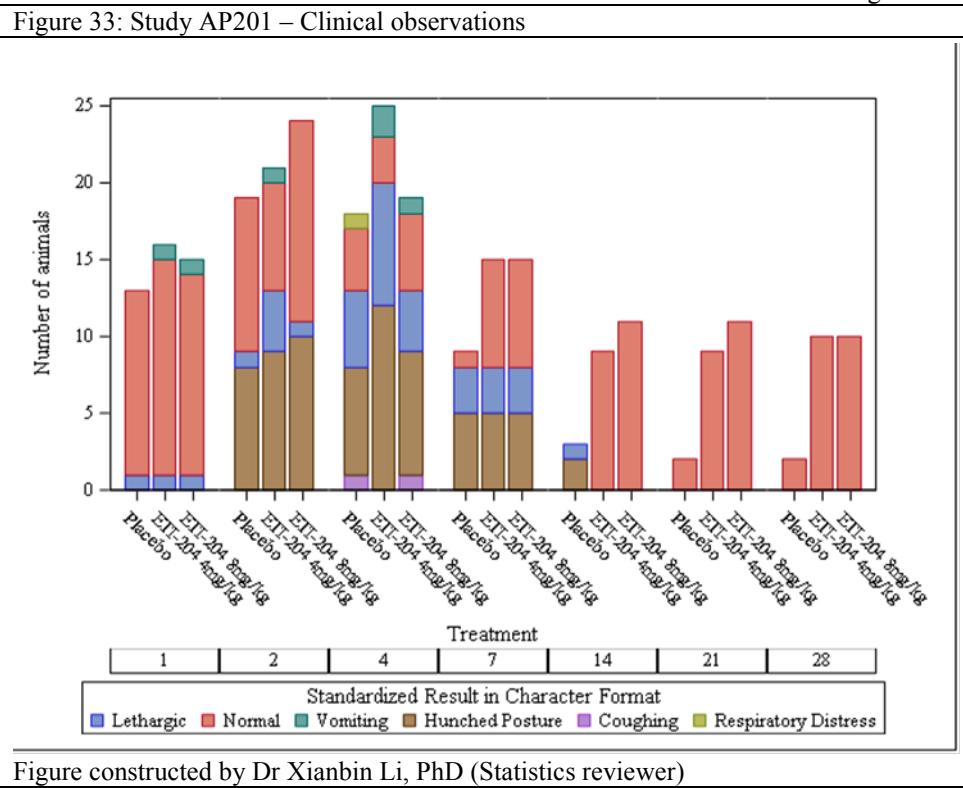


+=Survivors to Day 28; ○=Death before Day 28

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Hematological parameters: WBC count increased following challenge in all animals, as was reported for the natural history studies summarized above. Maximum increase occurred 24 hours post-treatment. CRP levels were elevated at most time points including post-treatment 24 and 96 hour time points in comparison with Day -7 values. Recovery or a trend towards recovery was evident for all of the WBC changes by Day 30 post challenge.

Clinical Observations: Clinical observations post-challenge were consistent with those reported in the natural history studies summarized above. There was no difference in increase in temperature (SIBT; measured as for the natural history studies) among the animals in the three groups prior to treatment and resolution of temperature post-treatment. Animals that survived following treatment were documented as having hunched posture, lethargic and not eating. Surviving animals treated with ETI-204 returned to normal (generally between 8-10 day post-challenge) but still had the occasional inappetence and/or diarrhea/soft stool notation, which is common with laboratory housed nonhuman primates (Figure 33).



In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included discoloration of the brain, lung, spinal cord and/or enlarged lymph nodes (bronchial and/or renal), and/or fluid in the thoracic or abdominal cavities (Table 48). These lesions are typical of acute fulminant anthrax in NHPs and similar to those reported in the natural history studies summarized above.

Monkeys that were found dead or were euthanized in moribund condition all had one or more microscopic lesions consistent with anthrax. Monkeys that survived until Day 30 had no findings attributable to anthrax or ETI-204 administration. Inflammatory reaction was more in animals treated with ETI-204 compared to the control animals that died (Table 48). Such an effect may be due to immune response to *B. anthracis* infection.

Neuropathological evaluations: The areas of the brain that were most affected tended to be those with the greatest surface area (cerebrum and cerebellum) and therefore with the most exposure to the meninges. Microscopic changes, indicative of a response by the host animal (inflammation including vasculitis and hemorrhage), were more pronounced in ETI-204 treated animals as compared to the vehicle controls. Bacteria were more commonly observed compared to the ETI-204-treated animals (4 or 8 mg/kg) among the non-surviving animals.

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Table 48: Study AP201- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)
Brain			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	2/14 (NA)	2/14 (NA)	3/15 (NA)
Microscopic Finding ^{2,3}			
# Necropsied/Total Infected	14/14	14/14	15/15
Brain, basal nuclei: Blood vessels, bacteria	3/14 (3.00)	1/14 (2.00)	2/15 (4.00)
Brain, basal nuclei: Hemorrhage(s)	0/14	0/14	1/15 (3.00)
Brain, basal nuclei: Mineralization	0/14	1/14 (3.00)	0/15
Brain, basal nuclei: Perivascular, inflammation	0/14	2/14 (2.00)	1/15 (4.00)
Brain, basal nuclei: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	0/15
Brain, basal nuclei: Perivascular, inflammation, 30%granulocytes	0/14	1/14 (NA)	0/15
Brain, basal nuclei: Perivascular, inflammation, 50%granulocytes	0/14	1/14 (NA)	0/15
Brain, basal nuclei: Perivascular, inflammation, 50%mononuclear cells	0/14	1/14 (NA)	0/15
Brain, basal nuclei: Perivascular, inflammation, 70%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, basal nuclei: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	0/15
Brain, basal nuclei: Perivascular, pigment	0/14	1/14 (3.00)	0/15
Brain, basal nuclei: Vasculitis	0/14	1/14 (3.00)	1/15 (4.00)
Brain, cerebellum: Blood vessels, bacteria	1/14 (3.00)	1/14 (3.00)	1/15 (2.00)
Brain, cerebellum: Hemorrhage(s)	1/14 (3.00)	2/14 (3.00)	3/15 (3.00)
Brain, cerebellum: Molecular layer, vacuoles	0/14	0/14	1/15 (3.00)
Brain, cerebellum: Perivascular giosis	0/14	0/14	1/15 (2.00)
Brain, cerebellum: Perivascular, inflammation	0/14	1/14 (2.00)	3/15 (2.67)
Brain, cerebellum: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)
Brain, cerebellum: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	1/15 (NA)
Brain, cerebellum: Perivascular, inflammation, 50%granulocytes	0/14	0/14	1/15 (NA)
Brain, cerebellum: Perivascular, inflammation, 50%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, cerebellum: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	1/15 (NA)
Brain, cerebellum: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, cerebellum: Vasculitis	0/14	1/14 (2.00)	1/15 (3.00)
Brain, cerebral cortexmotor/somatosensory/parietal: Blood vessels, bacteria	3/14 (3.00)	0/14	2/15 (3.00)
Brain, cerebral cortexmotor/somatosensory/parietal: Hemorrhage(s)	0/14	0/14	3/15 (3.33)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, infiltrates, mononuclear cells	0/14	0/14	1/15 (3.00)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation	0/14	2/14 (3.00)	3/15 (3.00)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	0/15
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 50%granulocytes	0/14	1/14 (NA)	2/15 (NA)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 50%mononuclear cells	0/14	1/14 (NA)	2/15 (NA)
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	0/15
Brain, cerebral cortexmotor/somatosensory/parietal: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, cerebral cortexmotor/somatosensory/parietal: Vacuoles	0/14	0/14	1/15 (3.00)
Brain, cerebral cortexmotor/somatosensory/parietal: Vasculitis, multifocal	0/14	2/14 (3.00)	3/15 (2.33)
Brain, hippocampus: Blood vessels, bacteria	1/14 (3.00)	0/14	1/15 (4.00)
Brain, hippocampus: Hemorrhage(s)	0/14	0/14	2/15 (2.50)
Brain, hippocampus: Perivascular, inflammation	0/14	0/14	3/15 (2.67)
Brain, hippocampus: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)
Brain, hippocampus: Perivascular, inflammation, 50%granulocytes	0/14	0/14	2/15 (NA)
Brain, hippocampus: Perivascular, inflammation, 50%mononuclear cells	0/14	0/14	2/15 (NA)
Brain, hippocampus: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, hippocampus: Vasculitis	0/14	0/14	2/15 (2.50)
Brain, medulla oblongata: Blood vessels, bacteria	1/14 (3.00)	1/14 (3.00)	0/15
Brain, medulla oblongata: Perivascular, inflammation	0/14	1/14 (3.00)	0/15
Brain, medulla oblongata: Perivascular, inflammation, 40%granulocytes	0/14	1/14 (NA)	0/15
Brain, medulla oblongata: Perivascular, inflammation, 60%mononuclear cells	0/14	1/14 (NA)	0/15
Brain, meninges: Bacteria	7/14 (3.00)	3/14 (3.67)	4/15 (4.25)
Brain, meninges: Hemorrhage(s)	1/14 (4.00)	2/14 (3.50)	4/15 (3.00)
Brain, meninges: Infiltrates, mononuclear cells	2/14 (3.00)	1/14 (3.00)	4/15 (3.00)
Brain, meninges: Inflammation	1/14 (4.00)	2/14 (4.00)	4/15 (4.25)
Brain, meninges: Inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)
Brain, meninges: Inflammation, 20%mononuclear cells	0/14	0/14	2/15 (NA)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at █ (b) (4)

²Neuropathology performed at █ (b) (4)

³All microscopic findings were graded at ⁽¹⁾⁽⁴⁾ according to the following scale, with the associated numerical score:
 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at █ (b) (4) according to the following scale, with the associated numerical score:
 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 48 (continued): Study AP201- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Placebo n/N (*)	Treatment Group		
		4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)	1/14 (NA)
Brain, meninges: Inflammation, 40%granulocytes	0/14	1/14 (NA)	1/15 (NA)	
Brain, meninges: Inflammation, 50%granulocytes	1/14 (NA)	1/14 (NA)	0/15	
Brain, meninges: Inflammation, 50%mononuclear cells	1/14 (NA)	1/14 (NA)	0/15	
Brain, meninges: Inflammation, 60%mononuclear cells	0/14	1/14 (NA)	1/15 (NA)	
Brain, meninges: Inflammation, 80%granulocytes	0/14	0/14	2/15 (NA)	
Brain, meninges: Inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, meninges: Thrombosis	0/14	0/14	1/15 (3.00)	
Brain, meninges: Vasculitis	1/14 (3.00)	2/14 (3.00)	3/15 (2.33)	
Brain, midbrain: Blood vessels, bacteria	1/14 (3.00)	0/14	1/15 (4.00)	
Brain, midbrain: Hemorrhage(s)	0/14	0/14	1/15 (3.00)	
Brain, midbrain: Perivascular, inflammation	0/14	1/14 (3.00)	1/15 (3.00)	
Brain, midbrain: Perivascular, inflammation, 20%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, midbrain: Perivascular, inflammation, 40%granulocytes	0/14	1/14 (NA)	0/15	
Brain, midbrain: Perivascular, inflammation, 60%mononuclear cells	0/14	1/14 (NA)	0/15	
Brain, midbrain: Perivascular, inflammation, 80%granulocytes	0/14	0/14	1/15 (NA)	
Brain, midbrain: Vasculitis	0/14	0/14	1/15 (3.00)	
Brain, occipital/visual cortex: Hemorrhage(s)	0/14	0/14	2/15 (2.50)	
Brain, occipital/visual cortex: Perivascular, inflammation	0/14	1/14 (2.00)	3/15 (2.67)	
Brain, occipital/visual cortex: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)	
Brain, occipital/visual cortex: Perivascular, inflammation, 40%granulocytes	0/14	1/14 (NA)	0/15	
Brain, occipital/visual cortex: Perivascular, inflammation, 50%granulocytes	0/14	0/14	2/15 (NA)	
Brain, occipital/visual cortex: Perivascular, inflammation, 50%mononuclear cells	0/14	0/14	2/15 (NA)	
Brain, occipital/visual cortex: Perivascular, inflammation, 60%mononuclear cells	0/14	1/14 (NA)	0/15	
Brain, occipital/visual cortex: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, occipital/visual cortex: Vacuoles	0/14	0/14	1/15 (3.00)	
Brain, occipital/visual cortex: Vasculitis	0/14	1/14 (2.00)	1/15 (2.00)	
Brain, pons region/pontine nuclei: Blood vessels, bacteria	1/14 (2.00)	0/14	0/15	
Brain, pons region/pontine nuclei: Perivascular, Inflammation	0/14	0/14	1/15 (3.00)	
Brain, pons region/pontine nuclei: Perivascular, inflammation	0/14	0/14	1/15 (2.00)	
Brain, pons region/pontine nuclei: Perivascular, inflammation, 100%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, pons region/pontine nuclei: Perivascular, inflammation, 50%granulocytes	0/14	0/14	1/15 (NA)	
Brain, pons region/pontine nuclei: Perivascular, inflammation, 50%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, pons region/pontine nuclei: Vasculitis	0/14	0/14	1/15 (2.00)	
Brain, prefrontal/cingulate/premotor cortex: Blood vessels, bacteria	2/14 (2.50)	1/14 (3.00)	3/15 (3.33)	
Brain, prefrontal/cingulate/premotor cortex: Hemorrhage(s)	0/14	0/14	3/15 (3.00)	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation	1/14 (3.00)	2/14 (3.00)	4/15 (2.75)	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation, 100%mononuclear cells	1/14 (NA)	0/14	1/15 (NA)	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	0/15	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation, 50%granulocytes	0/14	1/14 (NA)	3/15 (NA)	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation, 50%mononuclear cells	0/14	1/14 (NA)	3/15 (NA)	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	0/15	
Brain, prefrontal/cingulate/premotor cortex: Perivascular, pigment	0/14	1/14 (3.00)	1/15 (2.00)	
Brain, prefrontal/cingulate/premotor cortex: Vasculitis, multifocal	0/14	2/14 (3.00)	3/15 (2.67)	
Brain, temporal lobe: Blood vessels, bacteria	1/14 (3.00)	0/14	1/15 (4.00)	
Brain, temporal lobe: Hemorrhage(s)	0/14	1/14 (3.00)	2/15 (3.00)	
Brain, temporal lobe: Perivascular, inflammation	0/14	2/14 (2.50)	3/15 (2.33)	
Brain, temporal lobe: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)	
Brain, temporal lobe: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	1/15 (NA)	
Brain, temporal lobe: Perivascular, inflammation, 50%granulocytes	0/14	1/14 (NA)	1/15 (NA)	
Brain, temporal lobe: Perivascular, inflammation, 50%mononuclear cells	0/14	1/14 (NA)	1/15 (NA)	
Brain, temporal lobe: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	1/15 (NA)	
Brain, temporal lobe: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)	
Brain, temporal lobe: Vacuolation	0/14	0/14	1/15 (3.00)	
Brain, temporal lobe: Vasculitis	0/14	1/14 (3.00)	3/15 (2.33)	
Brain, thalamus/hypothalamus: Blood vessels, bacteria	2/14 (3.00)	0/14	2/15 (4.00)	
Brain, thalamus/hypothalamus: Hemorrhage(s)	0/14	0/14	1/15 (3.00)	
Brain, thalamus/hypothalamus: Mineralization	0/14	1/14 (3.00)	0/15	
Brain, thalamus/hypothalamus: Perivascular hemosiderin	0/14	1/14 (3.00)	0/15	
Brain, thalamus/hypothalamus: Perivascular, inflammation	1/14 (3.00)	1/14 (2.00)	2/15 (3.00)	
Brain, thalamus/hypothalamus: Perivascular, inflammation, 20%granulocytes	0/14	0/14	1/15 (NA)	

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:
 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:
 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 48 (continued): Study AP201-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)
Brain, thalamus/hypothalamus: Perivascular, inflammation, 20%mononuclear cells	0/14	1/14 (NA)	1/15 (NA)
Brain, thalamus/hypothalamus: Perivascular, inflammation, 80%granulocytes	0/14	1/14 (NA)	1/15 (NA)
Brain, thalamus/hypothalamus: Perivascular, inflammation, 80%mononuclear cells	0/14	0/14	1/15 (NA)
Brain, thalamus/hypothalamus: Vasculitis	0/14	1/14 (2.00)	2/15 (3.00)
Brain, ventricular system: Choroid plexus, bacteria	4/14 (3.00)	1/14 (2.00)	0/15
Brain, ventricular system: Choroid plexus, infiltrates, mononuclear cell	0/14	1/14 (3.00)	0/15
Brain, ventricular system: Perivascular, inflammation	1/14 (2.00)	0/14	2/15 (2.50)
Brain, ventricular system: Perivascular, inflammation, 30%granulocytes	1/14 (NA)	0/14	0/15
Brain, ventricular system: Perivascular, inflammation, 50%granulocytes	0/14	0/14	2/15 (NA)
Brain, ventricular system: Perivascular, inflammation, 50%mononuclear cells	0/14	0/14	2/15 (NA)
Brain, ventricular system: Perivascular, inflammation, 70%mononuclear cells	1/14 (NA)	0/14	0/15
Brain: Bacteria ^{1,4}	6/14 (2.33)	3/14 (2.67)	4/15 (3.00)
Brain: Hemorrhage(s) ^{1,4}	1/14 (3.00)	2/14 (2.50)	3/15 (3.00)
Brain: Inflammation ^{1,4}	1/14 (3.00)	2/14 (3.00)	3/15 (3.33)
Brain: Vasculitis ^{1,4}	0/14	0/14	2/15 (3.00)
Kidney			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	1/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Bacteria	6/14 (1.83)	1/14 (2.00)	1/15 (3.00)
Infarction	1/14 (2.00)	0/14	0/15
Renal tubular necrosis/mineralization	1/14 (2.00)	0/14	0/15
Liver			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	1/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Bacteria	8/14 (1.75)	1/14 (2.00)	1/15 (1.00)
Necrosis	5/14 (2.00)	1/14 (3.00)	1/15 (3.00)
Protozoan granuloma	2/14 (1.50)	0/14	0/15
Vasculitis	3/14 (2.00)	1/14 (2.00)	0/15
Lymph Node, Bronchial			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	6/14 (NA)	4/14 (NA)	2/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Atrophy, lymphoid follicle(s)	6/14 (4.00)	2/14 (3.50)	2/15 (3.50)
Bacteria	6/14 (1.83)	0/14	1/15 (3.00)
Congestion/hemorrhage	6/14 (3.17)	2/14 (2.00)	2/15 (2.00)
Lymphoid hyperplasia	0/14	2/14 (2.00)	0/15
Necrosis	5/14 (3.40)	0/14	1/15 (3.00)
Lymph Node, Mediastinal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	2/14 (NA)	3/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Atrophy, lymphoid follicle(s)	11/14 (3.73)	4/14 (2.50)	4/15 (4.00)
Bacteria	10/14 (2.20)	2/14 (1.50)	1/15 (2.00)
Congestion/hemorrhage	8/14 (2.50)	2/14 (1.00)	3/15 (2.33)
Fibrin deposition	3/14 (3.00)	0/14	0/15
Lymphoid hyperplasia	0/14	1/14 (2.00)	1/15 (2.00)
Necrosis	8/14 (3.25)	1/14 (2.00)	2/15 (2.50)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 48 (continued): Study AP201-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)
Lung			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	1/14 (NA)	3/14 (NA)	1/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Bacteria	5/14 (2.60)	1/14 (3.00)	1/15 (2.00)
Edema	1/14 (2.00)	2/14 (2.00)	0/15
Fibrosis	0/14	1/14 (1.00)	0/15
Hemorrhage(s), alveolar	0/14	0/14	1/15 (1.00)
Hemorrhage, peribronchial	0/14	1/14 (3.00)	0/15
Inflammation	0/14	1/14 (2.00)	0/15
Spleen			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	0/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	13/14	14/14	15/15
Bacteria	9/13 (2.44)	1/14 (3.00)	1/15 (1.00)
Fibrin deposition, red pulp	3/13 (2.67)	2/14 (2.50)	1/15 (2.00)
Lymphoid atrophy	12/13 (3.00)	3/14 (3.00)	4/15 (3.00)
Necrosis, mononuclear cells, red pulp	6/13 (3.50)	1/14 (3.00)	1/15 (2.00)
Cavity, Abdominal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	4/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	14/14	14/14	15/15
Cavity, Pericardial			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	1/14 (NA)	1/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	14/14	14/14	15/15
Cavity, Thoracic			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	2/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	14/14	14/14	15/15
Cecum			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	0/14 (NA)	0/14 (NA)	1/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Acute hemorrhage(s), muscularis and submucosa	0/14	0/14	1/15 (3.00)
Thymus			
Macroscopic Finding ¹			
# Necropsied/Total Infected	14/14	14/14	15/15
Gross Lesions	1/14 (NA)	0/14 (NA)	0/15 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	14/14	14/14	15/15
Edema, perithymic tissue	1/14 (2.00)	0/14	0/15

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Tissue bacterial assessments:

Histology: No bacteria were observed in any of the tissues from surviving animals. However, bacteria were observed in a majority of the tissues from non-survivors (Table 49).

Culture: Of the 19 animals that died on study, 95% (18/19) of the animals had a positive culture finding for either the spleen or bronchial/mediastinal lymph node. Seventy-nine percent (15/19) of the animals were culture positive for bronchial/mediastinal lymph node while 74% (14/19) of the animals had a positive bacteremia results for spleen. All of the spleen or lymph nodes from surviving animals were culture negative (Table 49).

Table 49: Study AP201 - Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 4mg/kg		ETI-204 8mg/kg	
	Survivors (N=2)	Non Survivors (N=12)	Survivors (N=11)	Non Survivors (N=3)	Survivors (N=11)	Non Survivors (N=4)
Presence of bacteria by microscopy [1, 5]						
Brain [3]						
Brain: Total [4] (Tox Path)	0/2	7/12	0/11	3/3	0/11	4/4
Brain, basal nuclei: Blood vessels (Tox Path)	0/2	3/12	0/11	1/3	0/11	2/4
Brain, cerebellum: Blood vessels (Tox Path)	0/2	1/12	0/11	1/3	0/11	1/4
Brain, cerebral cortex/motor/somatosensory/parietal: Blood vessels (Tox Path)	0/2	3/12	0/11	0/3	0/11	2/4
Brain, hippocampus: Blood vessels (Tox Path)	0/2	1/12	0/11	0/3	0/11	1/4
Brain, medulla oblongata: Blood vessels (Tox Path)	0/2	1/12	0/11	1/3	0/11	0/4
Brain, meninges: Bacteria (Tox Path)	0/2	7/12	0/11	3/3	0/11	4/4
Brain, midbrain: Blood vessels (Tox Path)	0/2	1/12	0/11	0/3	0/11	1/4
Brain, pons region/pontine nuclei: Blood vessels (Tox Path)	0/2	1/12	0/11	0/3	0/11	0/4
Brain, prefrontal/cingulate/premotor cortex: Blood vessels (Tox Path)	0/2	2/12	0/11	1/3	0/11	3/4
Brain, temporal lobe: Blood vessels (Tox Path)	0/2	1/12	0/11	0/3	0/11	1/4
Brain, thalamus/hypothalamus: Blood vessels (Tox Path)	0/2	2/12	0/11	0/3	0/11	2/4
Brain, ventricular system: Choroid plexus (Tox Path)	0/2	4/12	0/11	1/3	0/11	0/4
Brain: Bacteria (Batelle)	0/2	6/12	0/11	3/3	0/11	4/4
Kidney	0/2	6/12	0/11	1/3	0/11	1/4
Liver	0/2	8/12	0/11	1/3	0/11	1/4
Lymph Node, Bronchial	0/2	6/12	0/11	0/3	0/11	1/4
Lymph Node, Mediastinal	0/2	10/12	0/11	2/3	0/11	1/4
Lung	0/2	5/12	0/11	1/3	0/11	1/4
Spleen	0/1	9/12	0/11	1/3	0/11	1/4
Cavity, Abdominal	0/2	0/12	0/11	0/3	0/11	0/4
Cavity, Pericardial	0/2	0/12	0/11	0/3	0/11	0/4
Cavity, Thoracic	0/2	0/12	0/11	0/3	0/11	0/4
Cecum	0/2	0/12	0/11	0/3	0/11	0/4
Thymus	0/2	0/12	0/11	0/3	0/11	0/4
Presence of bacteria by culture [1, 2]						
Lymph Node, Bronchial	0/2	11/12	0/11	2/3	0/11	2/4
Spleen	0/2	9/12	0/11	1/3	0/11	4/4
[1] All treated animals irrespective of bacteremia status prior to treatment						
[2] Animal was considered positive if at least 1-5 colonies were present on plate						
[3] Neuropathology performed at (b) (4) and (b) (4)						
[4] Animals that were positive in at least one area for either extra- or intra-vascular bacteria						
[5] Histopathology performed at (b) (4) Not all animal were assessed microscopically; numbers examined are shown						
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: None						
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None						
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: C38277						

Comments:

The study showed that ETI-204 at a dose of 4 or 8 mg/kg was effective in improving survival in approximately 75% of the animals up to Day 28; 2 (14%) of the animals in the control group

survived. All of the non-surviving animals died within 7 days of challenge. Bacteremia and PA levels decreased after treatment with ETI-204.

All animals were bacteremic or PA positive at the time of initiation of treatment. Higher bacteremia levels and higher PA levels at the time of initiation of treatment appear to be associated with a lower survival rate. The study suggests that an animal with a higher level of bacteremia or PA prior to treatment is more likely to die after treatment with ETI-204.

Among monkeys that were found dead or euthanized, gross lesions were consistent with acute *B. anthracis* infection; additionally, the spleen and lymph node tissues from animals found dead or euthanized were positive for bacteria. No gross lesions were found in surviving animals; no bacteria were observed in any of the tissues examined by microscopy or culture (Table 49). Lungs were not tested by bacterial culture.

All of the non-surviving animals that were examined for neuropathology had intravascular and/or extravascular bacteria, suggesting the cause of death in these animals was a bacterial infection. Only ETI-204-treated non-surviving animals had evidence of an inflammatory response, and always in association with extravascular bacteria. None of the deaths were attributed to ETI-204.

6.2.2.3. Study AP203

This was a randomized, blinded, placebo-controlled GLP study to evaluate the efficacy of 8 mg/kg and 32 mg/kg dose of ETI-204 (Lonza product) administrated IV, to 50 cynomolgus monkeys challenged with *B. anthracis* spores (spore lot no. B37) by inhalational route.⁴⁸ Two animals served as replacements however, these animals were not used in the study [animal C38804 (diagnosed with *Trichomonas* by urinalysis) was listed as the extra female and animal C47858 (over the 5.0 kg protocol-defined weight limit) was listed as the extra male]. The study design was similar to that for Study AP202 except that ELISA for measuring free PA levels in serum was performed at [REDACTED]^{(b)(4)} the assay used for quantitation of free PA in serum was different from that used for Study AP202. Additionally, anti-ETI-204 antibodies were measured in serum at the end of the study, in addition to baseline measurements (Table 50). Also, CRP levels and neuropathological evaluations were performed. Anti-PA antibodies were not measured.

The age of the animals ranged between 3 and 5 years and body weight between 3.0 and 4.8 kg at the time of randomization. Monkeys were quarantined for 5.7 weeks at [REDACTED]^{(b)(4)} and confirmed to be in good health, free of malformations, free of intestinal parasites, and exhibiting no signs of clinical disease by the study veterinarian. None of the animals included in this study were stated to have *Klebsiella* infection.

ETI-204 or control material (saline) was administered post-challenge within 3 hours of determining a positive serum PA-ECL screening assay.

⁴⁸ [REDACTED]^{(b)(4)} Study Number 1219-100005989: AP203: Evaluating the efficacy of intravenously ETI-204 when administered therapeutically in the cynomolgus macaque with inhalational anthrax (December 20, 2012).

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Table 50: AP203 - Blood collection and assay schedule

Approximate Time Point	Blood Tube type/ Approximate Blood volume	Bacteremia (Culture)	CBC/ CRP	Serum for PA-ECL	Serum PA levels (via ELISA)	Serum for ETI-204 PK	Serum for Anti- ETI-204 Antibodies
Day -6	EDTA ~1.5 mL SST ~3.0 mL	X	X	X	X	X	X
^24hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
^30hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
^36hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
^42hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
^48hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
^54hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
prior to treatment ^a	EDTA ~0.5 mL SST ~1.5 mL SPS ~1.0 mL	X [#]		X	X		
15 min PT	SST ~1.0 mL				X	X	
2hr PT	SST ~1.0 mL					X	
6hr PT	SST ~2.0 mL					X	
24hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
48hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
96hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
5 days PT	SST ~1.5 mL					X	
7 days PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
16 days PC	EDTA ~1.5 mL SST ~1.5 mL	X	X		X	X	
23 days PC	EDTA ~1.5 mL SST ~4.0 mL	X	X		X	X	
28 days PC	EDTA ~1.5 mL SST ~4.0 mL	X	X		X	X	X
Terminal ^{b, c}	EDTA ~1.5 mL SST ~4.0 mL	X	CRP only		X	X	X

PC = Post-Challenge, PT = Post-Treatment

^a = Post-challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort. Post-treatment bleed times were calculated from the time each animal's IV treatment ends. Blood specimens occurred within ± 60 minutes of the calculated time, except for the 15 min PT, 2hr PT, 6hr PT and 96hr PT specimens which occurred within 5 min, 10 min, 15 min and 3 hours of their calculated times respectively. The Day -6, Day 5 PT, Day 7 PT, Day 16 PC, Day 23 PC and Day 28 PC bloods were relative to the day of treatment or challenge.

^b = When collection was possible.

^c = Terminal specimens were not collected on animals euthanized at the end of study as Day 28 PC's specimens were already scheduled.

[#] = Post-Challenge, pre-treatment sampling stopped once decision to treat had been made.

[#] = prior to treatment Bacteremia enrichment performed on specimen collected in SPS tube (see section 14.3.)

Results:

Baseline characteristics: All animals were culture negative as well as PA negative by the ECL assay and ELISA on Day-6 i.e., prior to challenge. Age, gender, body weight, and challenge dose (mean LD₅₀ 289; ^{(b) (4)} cfu) were comparable among the animals in the three groups; the LD₅₀ dose was ≥ 200 for approximately 94% of the animals and was comparable among the three groups (Table 51). The MMAD for each exposure day ranged from 1.17 - 1.24 μm which is consistent with the particle size range that would reach the alveoli.

All animals were PA positive by ECL assay between 24 and 48 hours post-challenge (Table 51). However, by ELISA one animal in the control group, one treated with ETI-204 8 mg/kg and 2 animals treated with ETI-204 32 mg/kg treated group were negative for PA (Table 51). All animals were bacteremic between 24 and 42 hours post-challenge (Table 51). At the time of initiation of therapy, PA and bacteremia levels were higher in animals treated with 8 mg/kg ETI-204 compared to the other two groups (Table 51). There was a positive correlation between quantitative bacteremia and free PA levels at 36 hours post-challenge, prior to treatment, 24 hours post-treatment, and 48 hours post-treatment suggesting a linear relationship between quantitative bacteremia and PA levels.

The mean time to trigger (about 33 hours) and treatment (37 hours) was similar in all the three groups (Table 51). The microbial burden (bacteremia and PA levels) were higher in the Group 2 animals compared to the other two groups.

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Table 51: Study AP203 - Baseline characteristics, inhaled dose of <i>B. anthracis</i> , time to treatment and survival			
Parameters	Placebo (Group 1) N=16	ETI-204: 8 mg/kg (Group 2) N=16	ETI-204: 32 mg/kg (Group 3) N=16
Baseline characteristics			
Age (years) Mean ± SD	4.4±0.6	4.3±0.6	4.4±0.6
Body weight (kg) Mean ± SD	3.88±0.56	3.83±0.64	3.99±0.62
Total Inhaled Dose: cfu x 10 ⁷ Mean ± SD (Range)			(b) (4)
LD ₅₀ dose Mean ± SD (Range)	294.6±76.7 (166-462)	279.4±59.2 (160-384)	291.8±79.7 (185-430)
<200 LD ₅₀ dose n (%)	1 (6.3)	1 (6.3)	1 (6.3)
≥200 LD ₅₀ dose n (%)	15 (93.8)	15 (93.8)	15 (93.8)
Trigger (PA by ECL) for treatment^a and microbial burden prior to treatment			
Positive screening PA/ECL Assay n (%)	16 (100)	16 (100)	16 (100)
Bacteremia (cfu/mL)			
Enriched (qualitative) bacteremia n (%)	16 (100)	16 (100)	16 (100)
Quantitative bacteremia n (%)	16 (100)	16 (100)	15 (93.8)*
Log ₁₀ bacteremia (cfu/mL) Mean±SD (Range)	4.77±1.08 (3.21-6.93)	5.07±1.30 (3.26-7.72)	4.39±1.66 (2.18-6.61)
Quantitative (cfu/mL x 10 ⁴) Geometric mean	5.9	11.9 [‡]	2.5
PA levels by ELISA (ng/mL)			
Log ₁₀ ±SD	1.89±0.72	2.12±0.87	1.96±0.75
Geometric mean	77.6	133.3	90.3
Time (hours) between challenge, trigger, and treatment			
Time to trigger (PA ^{+ve}) post challenge ^a Mean±SD (Range)	33.3±4.7 (27.9-45.1)	32.5±5.5 (22.8-45.5)	33.4±4.2 (28.5-42.7)
Time to bacteremia Mean±SD (Range)	29.98±4.9 (22.7-39.2)	28.3±5.0 (22.2-37.3)	29.9±4.7* (22.4-39.2)
Time to treatment ^b Mean±SD (Range)	37.1±4.2 (32.4-47.4)	36.2±5.2 (26.3-47.5)	37.5 ±4.0 (32.6-46.5)
Time from trigger to treatment	3.8±0.6	3.8±0.7	4.1±0.4
Survivors at the end of study (Day 28)			
Survivors*	2 (12.5)	1 (6.3)	6 (37.5)
Survivors in bacteremic animals*	2 (12.5)	1 (6.3)	5/15 (33.3)

[‡]Animal C40915 treated with 32 mg/kg had no bacteremia count and not included in the calculation of cfu. If this animal was excluded, the values were 4.64x10⁴ [8.97x10³, 2.40x10⁵]. However, by enriched culture, the animal was bacteremic prior to treatment.

SD Standard deviation

PTT Prior to treatment; Quantitative bacteremia LOD Limit of detection=3 cfu/mL; PA ELISA LLOQ Lower limit of quantification=9.68 ng/mL.

^a The trigger for treatment was defined as the time from challenge to a positive PA-ECL post-challenge.

^b The time to treatment was defined as the time from challenge to treatment.

*No statistically significant between the ETI-204 treated animals and the control group by exact method and Boschloo's one-sided test.

Detection of Bacteremia and PA: The variability among the results of two bacterial culture methods and the detection of PA by ECL and ELISA are shown in Table 52. The samples used for these assays were collected at the same time points post-challenge. Of the 48 monkeys in the study, all animals were positive by the qualitative enriched culture method and 47 (98%) were positive by the quantitative culture method. All animals were PA positive by the ECL assay and 44 (91.7%) by ELISA. Both enriched and quantitative culture methods for measuring

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bacteremia were equally sensitive. However, ECL assay was more sensitive than the ELISA for detection of PA.

Table 52: Study AP203 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture	Qualitative Enriched Culture*	Detection Method				Treatment Group			Total (n=48)
		Quantitative Culture‡	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)‡	Placebo (n=16)	8 mg/kg ETI-204 (n=16)	32 mg/kg ETI-204 (n=16)		
ND	+	+	+	+	15	15	14	44	
ND	+	+	+	-	1	1	1	3	
ND	+	-	+	-	0	0	1	1	

n = Number of treated animals. Results determined on a per animal basis, not for individual tests.
Animal with a positive test from any time prior to treatment is considered as positive.
ND = Not Done
Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative.
LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL); LOD 4 ng/mL
‡LLOQ by quantitative culture 100 cfu/mL; LLOQ by free PA ELISA 9.68 ng/mL

Effect of treatment on survival and microbial burden: The results show that ETI-204 at a dose of 32 mg/kg was more effective in improving survival compared to the lower dose (8 mg/kg) and the control group (Figure 34 and Table 51); however, the differences were not statistically significant. All the non-surviving animals died within 7 days of challenge.

Of the 16 animals in the control group, two (C49041 and C49058) survived the period of observation. Both animals were PA positive by the ECL assay and were bacteremic at the time of treatment. PA by ELISA was below the LLOQ for one animal (C49058) at the time of trigger. However, animal C49058 was PA positive between 15 minutes and 96 hours post-treatment. Both animals were PA and culture negative at Day 7 post-treatment and remained negative until the end of study. Brain of one of the surviving animal (C49041) was culture positive.

The PA and bacteremia levels decreased after initiation of treatment (Figures 34 and 35). PA was undetectable in a majority of the animals within 15 minutes of treatment. All animals, except one (C48922) treated with 32 mg/kg of ETI-204, remained PA negative until Day 28; Animal C48922 became PA negative within 15 minutes of treatment; this was followed by a PA positive finding at Day 7 post-treatment and remained positive until Day 16 post-challenge. Of the 25 animals that died after treatment with ETI-204, PA (107 ng/mL and 13 ng/mL) was detected by ELISA in only 2 animals (C44167 and C49020) at 15 minutes post-treatment.

All animals treated with ETI-204 that survived to the end of the study were culture negative by Day 4 following treatment (Figure 34 and 35).

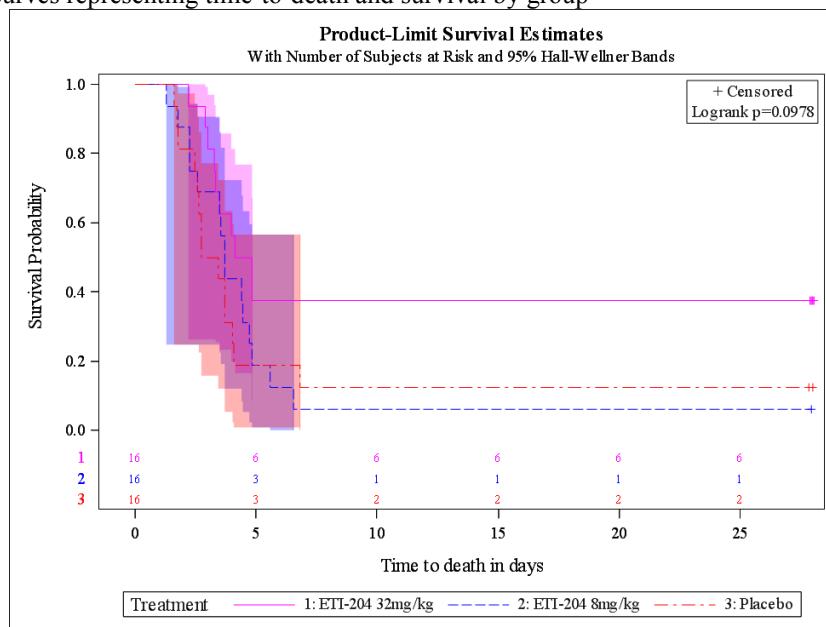
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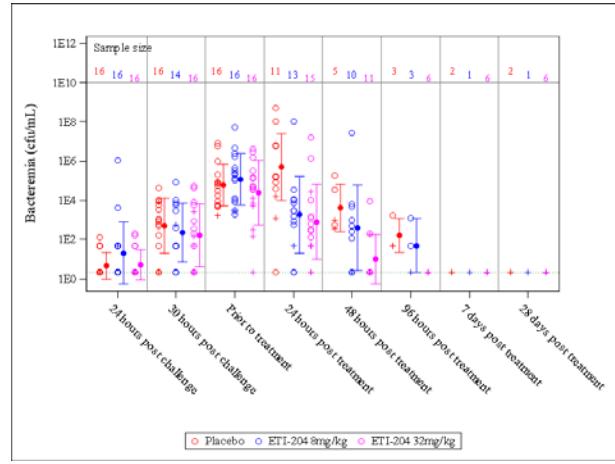
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Figure 34: Study AP203 - Survival and microbial burden at different time points post-challenge

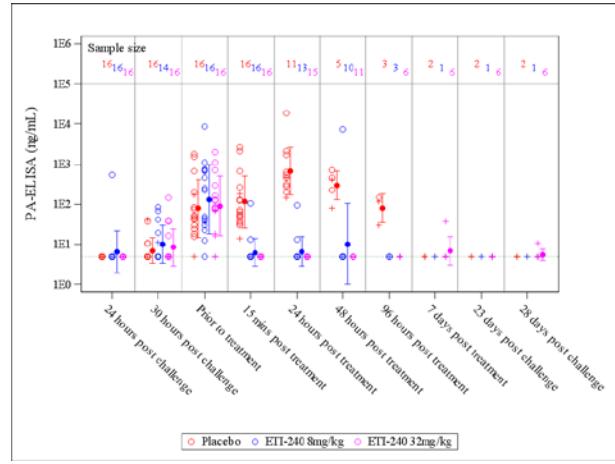
A: Kaplan-Meier curves representing time-to-death and survival by group



B: Bacteremia



C: PA levels



+=Survivors to Day 28; ○=Death before Day 28

Source: Statistics review

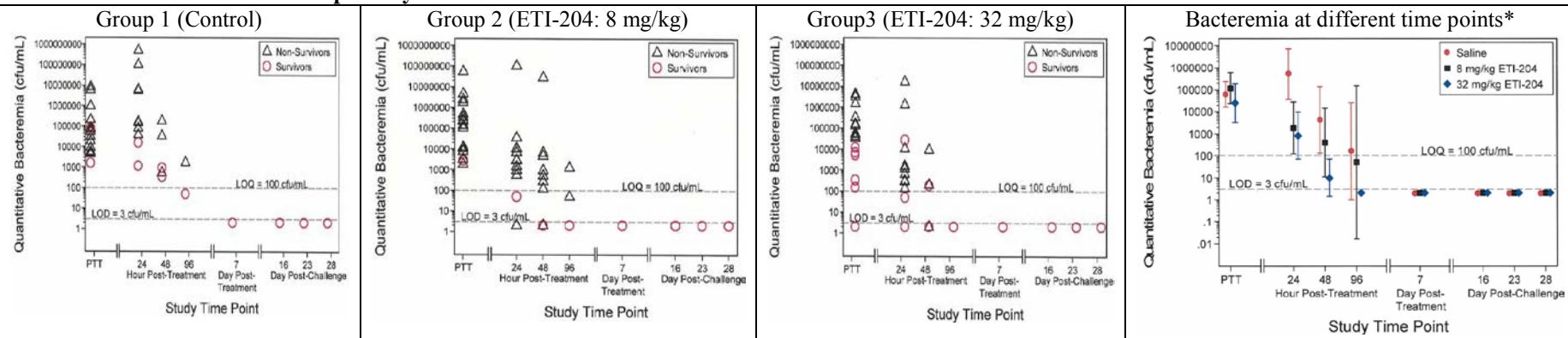
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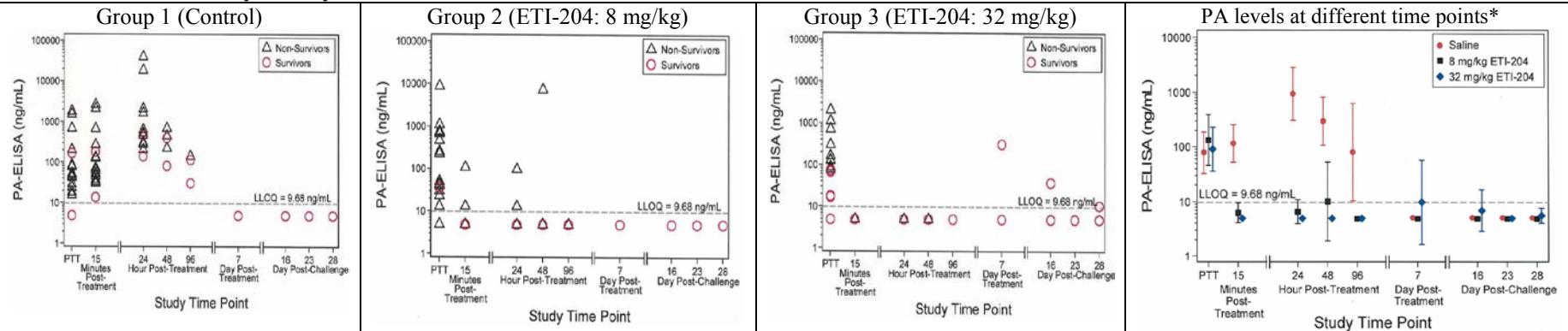
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Figure 35: Study AP203 - Bacteremia and free PA levels over time in survivors and non-survivors.

A: Bacteremia levels versus time point by survival status



B: PA levels versus study time by survival status



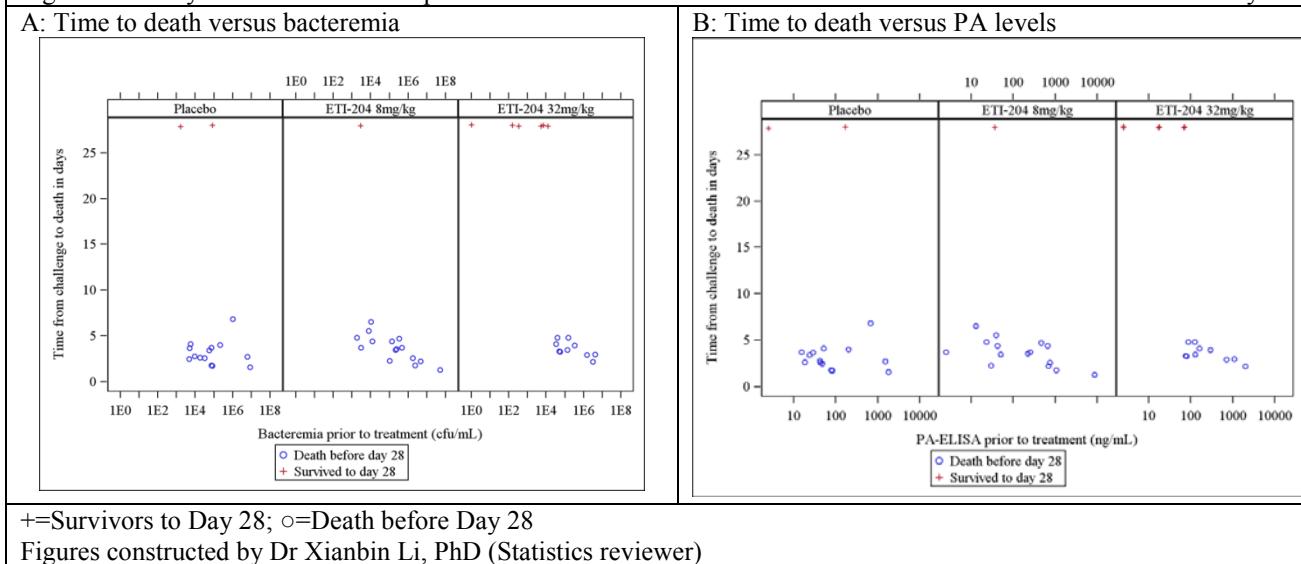
PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge. Results represent geometric means and 95 percent confidence intervals

*Note: Confidence intervals were not plotted or study time points with fewer than 3 observations or if all observations were the same.

Source: BLA submission

Effect of microbial burden on survival: Animals with higher bacteremia and PA levels at the time of initiation of therapy are less likely to survive after treatment with ETI-204 (Figure 36).

Figure 36: Study AP203 - Relationship between microbial burden at the time of treatment and survival status at Day 28



⊕=Survivors to Day 28; ○=Death before Day 28

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Hematological parameters: The hematological parameters were measured at Day-6 and Day 16 post-challenge. All animals had white blood cell counts within the normal range ($6.90\text{-}19.00 \times 10^3/\mu\text{L}$) or only slightly above the normal range on Day -6, except for one animal (C50265) in the control group. There was an extended period of time between the baseline collection for hematology and CRP data and the first post-challenge time point. These results should be interpreted with caution due to infrequent sampling time points to evaluate the effect of anthrax infection and treatment on hematological parameters and CRP.

Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge (Figure 37); these signs were consistent with those reported in the natural history studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is common with laboratory housed non-human primates).

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

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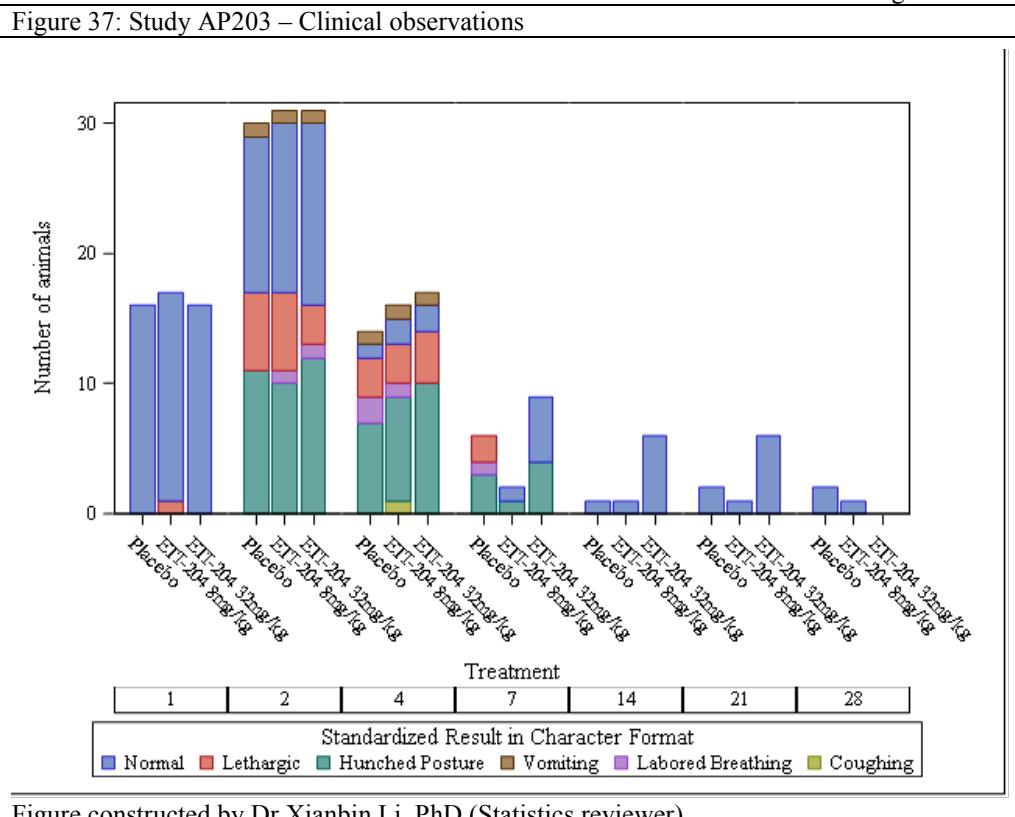


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included inflammation (suppurative to necrotizing), hemorrhage, edema, fibrin exudation, and necrosis (Table 53). Brain discoloration or foci were slightly more common among treated animals than those in the control group that died on study (3/14 for control animals, 12/15 for 8 mg/kg ETI-204 animals, and 5/10 for 32 mg/kg ETI-204 animals). These observations are similar to those observed in the natural history studies summarized above.

Animals surviving until Study Day 28 lacked significant microscopic lesions; some inflammation or lymphoid depletion were reported.

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Table 53: Study AP203-Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	8 mg/kg ETI-204 n/N (*)	32 mg/kg ETI-204 n/N (*)
Brain			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	3/16 (NA)	13/16 (NA)	6/16 (NA)
Microscopic Finding ^{2,3}			
# Necropsied/Total Infected	7/16	6/16	11/16
Brain, basal nuclei/striatum: Bacteria, extravascular	0/ 7	1/ 6 (2.00)	1/11 (2.00)
Brain, basal nuclei/striatum: Bacteria, intravascular	2/ 7 (1.50)	0/ 6	1/11 (2.00)
Brain, basal nuclei/striatum: Blood vessels, leucocytosis	0/ 7	0/ 6	1/11 (1.00)
Brain, basal nuclei/striatum: Gliosis	0/ 7	0/ 6	1/11 (1.00)
Brain, basal nuclei/striatum: Hemorrhage	0/ 7	2/ 6 (3.50)	2/11 (1.50)
Brain, basal nuclei/striatum: Parenchymal, vacuolation	0/ 7	1/ 6 (1.00)	1/11 (1.00)
Brain, basal nuclei/striatum: Perivascular, Infiltrates, mononuclear cells	0/ 7	1/ 6 (2.00)	0/11
Brain, basal nuclei/striatum: Perivascular, infiltrates, mononuclear cells	1/ 7 (2.00)	0/ 6	0/11
Brain, basal nuclei/striatum: Perivascular, inflammation	0/ 7	2/ 6 (2.00)	1/11 (3.00)
Brain, basal nuclei/striatum: Perivascular, inflammation, 100% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, basal nuclei/striatum: Perivascular, inflammation, 30% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, basal nuclei/striatum: Perivascular, inflammation, 50 % mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, basal nuclei/striatum: Perivascular, inflammation, 50%granulocytes	0/ 7	1/ 6 (NA)	0/11
Brain, basal nuclei/striatum: Perivascular, inflammation, 70% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, basal nuclei/striatum: Perivascular, pigment	1/ 7 (2.00)	0/ 6	1/11 (2.00)
Brain, basal nuclei/striatum: Vasculitis	0/ 7	1/ 6 (2.00)	1/11 (3.00)
Brain, cerebellum: Bacteria, extravascular	0/ 7	0/ 6	1/11 (1.00)
Brain, cerebellum: Bacteria, intravascular	1/ 7 (2.00)	0/ 6	0/11
Brain, cerebellum: Gliosis	0/ 7	2/ 6 (1.00)	1/11 (2.00)
Brain, cerebellum: Granular cell layer, neurons decreased	0/ 7	1/ 6 (1.00)	0/11
Brain, cerebellum: Hemorrhage	1/ 7 (2.00)	4/ 6 (1.50)	2/11 (2.50)
Brain, cerebellum: Inflammation	0/ 7	0/ 6	1/11 (2.00)
Brain, cerebellum: Inflammation, 50% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, cerebellum: Inflammation, 50% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, cerebellum: Molecular layer, vacuoles	0/ 7	1/ 6 (1.00)	1/11 (2.00)
Brain, cerebellum: Neuronal necrosis/neuron loss	0/ 7	1/ 6 (2.00)	0/11
Brain, cerebellum: Parenchymal, necrosis	0/ 7	1/ 6 (2.00)	0/11
Brain, cerebellum: Parenchymal, vacuolation	0/ 7	1/ 6 (1.00)	0/11
Brain, cerebellum: Perivascular, inflammation	0/ 7	1/ 6 (2.00)	0/11
Brain, cerebellum: Perivascular, inflammation, 50% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, cerebellum: Perivascular, inflammation, 50%granulocytes	0/ 7	1/ 6 (NA)	0/11
Brain, cerebellum: Vasculitis	0/ 7	1/ 6 (2.00)	0/11
Brain, cerebrum/neocortex: Bacteria, extravascular	0/ 7	2/ 6 (2.00)	2/11 (1.00)
Brain, cerebrum/neocortex: Bacteria, intravascular	3/ 7 (1.33)	1/ 6 (2.00)	1/11 (2.00)
Brain, cerebrum/neocortex: Blood vessels, leucocytosis	0/ 7	2/ 6 (2.00)	1/11 (1.00)
Brain, cerebrum/neocortex: Gliosis, focal/multifocal	0/ 7	4/ 6 (1.50)	3/11 (2.00)
Brain, cerebrum/neocortex: Hemorrhage	0/ 7	4/ 6 (1.25)	4/11 (2.00)
Brain, cerebrum/neocortex: Neuronal necrosis/neuron loss	0/ 7	2/ 6 (1.50)	1/11 (2.00)
Brain, cerebrum/neocortex: Parenchymal, necrosis	0/ 7	1/ 6 (1.00)	0/11
Brain, cerebrum/neocortex: Parenchymal, vacuolation	0/ 7	1/ 6 (1.00)	0/11
Brain, cerebrum/neocortex: Perivascular, Infiltrates, mononuclear cells	0/ 7	1/ 6 (2.00)	0/11
Brain, cerebrum/neocortex: Perivascular, inflammation	0/ 7	4/ 6 (2.00)	3/11 (1.67)
Brain, cerebrum/neocortex: Perivascular, inflammation, 30% granulocytes	0/ 7	1/ 6 (NA)	1/11 (NA)
Brain, cerebrum/neocortex: Perivascular, inflammation, 50% mononuclear cells	0/ 7	3/ 6 (NA)	2/11 (NA)
Brain, cerebrum/neocortex: Perivascular, inflammation, 50%granulocytes	0/ 7	3/ 6 (NA)	2/11 (NA)
Brain, cerebrum/neocortex: Perivascular, inflammation, 70% mononuclear cells	0/ 7	1/ 6 (NA)	1/11 (NA)
Brain, cerebrum/neocortex: Perivascular, pigment	0/ 7	1/ 6 (2.00)	1/11 (2.00)
Brain, cerebrum/neocortex: Vasculitis, multifocal	0/ 7	3/ 6 (1.67)	3/11 (1.67)
Brain, limbic system: Gliosis, focal/multifocal	0/ 7	1/ 6 (1.00)	0/11
Brain, limbic system: Hemorrhage	0/ 7	0/ 6	1/11 (2.00)
Brain, limbic system: Neuronal necrosis/neuron loss	0/ 7	1/ 6 (2.00)	0/11
Brain, limbic system: Perivascular, inflammation	0/ 7	1/ 6 (2.00)	1/11 (1.00)
Brain, limbic system: Perivascular, inflammation, 20% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, limbic system: Perivascular, inflammation, 30% granulocytes	0/ 7	1/ 6 (NA)	0/11

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:
1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 53 (continued): Study AP203-Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	8 mg/kg ETI-204 n/N (*)	32 mg/kg ETI-204 n/N (*)
Brain, limbic system: Perivascular, inflammation, 70% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, limbic system: Perivascular, inflammation, 80% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, limbic system: Vasculitis, multifocal	0/ 7	0/ 6	1/11 (1.00)
Brain, medulla oblongata: Bacteria, intravascular	1/ 7 (2.00)	0/ 6	0/11
Brain, meninges: Bacteria, extravascular	3/ 7 (1.67)	5/ 6 (3.00)	4/11 (2.25)
Brain, meninges: Bacteria, intravascular	5/ 7 (1.60)	3/ 6 (1.67)	2/11 (1.00)
Brain, meninges: Blood vessels, leucocytosis	1/ 7 (2.00)	0/ 6	0/11
Brain, meninges: Hemorrhage	4/ 7 (2.25)	6/ 6 (2.33)	7/11 (1.86)
Brain, meninges: Infiltrates, mononuclear cells	1/ 7 (1.00)	1/ 6 (2.00)	0/11
Brain, meninges: Inflammation	0/ 7	4/ 6 (4.00)	3/11 (3.33)
Brain, meninges: Inflammation, 30% granulocytes	0/ 7	2/ 6 (NA)	0/11
Brain, meninges: Inflammation, 30%mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, meninges: Inflammation, 50% granulocytes	0/ 7	2/ 6 (NA)	2/11 (NA)
Brain, meninges: Inflammation, 50% mononuclear cells	0/ 7	2/ 6 (NA)	2/11 (NA)
Brain, meninges: Inflammation, 70% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, meninges: Inflammation, 70% mononuclear cells	0/ 7	2/ 6 (NA)	0/11
Brain, meninges: Vasculitis	0/ 7	4/ 6 (2.25)	1/11 (2.00)
Brain, midbrain including substantia nigra: Bacteria, extravascular	0/ 7	1/ 6 (2.00)	1/11 (2.00)
Brain, midbrain including substantia nigra: Blood vessels, leucocytosis	0/ 7	0/ 6	1/11 (2.00)
Brain, midbrain including substantia nigra: Hemorrhage	0/ 7	1/ 6 (1.00)	2/11 (1.50)
Brain, midbrain including substantia nigra: Neuronal necrosis/neuron loss	0/ 7	1/ 6 (2.00)	0/11
Brain, midbrain including substantia nigra: Parenchymal, vacuolation	0/ 7	0/ 6	1/11 (2.00)
Brain, midbrain including substantia nigra: Perivascular, inflammation	0/ 7	2/ 6 (2.00)	1/11 (3.00)
Brain, midbrain including substantia nigra: Perivascular, inflammation, 20 % granulocytes	0/ 7	1/ 6 (NA)	0/11
Brain, midbrain including substantia nigra: Perivascular, inflammation, 30% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, midbrain including substantia nigra: Perivascular, inflammation, 50% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, midbrain including substantia nigra: Perivascular, inflammation, 50%granulocytes	0/ 7	1/ 6 (NA)	0/11
Brain, midbrain including substantia nigra: Perivascular, inflammation, 70% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, midbrain including substantia nigra: Perivascular, inflammation, 80% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, midbrain including substantia nigra: Vasculitis	0/ 7	1/ 6 (2.00)	1/11 (2.00)
Brain, thalamus/hypothalamus: Bacteria, extravascular	0/ 7	0/ 6	1/11 (1.00)
Brain, thalamus/hypothalamus: Bacteria, intravascular	1/ 7 (1.00)	0/ 6	1/11 (2.00)
Brain, thalamus/hypothalamus: Hemorrhage	0/ 7	0/ 6	2/11 (1.50)
Brain, thalamus/hypothalamus: Parenchymal, vacuolation	0/ 7	0/ 6	1/11 (2.00)
Brain, thalamus/hypothalamus: Perivascular hemosiderin	0/ 7	0/ 6	1/11 (2.00)
Brain, thalamus/hypothalamus: Perivascular, infiltrates, mononuclear cells	0/ 7	0/ 6	1/11 (2.00)
Brain, thalamus/hypothalamus: Perivascular, inflammation	0/ 7	0/ 6	1/11 (5.00)
Brain, thalamus/hypothalamus: Perivascular, inflammation, 50% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, thalamus/hypothalamus: Perivascular, inflammation, 50%granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, thalamus/hypothalamus: Vasculitis	0/ 7	0/ 6	1/11 (3.00)
Brain, ventricular system: Choroid plexus, infiltrates, mononuclear cell	0/ 7	1/ 6 (2.00)	1/11 (1.00)
Brain, ventricular system: Choroid plexus, inflammation	0/ 7	1/ 6 (2.00)	1/11 (1.00)
Brain, ventricular system: Choroid plexus, inflammation, 10% granulocytes	0/ 7	1/ 6 (NA)	0/11
Brain, ventricular system: Choroid plexus, inflammation, 50% granulocytes	0/ 7	0/ 6	1/11 (NA)
Brain, ventricular system: Choroid plexus, inflammation, 50% mononuclear cells	0/ 7	0/ 6	1/11 (NA)
Brain, ventricular system: Choroid plexus, inflammation, 90% mononuclear cells	0/ 7	1/ 6 (NA)	0/11
Brain, ventricular system: Inflammatory cells	0/ 7	0/ 6	1/11 (3.00)
Kidneys			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	0/16 (NA)	0/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	13/16 (1.54)	5/16 (1.20)	3/16 (1.67)
Degeneration/Necrosis, Tubular Epithelium	0/16	3/16 (1.67)	1/16 (2.00)
Inflammation	2/16 (1.00)	1/16 (1.00)	0/16

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 53 (continued): Study AP203-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	8 mg/kg ETI-204 n/N (*)	32 mg/kg ETI-204 n/N (*)
Liver			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	0/16 (NA)	0/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	12/16 (1.42)	3/16 (1.33)	4/16 (1.00)
Hepatocyte Sinusoidal Leukocytosis	1/16 (2.00)	0/16	0/16
Inflammation	0/16	0/16	1/16 (1.00)
Necrosis	1/16 (2.00)	1/16 (2.00)	1/16 (1.00)
Sinusoidal Leukocytosis	11/16 (1.09)	11/16 (1.36)	9/16 (1.33)
Lymph Nodes, Bronchial			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	1/16 (NA)	4/16 (NA)	0/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	10/16 (1.70)	3/16 (1.00)	2/16 (1.00)
Depletion/Necrosis, Lymphocytes	13/16 (2.69)	15/16 (1.87)	9/16 (1.78)
Edema	5/16 (1.60)	3/16 (1.33)	2/16 (1.00)
Fibrin	2/16 (1.00)	9/16 (1.11)	5/16 (1.20)
Hemorrhage	4/16 (1.75)	4/16 (1.50)	3/16 (1.33)
Inflammation	1/16 (1.00)	4/16 (1.75)	4/16 (1.50)
Lymph Nodes, Mediastinal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	0/16 (NA)	1/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	14/16 (1.71)	4/16 (1.00)	2/16 (1.00)
Depletion/Necrosis, Lymphocytes	14/16 (2.36)	15/16 (2.20)	9/16 (2.33)
Edema	6/16 (1.33)	4/16 (1.00)	5/16 (1.20)
Fibrin	6/16 (1.33)	4/16 (1.25)	2/16 (1.00)
Hemorrhage	9/16 (1.78)	8/16 (1.38)	3/16 (1.33)
Inflammation	5/16 (1.40)	2/16 (2.50)	1/16 (1.00)
Lungs			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	7/16 (NA)	1/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	12/16 (2.33)	6/16 (1.33)	3/16 (2.33)
Edema	4/16 (1.50)	10/16 (2.10)	2/16 (1.50)
Fibrin	3/16 (1.00)	3/16 (1.33)	0/16
Fibrosis	0/16	1/16 (2.00)	0/16
Hemorrhage	3/16 (1.00)	3/16 (1.67)	0/16
Inflammation	6/16 (1.17)	8/16 (1.25)	6/16 (1.00)
Pleura	0/16	1/16 (NA)	0/16
Spleen			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	0/16 (NA)	0/16 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Bacteria	14/16 (3.00)	5/16 (2.80)	4/16 (2.50)
Depletion/Necrosis, Lymphocytes	14/16 (2.36)	12/16 (1.50)	6/16 (1.83)
Fibrin	7/16 (1.29)	4/16 (1.25)	5/16 (1.60)
Hemorrhage	9/16 (1.44)	3/16 (1.00)	2/16 (2.00)
Inflammation	10/16 (2.10)	14/16 (2.14)	11/16 (2.00)
Neutrophilic	0/16	0/16	1/16 (NA)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 53 (continued): Study AP203-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	8 mg/kg ETI-204 n/N (*)	32 mg/kg ETI-204 n/N (*)
Spinal Cord			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	2/16 (NA)	0/16 (NA)
Microscopic Finding ^{1, 4}			
# Necropsied/Total Infected	0/16	2/16	0/16
Bacteria	0/ 0	1/ 2 (1.00)	0/ 0
Hemorrhage	0/ 0	1/ 2 (2.00)	0/ 0
Inflammation	0/ 0	2/ 2 (2.00)	0/ 0
Cavity, Abdominal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	0/16 (NA)	1/16 (NA)
Microscopic Finding ^{1, 4, 5}			
# Necropsied/Total Infected	16/16	16/16	16/16
Cavity, Pericardial			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	0/16 (NA)	1/16 (NA)	0/16 (NA)
Microscopic Finding ^{1, 4, 5}			
# Necropsied/Total Infected	16/16	16/16	16/16
Skin			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	1/16 (NA)	0/16 (NA)	0/16 (NA)
Microscopic Finding ^{1, 4}			
# Necropsied/Total Infected	16/16	16/16	16/16
Edema	1/16 (3.00)	0/16	0/16
Hemorrhage	1/16 (4.00)	0/16	0/16
Inflammation	1/16 (1.00)	0/16	0/16
Gross lesions			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	16/16
Gross Lesions	2/16 (NA)	5/16 (NA)	3/16 (NA)
Microscopic Finding ^{1, 4, 5}			
# Necropsied/Total Infected	16/16	16/16	16/16
NA, not applicable			
* Mean severity of lesion			
Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).			
¹ Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)			
² Neuropathology performed at [REDACTED] (b) (4)			
³ All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)			
⁴ All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)			
⁵ Tissues examined microscopically and found unremarkable			

Tissue bacterial assessments:

Histology: No bacteria were observed by microscopic examination of any of the tissues from the animals that survived; however, bacteria were reported in the tissues from many animals that died (Table 54).

Culture: At least three to six tissues, from all the animals treated with either 8 or 32 mg/kg dose of ETI-204 and died or were moribund and euthanized, were culture positive (Table 54). Of the 7 treated animals that survived, all tissues from one animal treated with the 8 mg/kg dose of ETI-204 were culture negative. Although spleen, liver, and kidney from six of the surviving animals treated with 32 mg/kg ETI-204 dose, were culture negative, lungs from 5 animals and brain from one animal were culture negative (Table 54).

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Of the two control animals that survived, all the tissues (lung, lymph nodes, spleen, kidney, and liver) except brain were culture negative from both the animals; brain from one animal (C49041) was culture positive but negative for animal C49058. One control animal (C49025) that died was negative for all the tissues tested (Table 54).

Table 54: Study AP203-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 8mg/kg		ETI-204 32mg/kg	
	Survivors (N=2)	Non Survivors (N=14)	Survivors (N=1)	Non Survivors (N=15)	Survivors (N=6)	Non Survivors (N=10)
Presence of bacteria by microscopy [1, 5]						
Brain: Total [3, 4]	0/2	5/5	0/1	5/5	0/6	5/5
Brain, basal nuclei/striatum: extravascular	0/2	0/5	0/1	1/5	0/6	1/5
Brain, basal nuclei/striatum: intravascular	0/2	2/5	0/1	0/5	0/6	1/5
Brain, cerebellum: extravascular	0/2	0/5	0/1	0/5	0/6	1/5
Brain, cerebellum: intravascular	0/2	1/5	0/1	0/5	0/6	0/5
Brain, cerebrum/neocortex: extravascular	0/2	0/5	0/1	2/5	0/6	2/5
Brain, cerebrum/neocortex: intravascular	0/2	3/5	0/1	1/5	0/6	1/5
Brain, medulla oblongata: intravascular	0/2	1/5	0/1	0/5	0/6	0/5
Brain, meninges: extravascular	0/2	3/5	0/1	5/5	0/6	4/5
Brain, meninges: intravascular	0/2	5/5	0/1	3/5	0/6	2/5
Brain, midbrain including substantia nigra: extravascular	0/2	0/5	0/1	1/5	0/6	1/5
Brain, thalamus/hypothalamus: extravascular	0/2	0/5	0/1	0/5	0/6	1/5
Brain, thalamus/hypothalamus: intravascular	0/2	1/5	0/1	0/5	0/6	1/5
Kidneys	0/2	13/14	0/1	5/15	0/6	3/10
Liver	0/2	12/14	0/1	3/15	0/6	4/10
Lymph Nodes, Bronchial	0/2	10/14	0/1	3/15	0/6	2/10
Lymph Nodes, Mediastinal	0/2	14/14	0/1	4/15	0/6	2/10
Lungs	0/2	12/14	0/1	6/15	0/6	3/10
Spleen	0/2	14/14	0/1	5/15	0/6	4/10
Spinal Cord	0/0	0/0	0/0	1/2	0/0	0/0
Cavity, Abdominal	0/2	0/14	0/1	0/15	0/6	0/10
Cavity, Pericardial	0/2	0/14	0/1	0/15	0/6	0/10
Skin	0/2	0/14	0/1	0/15	0/6	0/10
Gross lesions	0/2	0/14	0/1	0/15	0/6	0/10
Presence of bacteria by culture [1, 2]						
Brain	1/2	13/14	0/1	15/15	1/6	10/10
Kidneys	0/2	13/14	0/1	9/15	0/6	6/10
Liver	0/2	13/14	0/1	10/15	0/6	7/10
Lymph Nodes, Bronchial	0/2	13/14	0/1	12/15	1/6	7/10
Lungs	0/2	13/14	0/1	15/15	5/6	10/10
Spleen	0/2	13/14	0/1	13/15	0/6	8/10

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate

[3] Neuropathology performed at (b) (4)

[4] Animals that were positive in at least one area for either extra- or intra-vascular bacteria

[5] Histopathology performed at (b) (4) with exception of brain. Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: None

Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: None

Neuropathological evaluations: The brains of all 9 surviving animals and 15 (5 per group) non-surviving animals that were moribund or found dead were processed for microscopic examination (Table 55). Bacteria (intravascular and/or extravascular) were observed in all the 15 non-survivors. Hemorrhage and extravascular bacteria were noted in control animals, but inflammation was limited to the ETI-204 treated animals. None of the animals that survived to scheduled termination had any evidence of bacteria in their brain tissue. Microscopic changes noted in surviving animals treated with ETI-204 were similar to those noted in a surviving control animal, indicating the microscopic changes in the surviving ETI-204 treated animals were not related to treatment with ETI-204.

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Table 55: Study AP203 - Select microscopic findings in brain tissues of survivors and non-survivors

Group	Animal Number	Survival Outcome ²	Time to Death (days)	Presence of Lesions ¹				
				Bacteria (Intravascular)	Bacteria (Extravascular)	Inflammation	Hemorrhage	
Survivors—Males								
Saline Control (Group 1)	C49058	SS	28	-	-	-	P	
	C49041	SS	28	-	-	-	-	
	Non-Survivors—Males							
	C49025	FD	7	P	-	-	-	
	C48303	FD	3	P	-	-	-	
	C50265	FD	4	P	P	-	P	
	Non-Survivors—Females							
	C40043	FD	3	P	P	-	P	
	C41078	FD	3	P	P	-	P	
	Survivors—Female							
8 mg/kg ETI-204 (Group 2)	C44168	SS	28	-	-	-	P	
	Non-Survivors—Male							
	C46745	MS	3	P	P	P	P	
	C48253	MS	5	P	P	P	P	
	C49020	MS	3	P	P	P	P	
	Non-Survivors—Female							
	C40092	FD	5	-	P	P	P	
	30942	FD	4	-	P	P	P	
	Survivors—Males							
	C47896	SS	28	-	-	-	-	
32 mg/kg ETI-204 (Group 3)	C48922	SS	28	-	-	-	-	
	C47894	SS	28	-	-	-	P	
	C48903	SS	28	-	-	-	P	
	Survivors—Females							
	C40915	SS	28	-	-	-	P	
	C40067	SS	28	-	-	-	-	
	Non-Survivors—Males							
	C47893	FD	3	-	P	P	P	
	Non-Survivors Females							
	C40104	FD	3	-	P	P	P	

¹*Lesions marked with a “P” were present; lesions marked with a hyphen (-) were not present.

² SS = Scheduled sacrifice; MS = Moribund sacrifice; FD = Found dead

The applicant states that “In general, in the opinion of the study neuropathologist, animals that received ETI-204 were more likely to mount some sort of an inflammatory response (and thus have more evidence of inflammation) as compared to the animals that were given saline alone. Because the inflammatory reactions were always associated with extravascular bacteria, the bacteria (and not ETI-204) were interpreted to be the cause.” As this study lacked an uninfected control group of animals administered ETI-204, the applicant refers to another study in uninfected pregnant rabbits where administration of ETI-204 was shown to have no adverse morphologic effects in the brain (at least in uninfected animals). All the “morphologic lesions encountered in this study were either related to the bacteria (the majority of the findings, including hemorrhage and inflammation) or were spontaneous/incidental changes of no biologic significance (hemorrhage; occasional mononuclear cell infiltrates).”

Comments:

The study showed that ETI-204 at a dose of 32 mg/kg was more effective in improving survival (38%) up to Day 28 compared to the lower dose group of 8 mg/kg (6.3%) or untreated control group (13%). However, such differences were not significant. All of the non-surviving animals died within 7 days of challenge.

All animals were bacteremic and/or PA positive at the time of initiation of treatment. Higher bacteremia levels and higher PA levels at the time of initiation of treatment appear to be associated with a lower survival rate. The study suggests that an animal with a higher microbial burden prior to treatment is more likely to die after treatment with ETI-204.

*Among monkeys that were found dead or euthanized, gross lesions were consistent with acute *B. anthracis* infection; additionally, the tissues from animals found dead or euthanized were positive for bacteria. No gross lesions were found in surviving animals; some of the tissues especially lungs from the animals that survived showed evidence of bacteria by culture. This is consistent with the results from previous studies which have shown that spores can be found in the lung up to 56 days after challenge in surviving nonhuman primates (Henderson et al., 1956¹).*

All of the non-surviving animals that were examined for neuropathology had intravascular and/or extravascular bacteria, suggesting the cause of death in these animals was a bacterial infection. Only ETI-204-treated non-surviving animals had evidence of an inflammatory response, and always in association with extravascular bacteria. None of the deaths were attributed to ETI-204.

6.2.2.4. Study AP204

This was a randomized, placebo-controlled GLP study to evaluate the efficacy of ETI-204 (Baxter) when administered IV, in 48 cynomolgus monkeys infected with the spores (spore lot no. B36) of the Ames strain of *B. anthracis* by inhalational route.⁴⁹ The primary objective was to evaluate the efficacy of single IV doses of 4 or 16 mg/kg ETI204 (Baxter). The secondary object was to perform expanded microscopic evaluations of brain and meninges for non-surviving and surviving monkeys as well as neurological examinations pre-study and at 28 and 56 days post challenge.

The study design was same as for Study AP203. Blood was collected for microbiological measurements and other parameters at different time point (Table 56).

⁴⁹ (b) (4) Study Number 1121-G924204: AP204: Evaluating the efficacy of ETI-204 when administered therapeutically in the cynomolgus macaque inhalational anthrax model (December 20, 2012).

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Table 56: Study AP204 - Blood collection and assay schedule

Approximate Time Point	Blood Tube type/ Approximate Blood volume	Bacteremia (Culture)	CBC/ CRP	Serum for PA-ECL	Serum PA levels (via ELISA)	Serum for ETI-204 PK	Serum for Anti-ETI-204 Antibodies
Day -6	EDTA ~1.5 mL SST ~3.0 mL	X	X	X	X	X	X
~24hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
~30hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
~36hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
~42hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
~48hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
~54hr PC	EDTA ~0.5 mL SST ~1.5 mL	X		X	X		
PTT ^a	EDTA ~0.5 mL SST ~1.5 mL SPS ~1.0 mL	X [#]		X	X		
15 min PT	SST ~1.0 mL					X	
2hr PT	SST ~1.0 mL					X	
6hr PT	SST ~2.0 mL				X	X	
24hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
48hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
96hr PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
5 days PT	SST ~1.5 mL					X	
7 days PT	EDTA ~0.5 mL SST ~1.5 mL	X			X	X	
16 days PC	EDTA ~1.5 mL SST ~1.5 mL	X	X		X	X	
23 days PC	EDTA ~1.5 mL SST ~4.0 mL	X	X		X	X	
28 days PC	EDTA ~1.5 mL SST ~4.0 mL	X	X		X	X	X
56 days PC	EDTA ~1.5 mL SST ~4.0 mL	X	X		X		X
Terminal ^{b, d}	EDTA ~1.5 mL SST ~4.0 mL	X	CRP only		X	X	X

PC = Post-Challenge, PTT = Prior to Treatment, PT = Post-Treatment

^a = Post-challenge pre-treatment bleed time points were relative to a median challenge time for a challenge cohort. Post-treatment bleed times were calculated from the time each animal's IV treatment ended. Blood sampling occurred within ±60 minutes of the calculated time, except for the 15 min PT, 2hr PT, 6hr PT and 96hr PT samples which occurred within 5 min, 10 min, 15 min and 3 hours of their calculated times respectively. The Day -6, Day 5 PT, Day 7 PT, Day 16 PC, Day 23 PC, Day 28 PC and Day 56 PC bloods were relative to the day of treatment or challenge.

^b = If collection was possible.

^c = Terminal samples were not collected on animals euthanized at the end of study as Day 28 PC and Day 56 PC samples were already scheduled.

^d = Post-Challenge, pre-treatment sampling stopped once decision to treat had been made.

[#] = PTT Bacteremia enrichment performed on sample collected in SPS tube (see section 4.8)

Results:

Baseline characteristics: All animals were culture negative prior to challenge (Day -6). Although all animals, prior to challenge, were PA negative by ECL assay, one animal (C42724, saline group) was PA positive (10.7 ng/mL) by ELISA. Age, gender, body weight, and challenge dose (mean LD₅₀ 212; (b)(4) cfu) were comparable among the animals in all groups. The LD₅₀ dose was ≥ 200 for approximately 58% of the animals and was comparable among the three groups (Table 57). The MMAD was 1.14 µm which is consistent with the particle size range that would reach the alveoli.

All animals were treated between 25 and 59 hours post-challenge (Table 57). At the time of treatment, two animals in the 16 mg/kg group were PA negative by the ECL assay (Table 57). By ELISA the three animals in the control and 4 animals in the ETI-204 16 mg/kg group were negative for free PA (Table 57). A majority (47/48) of the animals were culture positive. The quantitative bacteremia and PA-ELISA levels though variable were comparable among animals across different groups (Table 57). Bacteremia and PA levels were slightly lower in all the animals in this study compared to other studies summarized above. The mean time to trigger and treatment was similar among the animals in all the three groups (Table 57).

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Table 57: Study AP204 - Baseline characteristics, inhaled dose of <i>B. anthracis</i> , time to treatment and survival			
Parameters	Placebo (Group 1) N=16	ETI-204: 4 mg/kg (Group 2) N=16	ETI-204: 16 mg/kg (Group 3) N=16
Baseline characteristics			
Age (years) estimated range	3.1±0.2 (2.6-3.3)	3.0±0.2 (2.7-3.3)	3.1±0.2 (2.8-3.3)
Body weight (kg) Mean ±SD	2.8±0.3	2.8±0.2	2.8±0.2
Total Inhaled Dose: cfu x 10 ⁷			(b) (4)
Mean ± SD (Range)			
LD ₅₀ dose	220.1±49.2	207.4±34.7	209.2±47.0
Mean ± SD (Range)	(136-327)	(155-279)	(136-325)
<200 LD ₅₀ dose n(%)	6 (37.5)	7 (43.8)	7 (43.8)
≥200 LD ₅₀ dose n(%)	10 (62.5)	9 (56.3)	9 (56.3)
Trigger for treatment (PA by ECL)^a and microbial burden prior to treatment			
Positive screening PA/ECL assay n (%)	16 (100)	16 (100)	14 (87.5)
Bacteremia prior to treatment			
Enriched (qualitative) bacteremia n (%)	16 (100)	16 (100)	15 (93.8)
Quantitative bacteremia n (%)	16 (100)	16 (100)	15 (93.8)*
Log ₁₀ bacteremia (cfu/mL) Mean±SD (Range)	4.09±1.06 (2.12-5.68)	4.17±0.88 (2.48-5.36)	3.50±1.34 (0.30-5.63)*
Quantitative bacteremia (cfu/mL x 10 ⁴) Geometric mean	1.22	1.46 [‡]	0.31
Free PA levels (ng/mL) prior to treatment			
N (%)	13 (81.3)	16 (100.0)	12 (75.0)
Log ₁₀ ± SD	1.58±0.59	1.78±0.41	1.49±0.55
Geometric mean	38.1	60.7	31.0
Time (hours) between challenge, trigger, and treatment			
Time trigger (PA^{+ve}) post challenge^a	35.7±5.3 (25.1-46.5)	37.1±6.2 (29.7-48.1)	41.4±9.0 (27.1-55.9)
Time to bacteremia	29.9±3.6 (21.9-34.8)	31.7±5.6 (23.6-42.3)	33.2±10.0 (21.6-58.7)
Time to treatment^b	39.2±5.0 (28.5-49.7)	40.4±6.0 (33.3-51.2)	44.4±8.7 (30.2-58.8)
Time from trigger to treatment	3.5±1.0	3.3±0.9	3.1±1.3
Survivors at the end of study (Day 28 and Day 56)			
Survivors	1 (6.3)	4 (25.0)	8 (50.0)**
Survivors in bacteremic animals	1 (6.3)	4 (25.0)	7/15 (46.7)**

*C43304's bacteremia level was 1 cfu/mL in the ADSL data set and 50 cfu/mL in the LB data set at the prior to treatment visit.

^aExcluding 3 and 4 animals in the placebo and ETI-204 groups, respectively, with a measurement ≤LLOD
SD Standard deviation

PTT Prior to treatment; Quantitative bacteremia LOD Limit of detection=3 cfu/mL; PA ELISA LLOQ Lower limit of quantification 9.68 ng/mL.

^aThe trigger for treatment was defined as the time from challenge to a positive PA-ECL post-challenge.

^bThe time to treatment was defined as the time from challenge to treatment.

**Statistically significant between the ET-204 32 mg/kg treated group and the control group by exact method and Boschloo's one-sided test; no difference between the ET-204 8 mg/kg treated group and the control group

Detection of Bacteremia and PA: The variability among the results of three bacterial culture methods and detection of PA by ECL and ELISA is shown in Table 58. The samples used for these three assays were collected at the same time post-challenge. Of the 48 animals, 47 (98%) were positive by both enriched and quantitative culture methods and 46 (96%) by all the three

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culture methods. Of the 48 monkeys in the study, 46 and 43 were PA positive by ECL assay or ELISA, respectively. All the culture methods for measuring bacteremia as well as detection of PA by ECL method were equally sensitive.

Table 58: Study AP204 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Detection Method					Placebo (n=16)	Treatment Group		Total (n=48)
	Qualitative Enriched Culture*	Quantitative Culture†	Screening PA* (ECL Assay)*	Quantitative PA* (ELISA Assay)†	4 mg/kg ETI-204 (n=16)		16 mg/kg ETI-204 (n=16)	Total (n=48)	
+	+	+	+	+	15	16	12	43	
+	+	+	+	-	1	0	2	3	
-	+	+	-	-	0	0	1	1	
-	-	-	-	-	0	0	1	1	

n = Number of treated animals. Results determined on a per animal basis, not for individual tests.
Animal with a positive test from any time prior to treatment is considered as positive.
ND = Not Done
*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; LOD by enriched culture: sample considered positive if at least 1-5 colonies were present in the primary streak after plating 40 µL of overnight culture of 1 mL of whole blood diluted 1:10 and thus LOD can be estimated as 1 cfu/mL of whole blood; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL)
†LLOQ by quantitative culture 100 cfu/mL; LLOQ by free PA ELISA 9.68 ng/mL

There was a significant positive correlation between quantitative bacteremia and free PA levels at 36 hours post-challenge, prior to treatment, 24 hours post-treatment, and 48 hours post-treatment. This suggests a linear relationship between bacteremia and the free PA levels at these study time points.

Effect of treatment on survival and microbial burden: The results show that ETI-204 at a dose of 16 mg/kg was effective in improving survival compared to the control group; the lower dose (4 mg/kg) of ETI-204 was less effective (Figure 38). All of the non-surviving animals died within 8 days of challenge. All animals except two (C41563 in the placebo group and C42573 treated with 16 mg/kg ETI-204) that were found dead or moribund were bacteremic at the unscheduled terminal time point.

One animal (C42861) that survived became PA negative and culture negative by Day 7; lung, collected at the time of necropsy, was culture positive whereas other tissues were culture negative. Animal C42724 in the control group, that was PA positive by ELISA but culture negative, prior to challenge, died on the study.

PA and bacteremia levels decreased after initiation of treatment (Figures 38 and 39). For all animals surviving to the end of the study, quantitative bacteremia levels at 7 days following treatment and thereafter (until 56 days post-challenge) were below than the LOD. PA was undetectable in a majority of the animals within 6 hours of treatment.

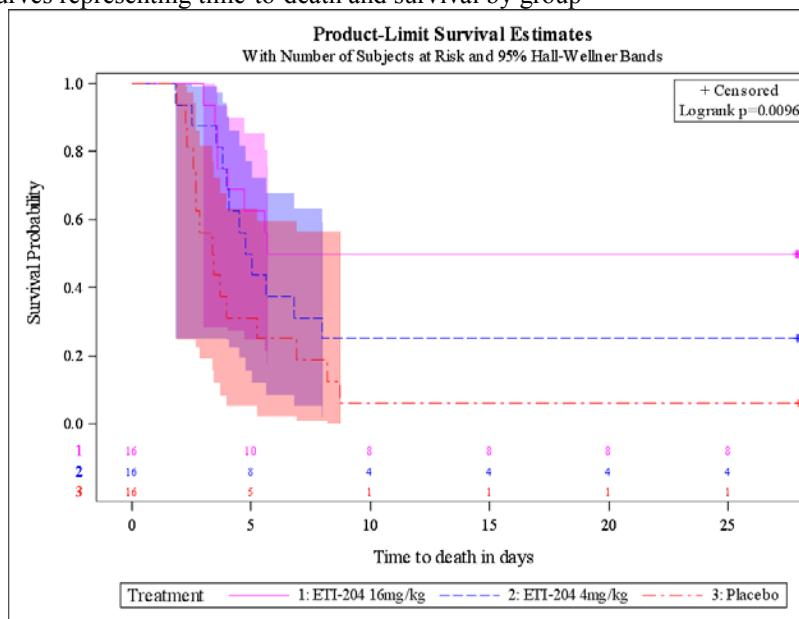
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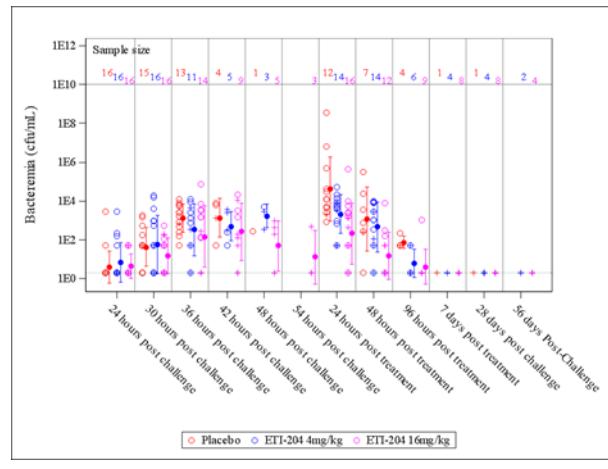
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Figure 38: Study AP204 - Survival and microbial burden at different time points

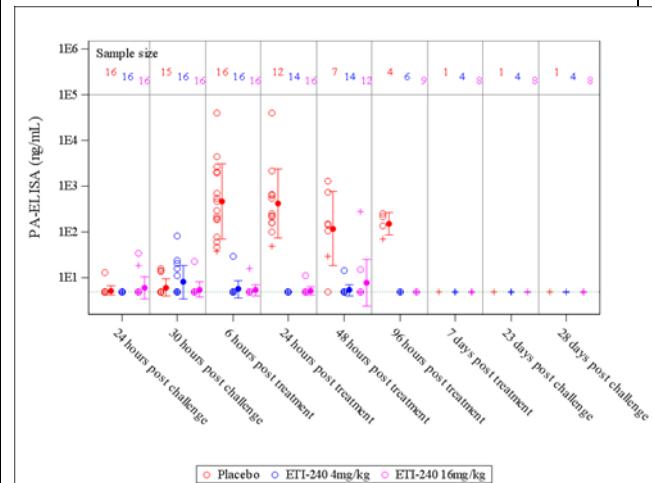
A: Kaplan-Meier curves representing time-to-death and survival by group



B: Bacteremia levels



C: PA levels



+=survivors; o=non-survivors

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

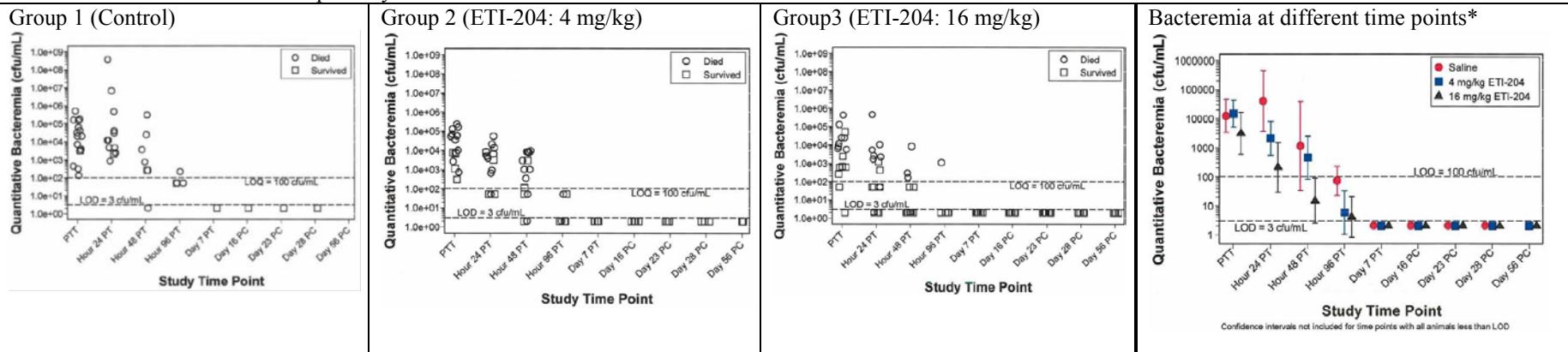
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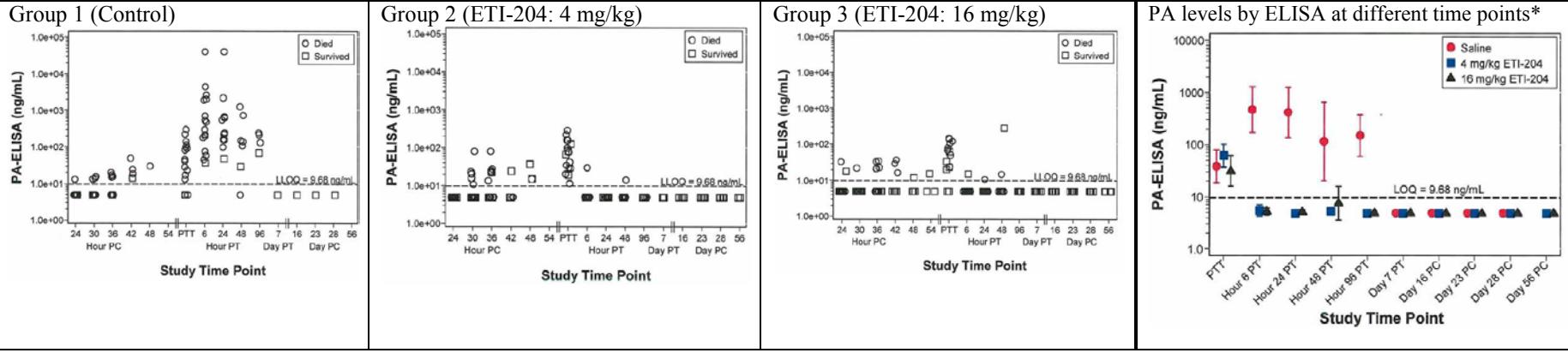
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Figure 39: Study AP204 - Observed bacteremia and free PA levels over time in survivors and non-survivors

A: Bacteremia levels versus time point by survival status



B: PA levels by ELISA versus study time by survival status



PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge

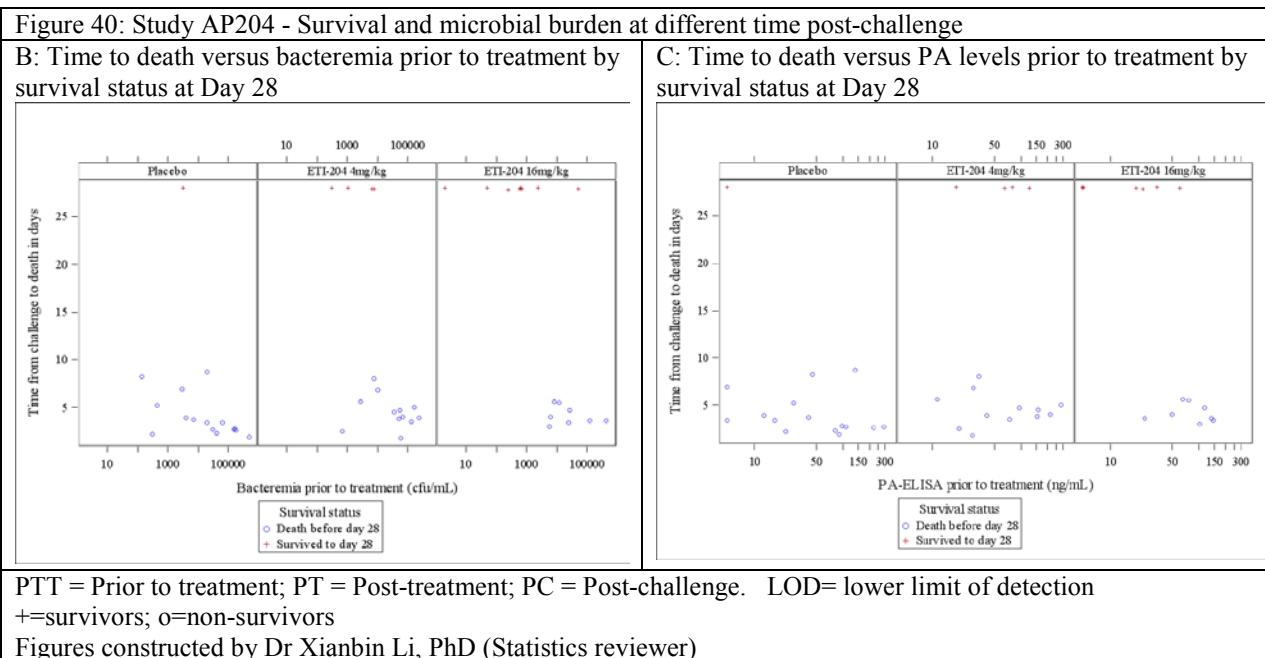
*Note: Plot of Group geometric means and 95 percent confidence intervals for bacteremia and PA levels versus study time; confidence intervals not included for time points with all animals less than LOD
Source: BLA submission

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Effect of microbial burden on survival: Animals with higher bacteremia and PA levels at the time of initiation of therapy are less likely to survive after treatment with ETI-204 (Figures 39 and 40).



Hematological parameters: The results for most of the hematological parameters measured were within the normal ranges at baseline and Day 16 post-challenge. Slightly elevated white blood cell counts and lymphocytes were observed post-challenge which is consistent with a recovery from *B. anthracis* infection. An increase in CRP levels on Day 16 and terminal samples were observed compared to baseline in all the 3 groups including the control group. However, these results should be interpreted with caution due to infrequent sampling times.

Clinical Observations: The majority of animals exhibited abnormal clinical signs consistent with anthrax following challenge; these signs were consistent with those reported in the natural history studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is considered to be common with laboratory housed NHPs) after Day 7 to 10 days post-challenge (Figure 41).

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Figure 41: Study AP204 – Clinical observations

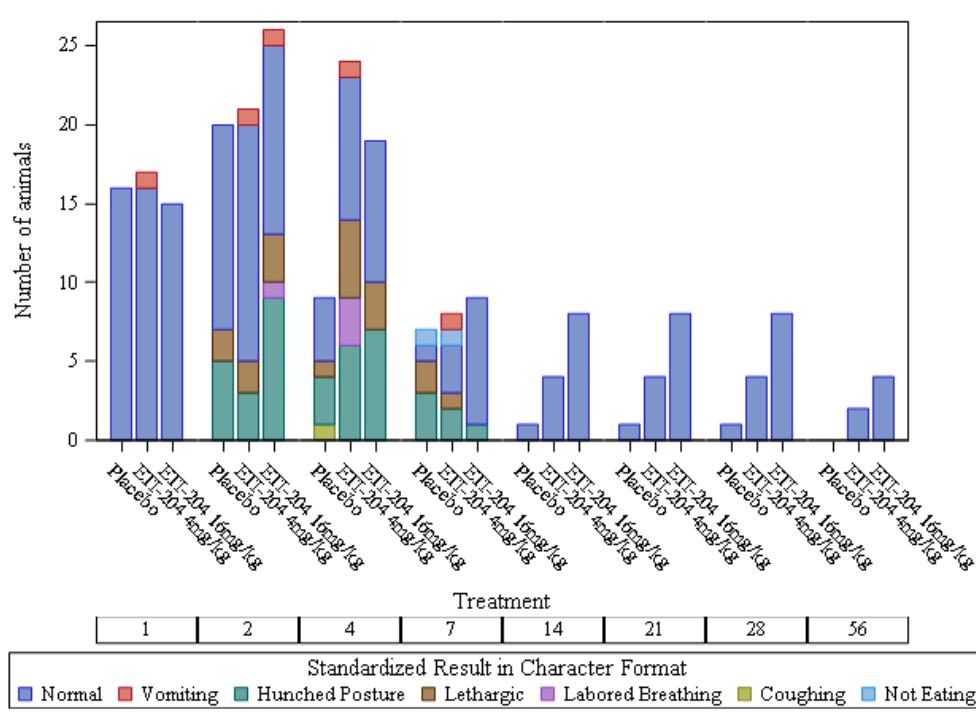
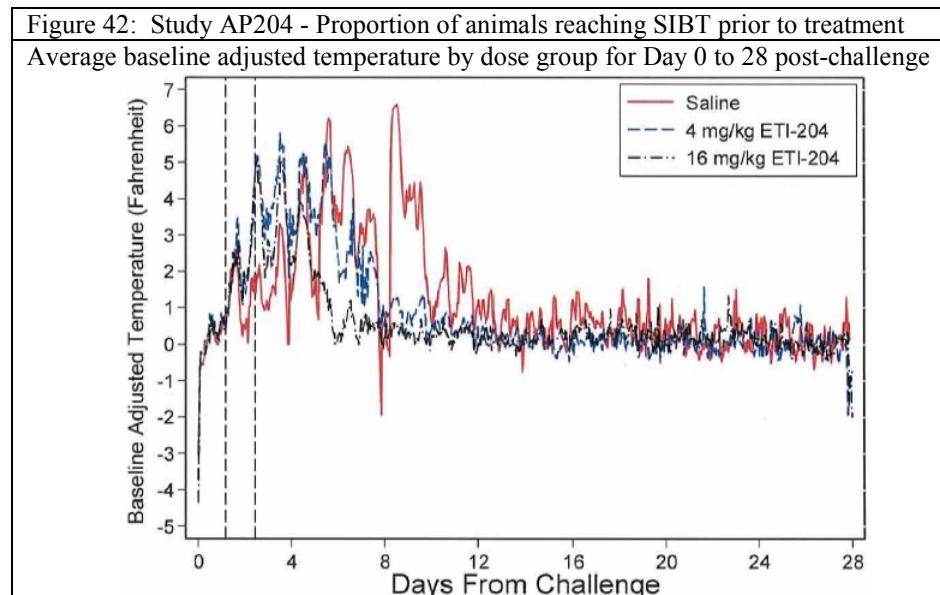


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

There was no difference in the proportion of animals reaching SIBT, prior to treatment, among the animals in the 3 groups (Figure 42). However, these observations should be interpreted with caution due to diurnal variability in non-human primates.



Proportion of animals reaching SIBT prior to treatment

Dose	Proportion Abnormal	Abnormal Rate (95% Confidence Interval)
Saline	9/16	0.56 (0.30, 0.80)
4 mg/kg ETI-204	6/16	0.38 (0.15, 0.65)
16 mg/kg ETI-204	5/16	0.31 (0.11, 0.59)

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 body weights.

Necropsy and Histopathology: Gross lesions and microscopic lesions in animals found dead or euthanized due to moribund condition were consistent with anthrax and included hemorrhage, necrosis or necrosis/atrophy, inflammation, edema/exudates, neutrophil infiltration, and fibrin deposition (Table 59). These observations are consistent with those summarized above for natural history studies. The applicant states that no gross lesions were found in animals that survived the period of observation.

The majority of ETI-204-treated monkeys dying on study, in either of the dose groups, showed marked morphologic/inflammatory changes. Microscopic changes were absent in monkeys that survived until their scheduled sacrifice (Day 28 or 56) except for occasional background changes, that include mononuclear cell infiltrates in the meninges and/or choroid plexus (changes not correlated with treatment).

Intravascular bacteria were observed in control animals; no morphologic/ inflammatory response was observed. The one female monkey that survived to scheduled sacrifice had no anthrax-related changes. The presence of bacteria without other morphologic signs suggests that the untreated animals died before they could mount an inflammatory response or were prevented from doing so by the bacteria/bacterial toxins.

Neurological and neuropathological evaluations: Neurological evaluations were performed in 19 animals. A majority of the animals were stated to be normal. A decreased range of motion in thoracic limbs was reported in six animals post-challenge and 2 animals prior to challenge. One animal had a head tilt; however, the neuropathology analysis of the brain for this animal was normal (Table 60). All these observations (decrease range of motion of thoracic limbs or a transient head tilt) can occur in captive non-human primates.

Although changes were noted in the meninges and brain, the predominant change was consistently in the meninges. Morphologic changes were relatively consistent and typically included:

- Bacteria, hemorrhage and inflammation in the meninges (bacterial meningitis).
- Vasculitis (inflammation/destruction of blood vessel walls) in the meninges and brain.
- Bacteria, hemorrhage and inflammation in the brain (bacterial encephalitis).

The meninges were most commonly and typically the most severely affected area, indicating meningitis was the main morphologic finding associated with inhalation anthrax in ETI-204 treated animals. In the brain, the areas most affected tended to be those with the greatest surface area (cerebrum and cerebellum) and therefore with the most exposure to the meninges.

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Table 59: Study AP204- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Brain			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	15/15
Gross Lesions	1/16 (NA)	7/16 (NA)	3/15 (NA)
Microscopic Finding ^{2,3}			
# Necropsied/Total Infected	16/16	16/16	15/15
Brain, Basal Ganglia, Blood Vessels: Leucocytosis	0/16	1/16 (2.00)	2/15 (2.50)
Brain, Basal Ganglia, Perivascular: Infiltrates, Mononuclear Cells	0/16	0/16	1/15 (3.00)
Brain, Basal Ganglia, Perivascular: Inflammation	0/16	4/16 (3.25)	2/15 (2.50)
Brain, Basal Ganglia: 30% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Basal Ganglia: 30% Mononuclear Cells	0/16	0/16	1/15 (NA)
Brain, Basal Ganglia: 50% Granulocytes	0/16	3/16 (NA)	1/15 (NA)
Brain, Basal Ganglia: 50% Mononuclear Cells	0/16	3/16 (NA)	1/15 (NA)
Brain, Basal Ganglia: 70% Granulocytes	0/16	0/16	1/15 (NA)
Brain, Basal Ganglia: 70% Mononuclear Cells	0/16	1/16 (NA)	0/15
Brain, Basal Ganglia: Extravascular Bacteria	0/16	3/16 (2.33)	1/15 (3.00)
Brain, Basal Ganglia: Giosis	0/16	1/16 (2.00)	0/15
Brain, Basal Ganglia: Hemorrhage	0/16	3/16 (3.00)	2/15 (2.50)
Brain, Basal Ganglia: Intravascular Bacteria	9/16 (2.89)	2/16 (3.50)	1/15 (3.00)
Brain, Basal Ganglia: Mineralization	0/16	1/16 (3.00)	1/15 (3.00)
Brain, Basal Ganglia: Vacuolation	0/16	1/16 (3.00)	0/15
Brain, Basal Ganglia: Vasculitis	0/16	4/16 (3.50)	3/15 (2.33)
Brain, Cerebellum, Blood Vessels: Leucocytosis	0/16	2/16 (2.50)	1/15 (3.00)
Brain, Cerebellum, Granular Cell Layer: Neurons Decreased	0/16	1/16 (2.00)	0/15
Brain, Cerebellum, Perivascular: Inflammation	1/16 (3.00)	7/16 (3.00)	3/15 (2.33)
Brain, Cerebellum, Purkinje Cell Layer: Vacuolation	0/16	0/16	1/15 (3.00)
Brain, Cerebellum, White Matter: Edema	0/16	0/16	1/15 (3.00)
Brain, Cerebellum: 30% Granulocytes	1/16 (NA)	0/16	0/15
Brain, Cerebellum: 40% Mononuclear Cells	0/16	0/16	1/15 (NA)
Brain, Cerebellum: 50% Granulocytes	0/16	7/16 (NA)	3/15 (NA)
Brain, Cerebellum: 50% Mononuclear Cells	0/16	7/16 (NA)	3/15 (NA)
Brain, Cerebellum: 60% Granulocytes	0/16	0/16	1/15 (NA)
Brain, Cerebellum: 70% Mononuclear Cells	1/16 (NA)	0/16	0/15
Brain, Cerebellum: Extravascular Bacteria	0/16	1/16 (2.00)	2/15 (3.50)
Brain, Cerebellum: Hemorrhage	1/16 (3.00)	7/16 (2.86)	4/15 (2.75)
Brain, Cerebellum: Inflammation	0/16	0/16	1/15 (4.00)
Brain, Cerebellum: Intravascular Bacteria	7/16 (3.00)	2/16 (3.00)	0/15
Brain, Cerebellum: Necrosis	0/16	2/16 (2.50)	3/15 (3.33)
Brain, Cerebellum: Neuronal Necrosis/Neuron Loss	0/16	0/16	1/15 (3.00)
Brain, Cerebellum: Vacuolation	0/16	1/16 (3.00)	0/15
Brain, Cerebellum: Vasculitis	0/16	3/16 (3.00)	4/15 (2.25)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal, Blood Vessels: Leucocytosis	1/16 (3.00)	2/16 (3.00)	3/15 (2.67)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal, Perivascular: Inflammation	1/16 (3.00)	9/16 (2.33)	6/15 (2.83)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal, Perivascular: Pigment	1/16 (3.00)	0/16	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 100% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 20% Mononuclear Cells	0/16	1/16 (NA)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 30% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 50% Granulocytes	1/16 (NA)	7/16 (NA)	6/15 (NA)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 50% Mononuclear Cells	1/16 (NA)	7/16 (NA)	6/15 (NA)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 70% Mononuclear Cells	0/16	1/16 (NA)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: 80% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Extravascular Bacteria	0/16	8/16 (2.13)	3/15 (3.33)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Giosis, Focal/Multifocal	0/16	0/16	1/15 (2.00)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Hemorrhage	2/16 (2.50)	6/16 (2.33)	1/15 (3.00)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Inflammation	0/16	1/16 (2.00)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Intravascular Bacteria	10/16 (2.80)	2/16 (3.50)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Neuronal Necrosis/Neuron Loss	0/16	0/16	1/15 (3.00)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Vacuolation	1/16 (2.00)	1/16 (2.00)	1/15 (3.00)
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Vacuoles	0/16	2/16 (2.50)	0/15
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Vasculitis, Multifocal	1/16 (3.00)	8/16 (2.25)	6/15 (2.83)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 59 (continued): Study AP204- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Brain, Hippocampus, Blood Vessels: Leucocytosis	0/16	1/16 (2.00)	1/15 (2.00)
Brain, Hippocampus, Perivascular: Inflammation	1/16 (3.00)	5/16 (2.60)	3/15 (2.67)
Brain, Hippocampus: 30% Granulocytes	0/16	0/16	1/15 (NA)
Brain, Hippocampus: 50% Granulocytes	1/16 (NA)	5/16 (NA)	2/15 (NA)
Brain, Hippocampus: 50% Mononuclear Cells	1/16 (NA)	5/16 (NA)	2/15 (NA)
Brain, Hippocampus: 70% Mononuclear Cells	0/16	0/16	1/15 (NA)
Brain, Hippocampus: Extravascular Bacteria	0/16	3/16 (2.33)	2/15 (2.00)
Brain, Hippocampus: Hemorrhage	0/16	3/16 (2.33)	1/15 (2.00)
Brain, Hippocampus: Intravascular Bacteria	7/16 (3.00)	2/16 (3.00)	0/15
Brain, Hippocampus: Vacuolation	0/16	0/16	1/15 (3.00)
Brain, Hippocampus: Vasculitis	0/16	2/16 (3.00)	3/15 (2.33)
Brain, Medulla Oblongata, Blood Vessels: Leucocytosis	0/16	1/16 (3.00)	0/15
Brain, Medulla Oblongata, Perivascular: Inflammation	0/16	2/16 (3.00)	1/15 (3.00)
Brain, Medulla Oblongata: 50% Granulocytes	0/16	2/16 (NA)	1/15 (NA)
Brain, Medulla Oblongata: 50% Mononuclear Cells	0/16	2/16 (NA)	1/15 (NA)
Brain, Medulla Oblongata: Gliosis, Focal	0/16	0/16	1/15 (3.00)
Brain, Medulla Oblongata: Intravascular Bacteria	8/16 (3.00)	2/16 (3.00)	1/15 (3.00)
Brain, Medulla Oblongata: Vasculitis	0/16	0/16	1/15 (3.00)
Brain, Meninges, Blood Vessels: Leucocytosis	2/16 (3.00)	6/16 (2.83)	5/15 (2.80)
Brain, Meninges: 20% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Meninges: 20% Mononuclear Cells	0/16	3/16 (NA)	0/15
Brain, Meninges: 30% Mononuclear Cells	1/16 (NA)	1/16 (NA)	3/15 (NA)
Brain, Meninges: 50% Granulocytes	1/16 (NA)	5/16 (NA)	4/15 (NA)
Brain, Meninges: 50% Mononuclear Cells	1/16 (NA)	5/16 (NA)	4/15 (NA)
Brain, Meninges: 70% Granulocytes	1/16 (NA)	1/16 (NA)	3/15 (NA)
Brain, Meninges: 80% Granulocytes	0/16	3/16 (NA)	0/15
Brain, Meninges: 80% Mononuclear Cells	0/16	1/16 (NA)	0/15
Brain, Meninges: Extravascular Bacteria	3/16 (3.00)	10/16 (3.90)	7/15 (4.00)
Brain, Meninges: Hemorrhage	3/16 (4.00)	11/16 (3.36)	6/15 (3.67)
Brain, Meninges: Infiltrates, Mononuclear Cells	1/16 (3.00)	1/16 (2.00)	2/15 (3.00)
Brain, Meninges: Inflammation	2/16 (3.50)	10/16 (3.90)	7/15 (4.14)
Brain, Meninges: Intravascular Bacteria	14/16 (2.57)	7/16 (3.29)	5/15 (2.80)
Brain, Meninges: Thrombosis	0/16	1/16 (3.00)	1/15 (3.00)
Brain, Meninges: Vasculitis	2/16 (2.50)	8/16 (3.00)	7/15 (3.00)
Brain, Midbrain, Blood Vessels: Leucocytosis	0/16	2/16 (2.50)	1/15 (2.00)
Brain, Midbrain, Perivascular: Infiltrates, Mononuclear Cells	0/16	1/16 (3.00)	0/15
Brain, Midbrain, Perivascular: Inflammation	1/16 (3.00)	9/16 (2.44)	4/15 (2.50)
Brain, Midbrain: 50% Granulocytes	1/16 (NA)	9/16 (NA)	4/15 (NA)
Brain, Midbrain: 50% Mononuclear Cells	1/16 (NA)	9/16 (NA)	4/15 (NA)
Brain, Midbrain: Extravascular Bacteria	0/16	5/16 (2.40)	1/15 (3.00)
Brain, Midbrain: Hemorrhage	0/16	6/16 (2.50)	0/15
Brain, Midbrain: Intravascular Bacteria	8/16 (2.75)	2/16 (3.00)	1/15 (3.00)
Brain, Midbrain: Vasculitis	0/16	6/16 (2.50)	4/15 (2.50)
Brain, Occipital/Visual Cortex, Blood Vessels: Leucocytosis	0/16	1/16 (3.00)	1/15 (2.00)
Brain, Occipital/Visual Cortex, Perivascular: Inflammation	2/16 (3.00)	6/16 (2.50)	6/15 (2.50)
Brain, Occipital/Visual Cortex: 50% Granulocytes	2/16 (NA)	6/16 (NA)	6/15 (NA)
Brain, Occipital/Visual Cortex: 50% Mononuclear Cells	2/16 (NA)	6/16 (NA)	6/15 (NA)
Brain, Occipital/Visual Cortex: Extravascular Bacteria	0/16	3/16 (2.67)	3/15 (2.00)
Brain, Occipital/Visual Cortex: Hemorrhage	1/16 (3.00)	5/16 (2.40)	2/15 (2.50)
Brain, Occipital/Visual Cortex: Inflammation	0/16	0/16	1/15 (2.00)
Brain, Occipital/Visual Cortex: Intravascular Bacteria	7/16 (3.00)	2/16 (3.00)	0/15
Brain, Occipital/Visual Cortex: Necrosis	0/16	0/16	1/15 (2.00)
Brain, Occipital/Visual Cortex: Vacuolation	1/16 (3.00)	2/16 (2.50)	0/15
Brain, Occipital/Visual Cortex: Vasculitis	2/16 (2.50)	6/16 (2.67)	5/15 (2.40)
Brain, Pons, Perivascular: Inflammation	0/16	5/16 (3.00)	1/15 (3.00)
Brain, Pons: 50% Granulocytes	0/16	5/16 (NA)	1/15 (NA)
Brain, Pons: 50% Mononuclear Cells	0/16	5/16 (NA)	1/15 (NA)
Brain, Pons: Hemorrhage	0/16	1/16 (3.00)	0/15
Brain, Pons: Intravascular Bacteria	8/16 (3.00)	2/16 (3.50)	0/15
Brain, Pons: Vasculitis	0/16	1/16 (3.00)	1/15 (3.00)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 59 (continued): Study AP204-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	4 mg/kg ETI-204 n/N (*)	16 mg/kg ETI-204 n/N (*)
Brain, Prefrontal/Cingulate/Premotor Cortex, Blood Vessels: Leucocytosis	1/16 (3.00)	6/16 (2.67)	3/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex, Perivascular: Inflammation	1/16 (3.00)	9/16 (2.56)	7/15 (2.57)
Brain, Prefrontal/Cingulate/Premotor Cortex, Perivascular: Pigment	0/16	1/16 (3.00)	1/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: 100% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Prefrontal/Cingulate/Premotor Cortex: 20% Mononuclear Cells	0/16	3/16 (NA)	0/15
Brain, Prefrontal/Cingulate/Premotor Cortex: 30% Granulocytes	0/16	0/16	1/15 (NA)
Brain, Prefrontal/Cingulate/Premotor Cortex: 50% Granulocytes	1/16 (NA)	6/16 (NA)	6/15 (NA)
Brain, Prefrontal/Cingulate/Premotor Cortex: 50% Mononuclear Cells	1/16 (NA)	6/16 (NA)	6/15 (NA)
Brain, Prefrontal/Cingulate/Premotor Cortex: 70% Mononuclear Cells	0/16	0/16	1/15 (NA)
Brain, Prefrontal/Cingulate/Premotor Cortex: 80% Granulocytes	0/16	3/16 (NA)	0/15
Brain, Prefrontal/Cingulate/Premotor Cortex: Extravascular Bacteria	0/16	6/16 (2.33)	5/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Gliosis, Focal/ Multifocal	0/16	2/16 (3.00)	1/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Hemorrhage	1/16 (3.00)	8/16 (2.63)	4/15 (2.50)
Brain, Prefrontal/Cingulate/Premotor Cortex: Inflammation	0/16	1/16 (3.00)	1/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Intravascular Bacteria	11/16 (2.82)	2/16 (3.00)	1/15 (3.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Necrosis	0/16	1/16 (3.00)	1/15 (2.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Vacuolation	1/16 (3.00)	5/16 (2.40)	2/15 (2.00)
Brain, Prefrontal/Cingulate/Premotor Cortex: Vasculitis, Multifocal	1/16 (3.00)	7/16 (2.71)	7/15 (2.86)
Brain, Temporal Lobe, Blood Vessels: Leucocytosis	0/16	3/16 (3.00)	2/15 (2.50)
Brain, Temporal Lobe, Perivascular: Infiltrates, Mononuclear Cells	1/16 (3.00)	0/16	0/15
Brain, Temporal Lobe, Perivascular: Inflammation	1/16 (3.00)	8/16 (2.75)	7/15 (2.29)
Brain, Temporal Lobe, Perivascular: Pigment	0/16	1/16 (2.00)	0/15
Brain, Temporal Lobe: 100% Granulocytes	0/16	1/16 (NA)	0/15
Brain, Temporal Lobe: 50% Granulocytes	1/16 (NA)	7/16 (NA)	7/15 (NA)
Brain, Temporal Lobe: 50% Mononuclear Cells	1/16 (NA)	7/16 (NA)	7/15 (NA)
Brain, Temporal Lobe: Extravascular Bacteria	0/16	4/16 (2.50)	1/15 (2.00)
Brain, Temporal Lobe: Gliosis, Focal/Multifocal	0/16	1/16 (3.00)	2/15 (2.50)
Brain, Temporal Lobe: Hemorrhage	1/16 (2.00)	6/16 (2.67)	2/15 (3.00)
Brain, Temporal Lobe: Intravascular Bacteria	8/16 (3.00)	2/16 (2.50)	0/15
Brain, Temporal Lobe: Vacuolation	1/16 (3.00)	0/16	0/15
Brain, Temporal Lobe: Vasculitis	1/16 (3.00)	8/16 (2.75)	6/15 (2.50)
Brain, Thalamus/Hypothalamus, Blood Vessels: Leucocytosis	0/16	1/16 (3.00)	1/15 (2.00)
Brain, Thalamus/Hypothalamus, Perivascular: Inflammation	0/16	5/16 (2.40)	1/15 (3.00)
Brain, Thalamus/Hypothalamus: 50% Granulocytes	0/16	5/16 (NA)	1/15 (NA)
Brain, Thalamus/Hypothalamus: 50% Mononuclear Cells	0/16	5/16 (NA)	1/15 (NA)
Brain, Thalamus/Hypothalamus: Extravascular Bacteria	0/16	2/16 (2.00)	1/15 (2.00)
Brain, Thalamus/Hypothalamus: Hemorrhage	0/16	3/16 (2.67)	1/15 (2.00)
Brain, Thalamus/Hypothalamus: Hemosiderin	1/16 (3.00)	0/16	0/15
Brain, Thalamus/Hypothalamus: Intravascular Bacteria	8/16 (3.00)	2/16 (3.50)	1/15 (3.00)
Brain, Thalamus/Hypothalamus: Vasculitis	0/16	5/16 (2.60)	1/15 (2.00)
Brain, Ventricular System, Blood Vessels: Leucocytosis	0/16	2/16 (3.00)	0/15
Brain, Ventricular System, Choroid Plexus: Bacteria	10/16 (2.80)	2/16 (3.50)	0/15
Brain, Ventricular System, Choroid Plexus: Infiltrates, Mononuclear Cells	2/16 (2.50)	1/16 (3.00)	1/15 (3.00)
Brain, Ventricular System, Choroid Plexus: Inflammation	1/16 (3.00)	1/16 (3.00)	3/15 (2.33)
Brain, Ventricular System: 20% Granulocytes	1/16 (NA)	0/16	1/15 (NA)
Brain, Ventricular System: 20% Mononuclear Cells	0/16	0/16	1/15 (NA)
Brain, Ventricular System: 50% Granulocytes	0/16	1/16 (NA)	1/15 (NA)
Brain, Ventricular System: 50% Mononuclear Cells	0/16	1/16 (NA)	1/15 (NA)
Brain, Ventricular System: 80% Granulocytes	0/16	0/16	1/15 (NA)
Brain, Ventricular System: 80% Mononuclear Cells	1/16 (NA)	0/16	1/15 (NA)
Brain, Ventricular System: Inflammation	0/16	1/16 (3.00)	0/15
Kidney			
Macroscopic Finding ¹			
# Necropsied/Total Infected	16/16	16/16	15/15
Gross Lesions	0/16 (NA)	0/16 (NA)	0/15 (NA)
Microscopic Finding ^{1, 4}			
# Necropsied/Total Infected	16/16	16/16	15/15
Kidney, Renal Tubules, Cortex: Necrosis	0/16	1/16 (1.00)	0/15
Kidney, Bacteria	12/16 (2.42)	3/16 (2.67)	2/15 (1.50)
NA, not applicable			
* Mean severity of lesion			
Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).			
¹ Gross necropsy and histopathology pathology performed at (b) (4)			
² Neuropathology performed at (b) (4)			
³ All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:			
1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)			
⁴ All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:			
1 (minimal); 2 (mild); 3 (moderate); 4 (marked)			
⁵ Tissues examined microscopically and found unremarkable			

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Table 59 (continued): Study AP204-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Placebo n/N (%)	Treatment Group		
		4 mg/kg ETI-204 n/N (%)	16 mg/kg ETI-204 n/N (%)	16 mg/kg ETI-204 n/N (%)
Liver				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	2/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Liver: Bacteria	13/16 (2.46)	3/16 (2.00)	2/15 (1.50)	
Liver: Necrosis	4/16 (2.00)	3/16 (1.00)	2/15 (1.50)	
Liver: Parasitic Granuloma (Incidental)	1/16 (2.00)	0/16	0/15	
Lymph Nodes, Mediastinal				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	0/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Lymph Node, Mediastinal, Lymphoid Follicles: Necrosis/Atrophy	15/16 (3.80)	12/16 (3.83)	8/15 (3.38)	
Lymph Node, Mediastinal, Paracortical Lymphocytes: Necrosis/Atrophy	10/16 (2.60)	6/16 (2.50)	4/15 (2.75)	
Lymph Node, Mediastinal: Bacteria	12/16 (2.42)	5/16 (1.80)	4/15 (1.50)	
Lymph Node, Mediastinal, Fibrin Deposition	3/16 (1.67)	3/16 (1.00)	0/15	
Lymph Node, Mediastinal: Infiltration, Neutrophils	1/16 (2.00)	0/16	0/15	
Lung				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	0/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Lung, Alveoli: Edema/Exudate	1/16 (2.00)	0/16	0/15	
Lung, Bronchus: Acute Inflammation	0/16	1/16 (2.00)	0/15	
Lung, Peribronchial Veins: Hemorrhage	2/16 (1.50)	3/16 (1.67)	1/15 (2.00)	
Lung: Bacteria	13/16 (2.62)	3/16 (2.33)	3/15 (1.33)	
Spleen				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	0/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Spleen, Lymphoid Follicles: Necrosis/Atrophy	15/16 (3.80)	10/16 (2.90)	8/15 (2.63)	
Spleen, Red Pulp: Fibrin Deposition	2/16 (1.50)	5/16 (1.80)	4/15 (1.25)	
Spleen: Bacteria	12/16 (3.08)	3/16 (2.33)	2/15 (1.00)	
Skin				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	2/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Skin: Bacteria	2/16 (1.00)	0/16	0/15	
Thymus				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	2/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4}				
# Necropsied/Total Infected	16/16	16/16	15/15	
Thymus: Bacteria	2/16 (1.00)	0/16	0/15	
Body Cavity				
Macroscopic Finding ¹				
# Necropsied/Total Infected	16/16	16/16	15/15	
Gross Lesions	2/16 (NA)	0/16 (NA)	0/15 (NA)	
Microscopic Finding ^{1,4,5}				
# Necropsied/Total Infected	16/16	16/16	15/15	

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

⁵Tissues examined microscopically and found unremarkable

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Table 60: Study AP204 - Comparison of published morphologic changes in the brain of anthrax infected (inhalation) monkeys with those in the current study

Diagnosis	Percentage Published Study	Percentage Current Study, Controls (Males and Females combined) Group Y	Percentage Current Study, 4.0 mg/kg ETI-204 (Males and Females Combined) Group X	Percentage Current Study, 16.0 mg/kg ETI-204 (Males and Females Combined) Group Z
Bacteria, intravascular/extravascular (Meninges, Bacteria)	71%	94% (15/16)	69% (11/16)	50% (8/16)
Necrotizing vasculitis (Meninges, Vasculitis)	14%	13% (2/16)	50% (8/16)	44% (7/16)
Hemorrhage, meningeal (Meninges, Hemorrhage)	57%	19% (3/16)	69% (11/16)	38% (6/16)
Hemorrhage, parenchymal (Hemorrhage, brain, any area)	29%	13% (2/16)	56% (9/16)	38% (6/16)
Meningitis, suppurative (Meninges, Inflammation)	21%	13% (2/16)	63% (10/16)	44% (7/16)
Leukomalacia (Parenchymal, Necrosis)	14%	0% (0/16)	19% (3/16)	25% (4/16)
(Parenchymal, Vacuolation)*	--	6% (1/16)	38% (6/16)	13% (2/16)
(Brain, Perivascular Inflammation)*	--	13% (2/16)	56% (9/16)	44% (7/16)
(Brain, Vasculitis)*	--	13% (2/16)	50% (8/16)	44% (7/16)
Overall mortality	Not provided	94% (15/16)	75% (12/16)	50% (8/16)

Note: The primary diagnosis is from the referenced publication. The diagnosis in parentheses is the comparable diagnosis in the current study.

*Diagnosis not used in the published study (Reference 3)

**Mortality figures taken from the Study Pathologist's draft report (75% = Group X [4.0 mg/kg ETI-204], 94% = Group Y [saline], 50% = Group Z [16.0 mg/kg ETI-204])

Tissue bacterial assessments:

Histology: No bacteria were reported in any of the tissues examined from the animals that survived; however, bacteria were observed in most of the tissues from a majority of the animals that died or were found moribund and euthanized (Table 61).

Culture: Most of the tissues including brain and lungs, from animals treated with either of the dose of ETI-204 and either died or were moribund/euthanized on study, were culture positive (Table 61). Of the 13 animals that survived, lung tissue was culture positive in 9 animals whereas the remaining tissues were culture negative; of these, one animal was in the control group (Table 61). The lungs from surviving animals were culture positive but no bacteria were observed by microscopic examination; this suggests the possibility of the presence of bacteria at a level that were not detected by light microscopic evaluation compared to cultures. Therefore, cultures are more sensitive than microscopic examination.

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Table 61: Study AP204-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 4mg/kg		ETI-204 16mg/kg	
	Survivors (N=1)	Non Survivors (N=15)	Survivors (N=4)	Non Survivors (N=12)	Survivors (N=8)	Non Survivors (N=8)
Presence of bacteria by microscopy [1, 5]						
Brain: Total [3, 4]	0/1	15/15	0/4	11/12	0/8	8/8
Brain, Basal Ganglia: Extravascular	0/1	0/15	0/4	3/12	0/8	1/8
Brain, Basal Ganglia: Intravascular	0/1	9/15	0/4	2/12	0/8	1/8
Brain, Cerebellum: Extravascular	0/1	0/15	0/4	1/12	0/8	2/8
Brain, Cerebellum: Intravascular	0/1	7/15	0/4	2/12	0/8	0/8
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Extravascular	0/1	0/15	0/4	8/12	0/8	3/8
Brain, Cerebral Cortex-Motor/Somatosensory/Parietal: Intravascular	0/1	10/15	0/4	2/12	0/8	0/8
Brain, Hippocampus: Extravascular	0/1	0/15	0/4	3/12	0/8	2/8
Brain, Hippocampus: Intravascular	0/1	7/15	0/4	2/12	0/8	0/8
Brain, Medulla Oblongata: Intravascular	0/1	8/15	0/4	2/12	0/8	1/8
Brain, Meninges: Extravascular	0/1	3/15	0/4	10/12	0/8	7/8
Brain, Meninges: Intravascular	0/1	14/15	0/4	7/12	0/8	5/8
Brain, Midbrain: Extravascular	0/1	0/15	0/4	5/12	0/8	1/8
Brain, Midbrain: Intravascular	0/1	8/15	0/4	2/12	0/8	1/8
Brain, Occipital/Visual Cortex: Extravascular	0/1	0/15	0/4	3/12	0/8	3/8
Brain, Occipital/Visual Cortex: Intravascular	0/1	7/15	0/4	2/12	0/8	0/8
Brain, Pons: Intravascular	0/1	8/15	0/4	2/12	0/8	0/8
Brain, Prefrontal/Cingulate/Premotor Cortex: Extravascular	0/1	0/15	0/4	6/12	0/8	5/8
Brain, Prefrontal/Cingulate/Premotor Cortex: Intravascular	0/1	11/15	0/4	2/12	0/8	1/8
Brain, Temporal Lobe: Extravascular	0/1	0/15	0/4	4/12	0/8	1/8
Brain, Temporal Lobe: Intravascular	0/1	8/15	0/4	2/12	0/8	0/8
Brain, Thalamus/Hypothalamus: Extravascular	0/1	0/15	0/4	2/12	0/8	1/8
Brain, Thalamus/Hypothalamus: Intravascular	0/1	8/15	0/4	2/12	0/8	1/8
Brain, Ventricular System, Choroid Plexus: Bacteria	0/1	10/15	0/4	2/12	0/8	0/8
Kidney	0/1	12/15	0/4	3/12	0/8	2/8
Liver	0/1	13/15	0/4	3/12	0/8	2/8
Lymph Nodes, Mediastinal	0/1	12/15	0/4	5/12	0/8	4/8
Lung	0/1	13/15	0/4	3/12	0/8	3/8
Spleen	0/1	12/15	0/4	3/12	0/8	2/8
Skin	0/1	2/15	0/4	0/12	0/8	0/8
Thymus	0/1	2/15	0/4	0/12	0/8	0/8
Body Cavity	0/1	0/15	0/4	0/12	0/8	0/8
Presence of bacteria by culture [1, 2]						
Brain	0/1	14/15	0/4	11/12	0/8	7/8
Kidney	0/1	13/15	0/4	7/12	0/8	4/8
Liver	0/1	15/15	0/4	11/12	0/8	4/8
Lung	1/1	15/15	2/4	12/12	6/8	8/8
Spleen	0/1	15/15	0/4	11/12	0/8	7/8
Lymph Nodes, Bronchial	0/1	14/15	0/4	9/12	1/8	7/8
[1] All treated animals irrespective of bacteremia status prior to treatment						
[2] Animal was considered positive if at least 1-5 colonies were present on plate						
[3] Neuropathology performed at (b) (4)						
[4] Animals that were positive in at least one area for either extra- or intra-vascular bacteria						
[5] Histopathology performed at (b) (4) with exception of brain; Not all animals were assessed microscopically; numbers examined are shown						
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: C43316						
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None						
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: C43304						

The applicant investigated if the *B. anthracis* contained in the lungs at the time of sacrifice was vegetative bacteria or spores; for this, the lung tissue was heat-shocked (60°C for 60 minutes) to kill any vegetative bacteria present and the samples were re-plated and incubated for >48 hours. Any growth after heat-shock would suggest the presence of spores in the lung tissue. The applicant states that "data suggest that there were *B. anthracis* spores present in the lung tissue for 71.4% (5/7) of the monkeys sacrificed at Day 28, and 33% (2/6) of the monkeys sacrificed at Day 56. This indicates that monkeys can survive with sporulated *B. anthracis* in their lung tissue up to 56 days after challenge without showing any signs of infection." However, these results should be interpreted with caution as data for animals prior to heat shock of the refrigerated samples were not available.

Comments:

The study showed that ETI-204 (Baxter) at a dose of 16 mg/kg was more effective in improving survival (47%) up to Day 56 compared to the lower dose group of 4 mg/kg (25%) or untreated control group (6%). All of the non-surviving animals died within 7 days of challenge. One animal in the control group survived until the end of study.

A majority of the animals were bacteremic and PA positive at the time of initiation of treatment. Higher bacteremia levels and higher PA levels at the time of initiation of treatment appear to be associated with a lower survival rate.

*Among monkeys that were found dead or euthanized, gross lesions were consistent with acute *B. anthracis* infection; additionally, the tissues from animals found dead or euthanized were positive for bacteria. No gross lesions were found in surviving animals; some of the tissues especially lungs from the animals that survived were culture positive consistent with *B. anthracis*. The applicant states that this is consistent with the results from previous studies which have shown that spores can be found in the lung up to 56 days after challenge in surviving nonhuman primates (Henderson et al., 1956¹). The applicant did examine the presence of spores or vegetative bacteria in the lung by heat treatment of the tissue suspensions at 60°C for 60 minutes; the applicant concluded that the spores were present in the lung of surviving animals at Day 28 or Day 56. However, the results should be interpreted with caution as the data for isolates prior to heat shock of the refrigerated samples were not available.*

The neuropathological examination of the non-surviving animals had intravascular and/or extravascular bacteria, suggesting the cause of death in these animals was a bacterial infection. Only ETI-204-treated non-surviving animals had evidence of an inflammatory response that was always in association with extravascular bacteria. None of the deaths were attributed to ETI-204.

ETI-204-treated animals that died or were sacrificed in a moribund condition (compared to controls) more commonly developed an appreciable morphologic/inflammatory response. It is possible that the affected ETI-204-treated animals' immune system was able to mount a response whereas the saline-treated animals lacked this ability.

6.3. Efficacy of ETI-204 in combination with an antibacterial drug

The efficacy of different products of ETI-204 (see Appendix-4) in combination with antibacterial drugs was measured in experimentally naive NZW rabbits and cynomolgus monkeys.

6.3.1. New Zealand White rabbits

The efficacy of ETI-204 in combination with levofloxacin (NIAID Study 1030, NIAID Study 1045, AR034, AR028, and AR007) or doxycycline (NIAID Study AP-10-055) was evaluated in six studies.

6.3.1.1. ETI-204 in combination with levofloxacin

All studies except one (Study AR028) were performed using human equivalent dose (50 mg/kg) of levofloxacin; the levofloxacin dose used for Study AR028 was 6.5 mg/kg.

6.3.1.1. Study 1030

This was an open-label, randomized, controlled, factorial design non-GLP study to assess the efficacy of ETI-204 (IV) and levofloxacin (oral) in 54 NZW rabbits 4 days post-challenge with the spores (spore lot no. B34) of the Ames strain of *B. anthracis* weighing 2.2 to 2.7 kg, conducted at [REDACTED] (b) (4) 50. The primary objective was to demonstrate that post-exposure administration of ETI-204 leads to increased survival above that of levofloxacin (50 mg/kg – human equivalent dose). Other objectives were to determine the efficacy of

- an anti-PA monoclonal antibody when administered following a SIBT.
- delayed treatment with levofloxacin or anti-PA monoclonal antibody in combination with levofloxacin.

Study design:

The study design was similar to the rabbit studies summarized above in section 6.2 except that animals were divided into four groups and treatment with ETI-204 (8 mg/kg) was initiated based on SIBT; treatment with levofloxacin (50 mg/kg) for 3 days or a combination of ETI-204 (8 mg/kg) and levofloxacin (50 mg/kg for 3 days) was initiated at a fixed time point (Day 4) post-challenge (Table 62). Microbiologic parameters measured include bacteremia by qualitative culture as well as PA by ECL assay and ELISA. Levofloxacin MICs were determined on isolates collected from positive blood cultures at the end of the study. MICs were determined by the broth dilution method using Mueller Hinton Broth (MHB) and cultures incubated for 16-20 hours. TNA was performed on residual serum samples collected at Study Day -1 and Day 28 at the applicant's discretion.

Table 62: Study 1030 - Study design and blood collection assay schedule

Group	Number of Rabbits	Treatment Type	Treatment Dose (SID)	Levofloxacin Regimen	Initial Treatment Time	Blood Collection Bacteremia and PA Levels (Relative to Median Challenge)
1	16	Antibody	8 mg/kg	N/A	SIBT	Hours -24^*, 24, 48, 72, 96 PTT Days 7, 14, 28*
2	16	Levofloxacin	50 mg/kg	3 days	96 hours post-median challenge +/- 1 hour	Hours -24^*, 24, 48, 72, 96 Days 7, 14, 28*
3	16	Antibody + Levofloxacin	Antibody - 8 mg/kg Levofloxacin - 50 mg/kg	3 days	96 hours post-median challenge +/- 1 hour	Hours -24^*, 24, 48, 72, 96 Days 7, 14, 28*
4	6	None	N/A	N/A	N/A	Hours -24^*, 24, 48, 72, 96 Days 7, 14, 28*

* = TNA analysis was performed based on Applicant's discretion

^ = a portion of the blood was used to determine levofloxacin baseline levels

PTT = A blood sample (EDTA and SST) was collected within 30 minutes prior to treatment.

EDTA = Ethylene diamine tetra acetic acid. N/A = not applicable. PA = protective antigen

PTT = prior to treatment. SIBT = significant increase in body temperature

SID = solution once daily. SST=serum separator tube

50 [REDACTED] (b) (4) Study Number 1030-G607604: NIAID 1030 - Determining the therapeutic efficacy of a novel anti-PA antibody administered alone or in combination with levofloxacin to New Zealand White rabbits following a *Bacillus anthracis* inhalation challenge (May 2, 2012).

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Animals in Group 1 were treated with ETI-204 within three hours of obtaining a positive PA-ECL assay finding. However, treatment of animals in Groups 2 and 3 was delayed and administered at 96 hours. Of the 54 rabbits randomized to the four study groups 23 died before treatment initiation in Groups 2 and 3 (Table 63); this was perhaps due to a delay in treatment after PA positive findings.

Table 63: Study 1030: Animal disposition before treatment initiation

	Group 4 Control	Group 1[‡] ETI-204 (8 mg/kg)	Group 2[‡] Levo (50 mg/kg)[§]	Group 3[‡] Levo (50 mg/kg)[§] + ETI-204 (8 mg/kg)	Total
Animals challenged	6	16	16	16	54
Animals who died before treatment	NA [‡]	0	11	12	23/48 (52%)
Animals who survived to be treated*	NA [‡]	16	5 [§]	4 [§]	34/40 (85%)

*Represent modified intent to treat (mITT)
[‡]No vehicle was administered to control group animals.
[§]Group 2: levofloxacin (50 mg/kg) treatment initiated by gavage at 96 hours, for 3 days.
Group 3: levofloxacin administered as for Group 2 animals + ETI-204 (single dose).
Levo=levofloxacin
Animal L23016 in Levo group was never bacteremic.
Animal L23040 in ETI-204 & Levo group became bacteremia post treatment.

Results:

Baseline characteristics: Prior to challenge, all animals were PA negative by the ECL assay; however one animal (L23009) in Group 4 (control group) was PA positive (1.22 ng/mL) by ELISA. Cultures were not done. Age, gender, body weight, and challenge dose (mean LD₅₀ 175.6; [redacted]^{(b)(4)} cfu) were comparable among different groups; the LD₅₀ was ≥ 200 for approximately 30% of the animals (Table 64). The MMAD for each exposure day was 1.11 µm which is consistent with the particle size range that would reach the alveoli.

Time between challenge and treatment initiation was approximately 31 hours for animals treated with ETI-204 and 96 hours for animals treated with levofloxacin or levofloxacin+ETI-204.

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Table 64: Study 1030 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 4 ^{\$} Control N = 6	Group 1 ETI-204 (8 mg/kg) N = 16	Group 2 Levo (50 mg/kg) N = 16	Group 3 ETI-204 (8 mg/kg) + Levo (50 mg/kg) N = 16
Baseline characteristics				
Age (months) estimated range	4.0±0	4.0±0	4.0±0	4.0±0
Body weight (kg)	2.5±0.1	2.5±0.1	2.5±0.1	2.5±0.1
PA-ECL n/N	0/6	0/16	0/16	0/16
PA-ELISA n/N (ng/mL)	1/6 (1.22)	0/16	0/16	0/16
Inhaled dose				
Total Inhaled Dose: cfux $\times 10^7$				
Mean ± SD (Range)				
LD ₅₀ Mean ± SD (Range)	183.8±20.1 (157-209)	178.9±68.9 (87-362)	185.4±42.4 (83-251)	159.3±37.6 (93-227)
<200 LD ₅₀ n (%)	5 (83.3)	11 (68.8)	9 (56.2)	13 (81.2)
≥200 LD ₅₀ n (%)	1 (16.7)	5 (31.2)	7 (43.8)	3 (18.8)
Microbial findings [PA (by ECL and ELISA), bacteremia] and SIBT prior to treatment				
PA				
PA/ECL Assay n (%)	6 (100) [§]	13 (81.3)	16 (100)	16 (100)
PA/ELISA n (%)	6 (100) [§]	8 (50.0)	16 (100)	15 (93.8)
Bacteremia				
Qualitative bacteremia n (%)	6 (100) [§]	12 (75.0)	15 (93.8)	16 (100)
SIBT				
n (%)	6 (100) [§]	15 (93.8)	16 (100)	16 (100)
Time (hours) to first PA⁺, bacteremia, SIBT and treatment post-challenge				
Time to first PA⁺ (ECL) post-challenge Mean±SD (Range)	40.9±24.1 [§] (22.2-72.6) n=6	33.4±12.9 (22.9-70.1) n=14	39.3±12.1 (21.9-50.8) n=16	37.3±14.6 (22.7-70.1) n=16
Time to first PA⁺ (ELISA) post-challenge Mean±SD (Range)	50.7±19.7 (26.7-87) n=6	46.5±42.5 (24.3-168.6) n=11	56.3±15.9 (44.7-97.7) n=16	45.3±23.4 (24.9-97.8) n=15
Time to bacteremia Mean±SD (Range)	43.9±21.7 [§] (23.3-70.3) n=6	29.8±8.4 (21.6-51.5) n=13	43.8±19.8 (20.9-73.2) n=15	49.8±36.8 (24.1-165) n=16
Time to SIBT Mean±SD (Range)	29.8±2.6 (27.7-34.2) n=6	31.8±6.7 (25.2-51.2) n=15	32±4.8 (26.6-46.5) n=16	30.4±4.7 (21.6-38.9) n=16
Time to treatment post challenge ; Mean ± SD	NA	31.4±7.0 ^a	95±1.4	94.9±2
Survivors at the end of study (Day 28)				
Survived to be treated	0 (0)	12/16 (75.0)**	2/5	4/4
Bacteremic animals that survived to be treated	0 (0)	8/12 (66.7)**	2/4	3/3

*Animal L23016 in levofloxacin group was never bacteremic.

** Animal L23040 in ETI-204 & levofloxacin group become bacteremia post treatment.

^{\$}All animals in the control group were PA⁺ by ECL at least one time between 24 and 72 hours post-challenge. By ELISA, all animals were PA⁺ at least at one time point post-challenge. All animals were bacteremic by quantitative culture at 48 hours. However, there is no PTT as no vehicle was administered.

SD=Standard deviation; PTT=Prior to treatment;

Qualitative culture LOD =25 cfu/mL; PA ELISA LOD 2 ng/mL and LLOQ=4.9 ng/mL. PA ECL LOD=4 ng/mL; however, a positive control of 2 ng/mL was positive.

^aThe time to treatment was defined as the time from challenge to treatment.

**Statistically significance by exact method and Boschloo's one-sided test between Groups 1 and 4; Differences between Groups 2 and 3 not significant (for details see statistics reviews by Drs Xianbin Li and Ling Lan)

Detection of Bacteremia and PA: The variability among the results of qualitative culture and the detection of PA by ELISA and ECL assays is shown in Table 65. The samples used for all these assays were collected at the same time points post-challenge. Of the 31 rabbits in the four Groups, 26 were culture positive; 28 were PA positive by ECL and 22 by ELISA. About 68 % (21/31) were culture positive as well as PA positive by both ELISA and ECL assays. ELISA for the detection of PA was the least sensitive in this study.

Table 65: Study 1030 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Qualitative Enriched Culture	Quantitative Culture	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay) †	Treatment Group				Total (n=31)
					Untreated Control (n=6)	8 mg/kg ETI-204 (n=16)	50 mg/kg Levo (n=5)	8 mg/kg ETI-204 + 50 mg/kg Levo (n=4)	
-	ND	ND	-	-	0	3	0	0	3
-	ND	ND	+	-	0	0	0	1	1
-	ND	ND	+	+	0	0	1	0	1
+	ND	ND	+	-	1	4	0	0	5
+	ND	ND	+	+	5	9	4	3	21

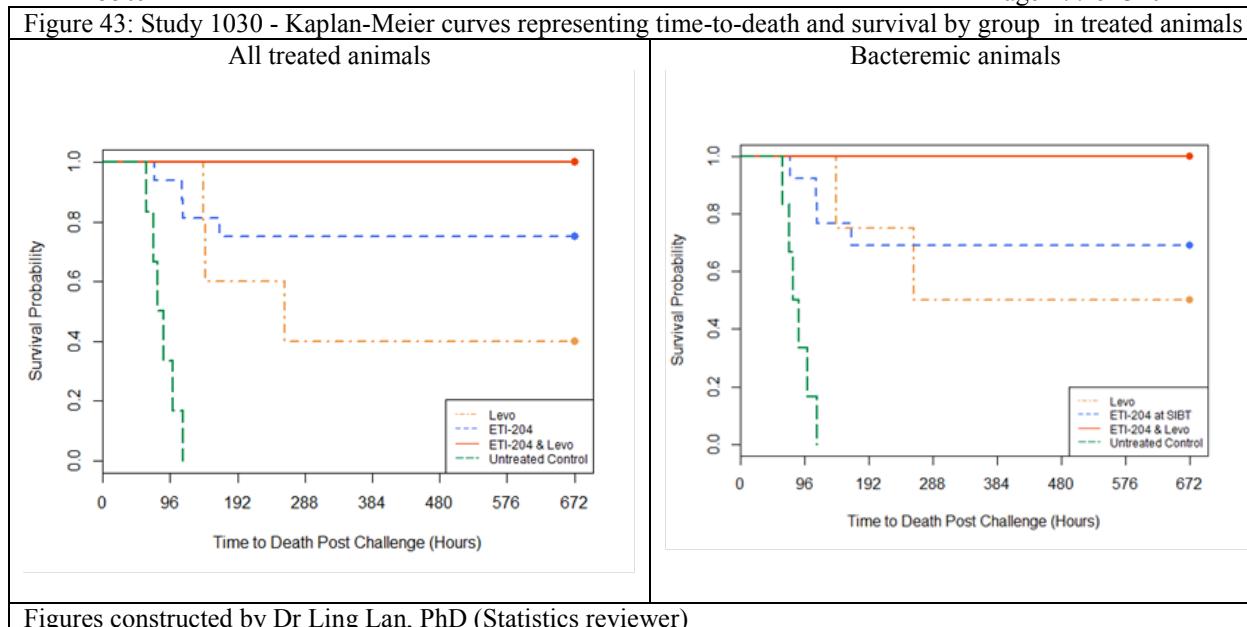
n = Number of treated animals. Control group was not treated and all control animals are included
Results determined on a per animal basis, not for individual tests. Animal with a positive test from any time prior to treatment is considered as positive.
ND = Not Done. NA=Not applicable
*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40 µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (1 ng/mL); LOD was 4.0 ng/mL
†LLOQ by free PA ELISA 4.9 ng/mL; the putative limit of detection (LOD) was stated to be 2.0 ng/mL.

Effect of ETI-204 treatment on survival: ETI-204 at a dose of 8 mg/kg was effective in improving survival in 75% of the animals until Day 28; 4 animals died by Day 10 (Table 64 and Figure 43). All the animals in the control untreated group died by Day 5.

About 75% of the animals in the ETI-204 treated group were bacteremic at the time of treatment. Culture negative findings were reported for three rabbits (L23003, L23035, and L20043) at all the time points in the ETI-204 treated group; two of these rabbits (L23003 and L23035) were also PA negative by ECL assay at all the time points tested. One rabbit (L23035) was PA negative by ELISA as well.

Effect of ETI-204 + levofloxacin treatment on survival: There were 5 animals in levofloxacin (Group 2) and 4 animals in the ETI-204 + levofloxacin (Group 3) treated groups that survived until the time of treatment initiation (96 hours). Of these, one animal in the levofloxacin group and one animal in the ETI-204 + levofloxacin group was culture negative at the time of treatment administration.

Two of the animals in the levofloxacin group and all the 4 animals in the ETI-204 + levofloxacin survived the period of observation (Table 64 and Figure 43); these observations should be interpreted with caution due to a small number of animals in each group.



Effect of treatment on PA and bacteremia at different time points: All the surviving animals treated with ETI-204 (Group 1) were PA negative by the ECL assay by Day 7; all animals were PA negative by ELISA as well as culture negative by Day 14 (Table 66). In the levofloxacin treated group, the surviving animals were PA negative (by ECL and ELISA) by Day 14 and culture negative by Day 7. The four animals treated with ETI-204 + levofloxacin, were PA negative and culture negative by Day 14 (Table 66).

The changes in PA levels over time were similar in the animals treated with ETI-204 + levofloxacin or levofloxacin treated groups (Figure 44). PA levels in animals treated with ETI-204 were lower than the control group animals or those treated with a combination of ETI-204 + levofloxacin or levofloxacin at 96 hours and Day 7.

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Table 66: Study 1030 – Number of animals PA positive (by ECL and ELISA) and bacteremic at different time points post-challenge

Study Time	Group 4 Control	Group 1 ETI-204 (8 mg/kg)	Group 2 Levo (50 mg/kg)	Group 3 ETI-204 (8 mg/kg) + Levo (50 mg/kg)
Number of animals PA^{+ve} by ECL				
-24 hours	0/6	0/16	0/16	0/16
24 hours	4/6	5/16	6/16	9/16
48 hours	5/6	2/16	16/16	15/16
72 hours	5/5	2/16	9/10	9/9
96 hours	2/2	3/15	5/5	4/4
Day 7	*	0/12	1/3	1/4
Day 14, 28	*	0/12	0/2	0/4
PTT	*	13/16	NA	NA
Unscheduled terminal	*	ND	ND	ND
Number of animals PA^{+ve} (Range) by ELISA				
-24 hours	0/6	0/16	0/16	0/16
24 hours	1/6	1/16 (17)	0/16	6/16 (2-86)
48 hours	5/5	9/16 (2-275)	11/16 (2-434)	13/15 (2-232)
72 hours	4/5	9/16 (2-1105)	8/10 (3-480)	6/9 (6-7737)
96 hours	2/2	5/13 (2-1049)	5/5 (8-314)	3/4 (30-629)
Day 7	*	1/12 (4)	0/3	2/4 (241-261)
Day 14, 28	*	0/12	0/2	0/4
PTT	*	9/16	NA	NA
Unscheduled terminal	5/5	0/1	11/12 (7-21220)	10/10 (67-19604)
Number of animals bacteremic				
24 hours	3/6	6/16	6/16	7/16
48 hours	4/6	9/16	11/16	13/16
72 hours	5/5	10/16	9/10	7/9
96 hours	2/2	9/15	4/5	3/4
Day 7	*	5/12	0/3	1/4
Day 14, 28	*	0/12	0/2	0/4
PTT	*	12/16	*	*
Unscheduled terminal	6/6	3/4	11/14	12/12
TNA in animals that survived n/N Mean±SD (Range)				
Day -1 ED ₅₀	!	0/12	0/2	0/4
		12/12	2/2	4/4
Day 28 ED ₅₀	!	1682.6±1275.9 (155.9-4625.6)	(3380.6-4106.2)	1664.5±832.3 (452.0-2305.9)
Day -1 NF ₅₀	!	0/12	0/2	0/4
		12/12	2/2	4/4
Day 28 NF ₅₀	!	3.53±2.71 (0.32-9.92)	(7.2-8.8)	3.5±1.8 (0.9-4.9)

*No animals survived; ND=not done; NA=not applicable as animals were treated at 96 hours; !No survivors
ED₅₀ - effective dilution-50 is defined as the reciprocal of the dilution corresponding to the inflection point ('c' parameter) of a 4-parameter logistic log fit of the curve.

NF₅₀ - neutralization factor-50 is a quotient of the ED₅₀ of the test sample and the ED₅₀ of the reference serum.

Figure 44: Study 1030 - PA levels at different time points

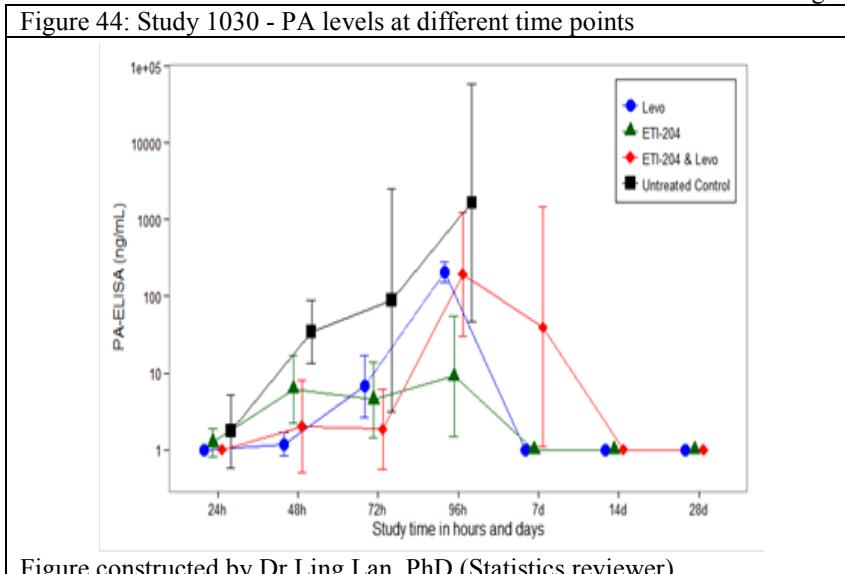


Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

In vitro susceptibility testing: The *in vitro* susceptibility of the isolates to levofloxacin collected at the time animals died or last positive culture prior to death (0.5 and 4 µg/mL) were similar to challenge strain used (spore lot no. B34 MIC 2 and 4 µg/mL) for aerosolization. The levofloxacin MIC for another spore lot (no. B21) was 4.00 µg/mL.

Toxin neutralizing antibodies: Toxin neutralizing antibodies were measured by the TNA assay using J774A.1 cells and serum from rabbits surviving until Day 28. The results were expressed as ED₅₀⁴⁷ and neutralization factor-50 (NF₅₀)⁵¹. The results showed that neutralizing antibodies were present at Day 28; all the animals at Day -1 were antibody negative (Table 66). Antibody response in non-survivors at different time interval during the study was not measured.

Clinical Observations: Abnormal clinical signs were observed starting approximately 48 hours post-challenge; these signs include lethargy, inappetence, respiratory and stool (soft stool; diarrhea) abnormalities that were similar to those reported in the natural history studies summarized above. After Day 7 post-challenge, clinical signs of illness were not observed, except for sporadic documentation of decreased appetite or diarrhea (with the exception of Animal L23004). Animal L23004 (Group 2) had continual lethargy, persistent decreased appetite and respiratory abnormalities through Day 15 post-exposure; however, this animal was documented as normal after Day 15 and survived to the scheduled euthanasia on Day 28 post-challenge.

Body temperatures returned to normal in all the treated animals in Groups 1 to 3. The time from treatment until resolution appears to be shorter in levofloxacin-treated animals compared to those treated with ETI-204 alone; however, such differences should be interpreted with caution due to a small number of animals in Groups 2 and 3.

Necropsy and Histopathology: Gross lesions in rabbits were similar to those reported in the natural history and other studies summarized above and include discoloration of the brain or

⁵¹ Neutralization factor-50 (NF₅₀) is a relative measure of toxin neutralization and represents a quotient of the ED₅₀ of the test sample and the ED₅₀ of the reference serum.

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lung, foci in the appendix or large intestine, enlargement of the mediastinal and/or bronchial lymph nodes, fluid in the thymus, and fluid (effusion) in the thoracic cavity or pericardial cavity. These lesions correlated histologically with hemorrhage, necrosis, edema, and acute inflammation. No rabbits surviving to scheduled euthanasia on Day 28 had gross lesions (Table 67).

Microscopic findings considered consistent with anthrax infection were present in all rabbits that died or became moribund during the study (Table 67). Lesions typical of anthrax included acute suppurative inflammation, necrosis, hemorrhage, edema, and the presence of large rod-shaped bacteria in the appendix, brain, liver, lung, thymus, bronchial and/or mediastinal lymph nodes, kidney and/or spleen. Brain lesions in these rabbits occasionally had minimal areas of necrosis in the submeningeal parenchyma, in areas of the most severe inflammation, and rare fibrinoid necrosis of associated meningeal vessels. Rabbits that survived until Day 28 often had minimal chronic inflammation in the lung. The applicant stated that most of the animals that survived to scheduled euthanasia on Day 28 also had variable clusters of hemosiderin or eosinophilic granular debris-laden macrophages within the spleen supporting inflammation/infection of these animals prior to Day 28. Most lesions of anthrax were qualitatively similar across study groups, regardless of treatment.

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Table 67: Study 1030- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	50 mg/kg Levo + 50 mg/kg Levo n/N (*)	8 mg/kg ETI-204 n/N (*)
Brain				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	1/ 6 (NA)	1/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	2/ 6 (2.00)	1/13 (4.00)	0/ 4	0/ 3
Hemorrhage	1/ 6 (4.00)	2/13 (2.50)	0/ 4	0/ 3
Inflammation, acute	0/ 6	1/13 (2.00)	0/ 4	0/ 3
Necrosis	1/ 6 (1.00)	1/13 (1.00)	0/ 4	0/ 3
Vasculitis	0/ 6	1/13 (1.00)	0/ 4	0/ 3
Kidney				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	6/ 6 (2.33)	0/13	0/ 4	0/ 3
Hemorrhage	2/ 6 (1.50)	0/13	0/ 4	1/ 3 (1.00)
Lymph node, Bronchial				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2, 3}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Lymph Node, Mediastinal				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	4/ 6 (NA)	2/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	6/ 6 (3.17)	2/13 (2.50)	0/ 4	0/ 3
Depletion/necrosis, lymphocytes	5/ 6 (3.20)	4/13 (3.25)	2/ 4 (1.50)	0/ 3
Fibrin accumulation	6/ 6 (2.67)	4/13 (2.75)	1/ 4 (2.00)	0/ 3
Hemorrhage	3/ 6 (3.00)	4/13 (2.25)	1/ 4 (3.00)	0/ 3
Inflammation, acute	2/ 6 (2.00)	0/13	0/ 4	0/ 3
Lung				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	6/ 6 (2.67)	2/13 (2.00)	0/ 4	0/ 3
Fibrin accumulation	4/ 6 (2.75)	3/13 (1.00)	1/ 4 (1.00)	0/ 3
Hemorrhage	2/ 6 (2.50)	0/13	0/ 4	0/ 3
Inflammation, acute	5/ 6 (1.60)	4/13 (2.00)	1/ 4 (1.00)	1/ 3 (1.00)
Inflammation, chronic	0/ 6	6/13 (1.17)	1/ 4 (2.00)	0/ 3
Inflammation, acute	1/ 6 (1.00)	0/13	0/ 4	0/ 3
Necrosis	3/ 6 (2.33)	1/13 (2.00)	0/ 4	0/ 3
Thrombosis	0/ 6	1/13 (3.00)	2/ 4 (2.50)	0/ 3
Spleen				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	5/ 6 (3.20)	2/13 (2.50)	0/ 4	0/ 3
Depletion/necrosis, lymphocytes	4/ 6 (2.25)	1/13 (2.00)	1/ 4 (1.00)	0/ 3
Fibrin accumulation	3/ 6 (2.00)	2/13 (2.00)	0/ 4	0/ 3
Hemorrhage	2/ 6 (2.50)	1/13 (3.00)	0/ 4	0/ 3
NA, not applicable				
* Mean severity of lesion				
Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).				
1Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)				
2All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)				
3Tissues examined microscopically and found unremarkable				

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Table 67 (continued): Study 1030- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	50 mg/kg Levo + 50 mg/kg Levo n/N (*)	8 mg/kg ETI-204 n/N (*)
Cavity, Pericardial				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2, 3}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Cavity, Thoracic				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	2/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2, 3}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Intestine, large				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	0/ 6 (NA)	0/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2, 3}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Appendix				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	1/ 6 (NA)	1/13 (NA)	0/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	1/ 6 (4.00)	0/13	0/ 4	0/ 3
Hemorrhage	1/ 6 (4.00)	0/13	0/ 4	0/ 3
Necrosis	1/ 6 (4.00)	0/13	0/ 4	0/ 3
Thymus				
Macroscopic Finding ¹				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Gross Lesions	1/ 6 (NA)	0/13 (NA)	1/ 4 (NA)	0/ 3 (NA)
Microscopic Finding ^{1, 2}				
# Necropsied/Total Infected	6/ 6	13/13	4/ 4	3/ 3
Bacteria	1/ 6 (2.00)	0/13	0/ 4	0/ 3
Edema	1/ 6 (3.00)	0/13	1/ 4 (4.00)	0/ 3

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

1Gross necropsy and histopathology pathology performed at (b) (4)

2All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

3Tissues examined microscopically and found unremarkable

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the surviving animals irrespective of the treatment. However, bacteria were observed in almost all the tissues from all the animals in the control group and some of the tissues from animals treated with ETI-204 or levofloxacin that died (Table 68).

Cultures: Cultures not done.

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Table 68: Study 1030 - Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Untreated Control		ETI-204 8mg/kg		Levo 50mg/kg		ETI-204 8mg+Levo 50mg/kg	
	Non Survivors (N=0)		Non Survivors (N=6)		Non Survivors (N=2)		Non Survivors (N=4)	
	Survivors (N=0)	Non Survivors (N=6)	Survivors (N=12)	Non Survivors (N=4)	Survivors (N=2)	Non Survivors (N=3)	Survivors (N=4)	Non Survivors (N=0)
Presence of bacteria by microscopy [1, 3, 4]								
Appendix	0/ 0	1/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Brain	0/ 0	2/ 6	0/12	1/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Cavity, Pericardial	0/ 0	0/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Cavity, Thoracic	0/ 0	0/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Intestine, large	0/ 0	0/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Kidney	0/ 0	6/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Lung	0/ 0	6/ 6	0/12	2/ 4	0/ 2	1/ 3	0/ 4	0/ 0
Lymph Node, Mediastinal	0/ 0	6/ 6	0/12	2/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Lymph node, Bronchial	0/ 0	0/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Spleen	0/ 0	5/ 6	0/12	2/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Thymus	0/ 0	1/ 6	0/12	0/ 4	0/ 2	0/ 3	0/ 4	0/ 0
Presence of bacteria by culture [1, 2]								
No culture assessment	ND	ND						
ND=Not Done								
[1] All treated animals irrespective of bacteremia status prior to treatment								
[2] Animal was considered positive if at least 1-5 colonies were present on plate								
[3] Histopathology performed at (b) (4)								
[4] Not all animals were assessed microscopically; numbers examined are shown								
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: L23003, L23035, L23043								
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: L23016, L23040								
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: None								

Comments:

The study showed that treatment with ETI-204 at a dose of 8 mg/kg administered after a significant increase in body temperature was effective in improving survival. A delayed treatment with ETI-204 + levofloxacin improved survival in all the 4 animals that survived until Day 4 post-challenge. Treatment with levofloxacin improved survival in 2 of the 5 animals. Overall, the study suggests that treatment with ETI-204 following exposure to *B. anthracis* is effective in improving survival and decreasing bacteremia. Bacteria were observed by microscopic examination of many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation.

Two of the four bacteremic animals in the levofloxacin group and 3 of the 3 bacteremic animals in the ETI-204 + levofloxacin survived the period of observation; the number of animals was too small to evaluate added benefit.

TNAs were present in the animals that survived. It would have been useful to measure TNA in non-surviving animals.

Clinical and necropsy findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above.

6.3.1.1.2. Study 1045

This was an open-label, randomized, controlled, factorial design non-GLP study to assess the efficacy of ETI-204 (IV) and levofloxacin (oral) in 54 NZW rabbits 3 days post-challenge with the spores (spore lot no. B34) of the Ames strain of *B. anthracis* weighing 2.5 to 3.1 kg, conducted at [REDACTED]^{(b) (4)}⁵². The primary objective was to demonstrate that post-exposure administration of ETI-204 leads to increased survival above that of levofloxacin (50 mg/kg – human equivalent dose).

Other objectives were to determine the efficacy of

- an anti-PA monoclonal antibody when administered following a SIBT,
- delayed treatment with levofloxacin or anti-PA monoclonal antibody in combination with levofloxacin.

Study design:

The study design was similar to that of the Study 1030 summarized above except that treatment with levofloxacin (Group 1) or ETI-204 + levofloxacin (Group 2) was initiated at 72 hours post-challenge instead of 96 hours post-challenge (Table 69).

Table 69: Study 1045 - Study design and blood collection assay schedule

Group	Number of Rabbits	Treatment Type	Treatment Dose (SID)	Levofloxacin Regimen	Initial Treatment Time	Blood Collection Bacteremia and PA Levels (Relative to Median Challenge)
1	16	levofloxacin	50 mg/kg	3 days	72 hours post-median challenge (\pm 1 hour)	Hours -24^, 24, 48, 72, 96 Days 7, 14, 28
2	16	Anti-toxin + levofloxacin	Anti-toxin – 8 mg/kg levofloxacin – 50 mg/kg	3 days	72 hours post-median challenge (\pm 1 hour)	Hours -24^, 24, 48, 72, 96 Days 7, 14, 28
3	16	Anti-toxin	8 mg/kg	N/A	72 hours post-median challenge (\pm 1 hour)	Hours -24^, 24, 48, 72, 96 Days 7, 14, 28
4	6	0	N/A	N/A	N/A	Hours -24^, 24, 48, 72, 96 Days 7, 14, 28

[^]= a portion of the blood will be used to determine levofloxacin baseline levels (no bacteremia analysis)

Of the 54 rabbits randomized to the four study groups, 17 died before treatment initiation in Groups 1, 2 and 3 (Table 70).

⁵² [REDACTED] (b) (4) Study Number 1045-G607604: NIAID 1045 - Determining the therapeutic efficacy of a novel anti-toxin administered alone or in combination with levofloxacin to New Zealand White rabbits following a *Bacillus anthracis* inhalation challenge (September 27, 2012).

Table 70: Study 1045: Animal disposition before treatment initiation

	Group 4 Control	Group 3 ETI-204 (8 mg/kg)	Group 1[§] Levo (50 mg/kg)	Group 2[§] Levo (50 mg/kg) + ETI-204 (8 mg/kg)	Total
Animals challenged	6	16	16	16	54
Animals who died before treatment	NA [‡]	5	7	5	17/48 (35.4%)
Animals who survived to be treated*	NA [‡]	11	9 [§]	11 [§]	31/48 (64.6%)

*Represent modified intent to treat (mITT)
†No vehicle was administered to control group animals.
§ Group 2: levofloxacin (50 mg/kg) treatment initiated by gavage 72 hours, for 3 days.
Group 3: levofloxacin administered as for Group 2 animals + ETI-204 (single dose).
Levo=levofloxacin

Results:

Baseline characteristics: Prior to challenge, all animals were PA negative by the ECL assay and ELISA; however, blood cultures were not done. Age, gender, body weight, and challenge dose (mean LD₅₀ 196.3) were comparable among the animals in the three groups; the LD₅₀ was ≥ 200 for approximately 46% of the animals (Table 71). The MMAD for each exposure day was 1.11 µm which is consistent with the particle size range that would reach the alveoli.

Time between challenge and treatment initiation was between 69 and 76 hours.

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Table 71: Study 1045 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 4 [§] Control N = 6	Group 3 ETI-204 (8 mg/kg) N = 16	Group 1 Levo (50 mg/kg) N = 16	Group 2 ETI-204 (8 mg/kg) + Levo (50 mg/kg) N = 16
Baseline characteristics				
Age (months) estimated range	5±0	5±0	5±0	4.8±1
Body weight (kg)	2.8±0.2	2.7±0.2	2.7±0.1	2.8±0.1
PA by ECL and ELISA	0/6	0/16	0/16	0/16
Inhaled dose				(b) (4)
Total Inhaled Dose: cfu x 10 ⁷				
Mean ± SD (Range)				
LD ₅₀ Mean ± SD (Range)	202.3±30.3 (164-247)	178.5±30.4 (120-229)	213.9±38.3 (150-289)	194.4±57.9 (108-289)
<200 LD ₅₀ n(%)	4 (66.7)	11 (68.8)	6 (37.5)	8 (50.0)
≥200 LD ₅₀ n(%)	2 (33.3)	5 (31.2)	10 (62.5)	8 (50.0)
Microbial findings [(PA (by ECL and ELISA), bacteremia] and SIBT prior to treatment (72 hours)				
PA				
PA/ECL Assay n (%)	6 (100)	14 (87.5)	16 (100)	16 (100)
PA/ELISA n (%)	6 (100)	13 (81.3)	12 (75.0)	15 (93.8)
Bacteremia				
Qualitative bacteremia n (%)	6 (100)	13 (81.3)	16 (100)	16 (100)
SIBT				
n (%)	6 (100)	15 (93.8)	16 (100)	16 (100)
Time (hours) to first PA^{+ve}, bacteremia, SIBT, and treatment post-challenge				
Time to first PA⁺ (ECL) post-challenge	36.1±13.2 (23.1-49.2)	43.6±13.3 (22.2-70.7)	36.5±11.8 (21.9-50.5)	40.9±11.1 (23.4-50.5)
Mean±SD (Range)	N=6	N=14	N=16	N=16
Time to first PA^{+ve} (ELISA) post-challenge	60±19.9 (47-96.3)	48.9±12.8 (26.9-72.2)	43.5±10.9 (24.6-60.2)	51.4±16.8 (25.7-74.3)
Mean±SD (Range)	N=6	N=13	N=12	N=12
Time to bacteremia	55.4±54.5 (23.1-164)	48.8±15.9 (25.7-72.9)	38±16.7 (21.9-73.6)	40.9±14.4 (23.4-74.3)
Mean±SD (Range)	N=6	N=13	N=16	N=16
Time to SIBT	33.6 (25.7-43.9)	33.2 (29.9-36.9)	31.45 (27.5-36.4)	31.3 (28.7-34.1)
Mean (Range)				
Time to treatment post challenge	NA	73.2±2.1 (69.8-75.9)	73.2±2.1 (69.5, 75.3)	72.2±1.8 (69.8, 74.8)
Mean ± SD (Range)		N=11	N=9	N=11
Survivors at the end of study (Day 28)				
Survived to be treated	0/6 (0)	7/11 (63.6)*	7/9 (77.8%)	9/11 (81.8%)
Bacteremic animals survived to be treated	0/3 (0)	5/9 (55.6%)*	7/9 (77.8%)	9/11 (81.8%)

[§] No vehicle was administered in the control group animals.

SD=Standard deviation; PTT=Prior to treatment;

Qualitative culture LOD =25 cfu/mL; quantitative culture LLOQ 250 cfu/mL; PA ELISA LLOQ=2.4 ng/mL. PA ECL positive based on positive control 2 ng/mL; LOD 4 ng/mL

(1) ETI-204 group: Animals L20651 and L20655 were negative for PA-ECL, animals L20646, L20651 and L20655 were negative for PA- ELISA, and animals L20602, L20651 and L20655 were negative for bacteremia. Animals L20602 and L20651 both survived through Day 28, but Animal L20655 died prior to the 48 hour post-challenge blood collection and exhibited a negative culture at death.

(2) Levo group: Animals L20614, L20625, L20644 and L20652 were negative of PA-ELISA.

(3) ETI-204 & Levo group: L20624 was negative for PA-ELISA.

*Statistically significance by exact method and Boschloo's one-sided test between Groups 1 and 4; Differences between Groups 2 and 3 not significant (for details see statistics reviews by Drs Xianbin Li and Ling Lan)

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Detection of Bacteremia and PA: Of the 54 rabbits, 31 were treated with ETI-204, levofloxacin, or ETI-204+levofloxacin and 6 were in the control group. The variability among the results of qualitative culture and the detection of PA by ELISA and ECL assays in 37 rabbits at the time of treatment is shown in Table 72. The samples used for all these assays were collected at the same time points post-challenge. Of the 37 animals, 34 were culture positive, 36 were PA positive by the ECL assay and 29 by ELISA (Table 72). All the culture positive animals were PA positive by ECL. PA detection by ELISA was the least sensitive in this study.

Table 72: Study 1045 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture	Detection Method				Untreated Control (n=6)	Treatment Group				Total (n=37)		
	Qualitative Enriched Culture	Quantitative Culture	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)†		8 mg/kg ETI-204		50 mg/kg Levo				
						n=11	n=9	n=11	n=11			
-	ND	ND	-	-	0	1	0	0	0	1		
-	ND	ND	+	-	1	0	0	0	0	1		
-	ND	ND	+	+	0	1	0	0	0	1		
+	ND	ND	+	-	0	1	4	1	6	6		
+	ND	ND	+	+	5	8	5	10	28	28		

n = Number of treated animals. Control group was not treated and all control animals are included

Results determined on a per animal basis, not for individual tests. Animal with a positive test from any time prior to treatment is considered as positive.

ND = Not Done. NA=Not applicable

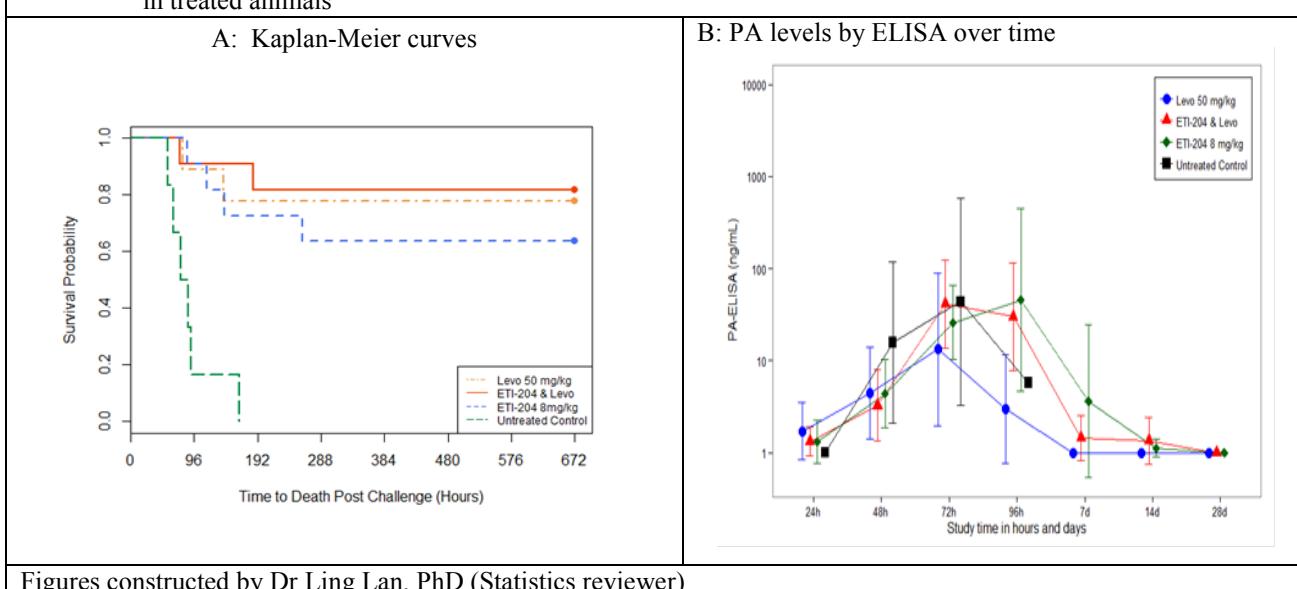
* Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40 µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (1 ng/mL)

†LLOQ by free PA ELISA 4.9 ng/mL.

Effect of treatment with ETI-204 on survival: ETI-204 was effective in improving survival in about 64% of the animals until Day 28; 7 animals died by Day 8 (Table 71 and Figure 45). All the animals in the control untreated group died by Day 6. Two of the animals in the ETI-204 treated group were culture negative at the time of treatment.

Effect of treatment with levofloxacin + ETI-204 on survival: There were 9 animals in levofloxacin and 11 animals in the ETI-204 + levofloxacin treated groups that survived until ~72 hours i.e., the time of initiation of treatment. All the animals were culture positive at the time of treatment administration. Seven (78%) of the animals in the levofloxacin group and 9 (82%) animals in the ETI-204 + levofloxacin survived the period of observation (Table 71 and Figure 45).

Figure 45: Study 1045 – (A) Kaplan-Meier curves representing time-to-death and survival and (B) PA levels by group in treated animals



Figures constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on PA and bacteremia at different time points: All the surviving animals treated with ETI-204 (Group 3) or ETI-204 + levofloxacin (Group 2) were PA negative by the ECL assay by Day 4 (Table 73). In the levofloxacin treated group, the surviving animals were PA negative by the ECL assay by Day 7 (Table 73).

The changes in PA levels by ELISA over time were similar in the animals treated with ETI-204, ETI-204 + levofloxacin, or levofloxacin alone (Figure 45B).

All animals treated with ETI-204 or levofloxacin were culture negative by Day 7; all animals treated with a combination of ETI-204 and levofloxacin were culture negative by Day 4 (Table 73).

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Table 73: Study 1045 – Number of animals PA positive findings (by ECL and ELISA) and bacteremic at different time points post-challenge

Study Time	Group 4 Control	Group 3 ETI-204 (8 mg/kg)	Group 1 Levo (50 mg/kg)	Group 2 ETI-204 (8 mg/kg) + Levo (50 mg/kg)
Number of animals PA^{+ve} by ECL				
-24 hours	0/6	0/16	0/16	0/16
24 hours	3/6	4/16	8/16	5/16
48 hours	6/6	13/15	16/16	16/16
72 hours	4/4	10/11	9/9	11/11
96 hours	1/1	0/10	6/8	0/10
Day 7	*	1/7	0/7	0/10
Day 14, 28	*	1/7	0/7	0/9
PTT	*	NA	NA	NA
Unscheduled terminal	*	ND	ND	ND
Number of animals PA^{+ve} (Range) by ELISA				
-24 hours	0/6	0/16	0/15	0/16
24 hours	0/6	2/16 (21-60)	3/16 (8-19)	3/16 (4.4-17)
48 hours	4/6 (7-384)	11/15 (4-217)	11/15 (4-428)	10/16 (4-407)
72 hours	3/4 (50-280)	9/9 (10-290)	5/5 (10-1672)	10/10 (8-758)
96 hours	1/1 (6)	6/9 (34-22767)	2/6 (18-37)	7/8 (7-636)
Day 7	*	2/7 (9-900)	0/7	2/10 (3-17)
Day 14	*	1/7 (2.2)	0/7	1/9 (15)
Day 28	*	0/7	0/7	0/9
Unscheduled terminal	3/3	5/7 (44-7792)	6/6 (41-8042)	4/4 (3-12273)
Bacteremia				
Baseline	ND	ND	ND	ND
24 hours	3/6	3/16	9/16	6/16
48 hours	4/6	10/15	14/16	15/16
72 hours	3/4	9/11	9/9	11/11
96 hours	0/1	8/10	2/8	0/10
Day 7	*	0/7	0/7	0/10
Day 14, 28	*	0/7	0/7	0/9
PTT	*	NA	NA	NA
Unscheduled terminal	5/6	7/9	8/9	5/6

*No animals available at this time point; ND=not done; NA=not applicable as animals were treated at 72 hours

In vitro susceptibility testing: The levofloxacin MICs (MICs <0.06 and 1.00 µg/mL) of the isolates, collected at the terminal time point i.e., time animal died or last positive culture prior to death, were similar to that of the challenge strain used (spore lot no. B34 0.5 µg/mL) for aerosolization. The levofloxacin MIC for a standard control spore lot (no. B35) was 0.25 µg/mL.

Clinical Observations: Abnormal clinical signs were observed starting approximately 48 hours post-challenge; the signs include lethargy, inappetence, respiratory and stool (soft stool; diarrhea) abnormalities were the most common abnormal clinical observations post-challenge. After Day 7 post-challenge, clinical signs of illness were not observed, except for sporadic documentation of decreased appetite or diarrhea (with the exception of Animal L20635). Animal L20635 (Group 3; treated with ETI-204) was observed to have continual lethargy, persistent decreased appetite and respiratory abnormalities through Day 15 post-exposure. Overall, these observations are consistent with those reported for the natural history and other studies summarized above. The time from treatment until resolution was similar in all the treatment groups.

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Necropsy and Histopathology: Gross lesions in rabbits dying post-challenge included fluid within the thoracic cavity and thymus; discoloration of the meninges, large intestine, liver and cecum (appendix); and foci of the cecum (appendix) and large intestine. There were no gross findings reported in animals that survived to scheduled euthanasia on Day 28 (Table 74).

Microscopic findings considered consistent with anthrax infection were present in all rabbits that died or became moribund during the study (Table 74). These findings included tissue necrosis, hemorrhage, edema, inflammation and the presence of large rod-shaped bacteria (consistent with *B. anthracis*) in one or more organs. In the brain, bacteria were often restricted to the meninges and often accompanied by meningeal hemorrhage and/or inflammation. In these rabbits, bacteria were also found in the vasculature of additional organs in association with hemorrhage, necrosis and/or inflammation. It should be noted that meningeal inflammation was only noted in animals treated with ETI-204 and the most number of cases were noted in animals treated with ETI-204 alone. Other pathological findings appear to be consistent across treatment groups. There were no anthrax-related microscopic findings in rabbits surviving to scheduled euthanasia on Day 28, regardless of treatment assignment. A few additional microscopic findings were observed, but these findings did not correlate with typical lesions associated with anthrax and were considered incidental to infection and treatment.

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Table 74: Study 1045- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	50 mg/kg Levo + 50 mg/kg Le n/N (*)	8 mg/kg ETI-204 n/N (*)
Adrenal Gland				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Hemorrhage	3/ 5 (2.00)	0/ 9	2/ 9 (2.50)	1/11 (1.00)
Aorta				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Appendix				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	3/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	1/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	3/ 5 (2.00)	0/ 9	0/ 9	0/11
Hemorrhage	3/ 5 (2.00)	0/ 9	0/ 9	1/11 (2.00)
Necrosis	3/ 5 (2.00)	0/ 9	0/ 9	1/11 (3.00)
Bone Marrow				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Hyperplasia	0/ 5	1/ 9 (3.00)	0/ 9	0/11
Brain				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	2/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	4/ 5 (1.25)	3/ 9 (2.00)	0/ 9	1/11 (3.00)
Hemorrhage	2/ 5 (1.50)	3/ 9 (2.00)	0/ 9	1/11 (2.00)
Meningeal Inflammation	0/ 5	2/ 9 (1.50)	0/ 9	1/11 (1.00)
Necrosis	0/ 5	1/ 9 (2.00)	0/ 9	0/11
Kidney				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	2/ 5 (1.00)	1/ 9 (1.00)	1/ 9 (1.00)	0/11
Inflammation	0/ 5	2/ 9 (3.00)	1/ 9 (1.00)	2/11 (1.50)
Liver				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	1/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	3/ 5 (1.67)	1/ 9 (1.00)	0/ 9	0/11
Hepatocellular Necrosis	1/ 5 (4.00)	0/ 9	1/ 9 (2.00)	0/11
Hyperplasia, Bile Duct	0/ 5	3/ 9 (1.00)	1/ 9 (1.00)	4/11 (1.00)
Mixed Cell Infiltrate	0/ 5	2/ 9 (1.00)	0/ 9	2/11 (1.00)
Sinusoidal Leukocytosis	5/ 5 (1.00)	4/ 9 (1.00)	2/ 9 (1.00)	2/11 (1.50)

NA, not applicable * Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

1Gross necropsy and histopathology pathology performed at (b) (4)

2All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

3Tissues examined microscopically and found unremarkable

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Table 74 (continued): Study 1045- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	50 mg/kg Levo + 50 mg/kg Levo ETI-204 n/N (*)	8 mg/kg ETI-204 n/N (*)
Lung				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
BALT Necrosis	2/ 5 (2.00)	1/ 9 (2.00)	1/ 9 (1.00)	2/11 (2.00)
Bacteria	4/ 5 (1.25)	1/ 9 (2.00)	1/ 9 (3.00)	1/11 (1.00)
Fibrinosuppurative Bronchopneumonia	3/ 5 (1.33)	4/ 9 (1.50)	0/ 9	2/11 (1.00)
Hemorrhage	1/ 5 (1.00)	2/ 9 (2.50)	0/ 9	2/11 (1.50)
Interstitial Inflammation	1/ 5 (1.00)	3/ 9 (1.33)	2/ 9 (1.00)	3/11 (1.33)
Lymph Node, Mandibular				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	4/ 5	9/ 9	9/ 9	11/11
Bacteria	3/ 4 (3.00)	1/ 9 (4.00)	1/ 9 (3.00)	0/11
Edema	2/ 4 (2.00)	3/ 9 (1.33)	0/ 9	0/11
Hemorrhage	3/ 4 (2.00)	1/ 9 (2.00)	2/ 9 (1.50)	2/11 (1.50)
Inflammation	1/ 4 (2.00)	1/ 9 (1.00)	0/ 9	0/11
Lymphoid Necrosis/Depletion	3/ 4 (3.33)	3/ 9 (1.67)	2/ 9 (2.00)	2/11 (2.00)
Lymph Node, Mediastinal				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	4/ 5 (3.00)	1/ 9 (4.00)	1/ 9 (4.00)	0/11
Edema	0/ 5	0/ 9	1/ 9 (1.00)	1/11 (1.00)
Hemorrhage	4/ 5 (3.00)	3/ 9 (2.67)	2/ 9 (2.50)	2/11 (2.00)
Inflammation	0/ 5	4/ 9 (1.25)	1/ 9 (1.00)	1/11 (1.00)
Lymphoid Necrosis/Depletion	4/ 5 (3.50)	3/ 9 (2.33)	3/ 9 (2.67)	2/11 (3.50)
Lymph Node, Mesenteric				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	8/ 9	11/11
Bacteria	5/ 5 (3.40)	1/ 9 (2.00)	1/ 8 (4.00)	0/11
Edema	1/ 5 (2.00)	3/ 9 (1.67)	1/ 8 (1.00)	1/11 (1.00)
Hemorrhage	5/ 5 (3.60)	2/ 9 (3.00)	2/ 8 (3.50)	2/11 (1.00)
Inflammation	1/ 5 (1.00)	0/ 9	1/ 8 (1.00)	0/11
Lymphoid Necrosis/Depletion	5/ 5 (4.00)	3/ 9 (3.00)	2/ 8 (3.00)	2/11 (2.00)
Spleen				
Macroscopic Finding ¹				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Gross Lesions	0/ 5 (NA)	0/ 9 (NA)	0/ 9 (NA)	0/11 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	5/ 5	9/ 9	9/ 9	11/11
Bacteria	3/ 5 (1.33)	1/ 9 (1.00)	1/ 9 (4.00)	0/11
Fibrin Exudation	4/ 5 (4.00)	2/ 9 (3.00)	2/ 9 (3.00)	2/11 (3.00)
Hemosiderin Pigmentation	0/ 5	0/ 9	1/ 9 (3.00)	0/11
Lymphoid Necrosis/Depletion	4/ 5 (4.00)	4/ 9 (1.75)	2/ 9 (3.00)	2/11 (2.00)

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

1Gross necropsy and histopathology pathology performed at (b) (4)

2All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

3Tissues examined microscopically and found unremarkable

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Tissue bacterial assessments:

Histology: No bacteria were observed in any of the surviving animals irrespective of the treatment. However, bacteria were observed in almost all the tissues from all the animals in the control group and some of the tissues from animals treated with ETI-204, levofloxacin or a combination of ETI-204+levofloxacin that died (Table 75).

Cultures: Cultures not done.

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Table 75: Study 1045- Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Untreated Control		ETI-204 8mg/kg		Levo 50mg/kg		ETI-204 8mg/kg+Levo 50mg/kg			
			Non Survivors (N=0)	Non Survivors (N=6)	Survivors (N=7)	Non Survivors (N=4)	Survivors (N=7)	Non Survivors (N=2)	Survivors (N=9)	Non Survivors (N=2)
	Presence of bacteria by microscopy [1, 3, 4]									
Adrenal Gland	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Aorta	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Appendix	0/0	3/6	0/7	0/4	0/7	0/2	0/9	0/2		
Bone Marrow	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Brain	0/0	4/6	0/6	3/4	0/7	0/2	0/9	1/2		
Cecum	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Cervix	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Colon	0/0	1/6	0/7	0/4	0/7	0/2	0/9	0/2		
Duodenum	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Epididymis	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Esophagus	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Eye	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Femur	0/0	0/5	0/6	0/4	0/7	0/2	0/9	0/2		
Harderian Gland	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Heart	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Ileum	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Intestine, Large	0/0	1/6	0/7	0/4	0/7	0/2	0/9	0/2		
Jejunum	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Kidney	0/0	3/6	0/7	1/4	0/7	1/2	0/9	0/2		
Liver	0/0	3/6	0/7	1/4	0/7	0/2	0/9	0/2		
Lung	0/0	5/6	0/7	1/4	0/7	1/2	0/9	1/2		
Lymph Node, Mandibular	0/0	3/4	0/7	1/4	0/7	1/2	0/9	0/2		
Lymph Node, Mediastinal	0/0	5/6	0/7	1/4	0/7	1/2	0/9	0/2		
Lymph Node, Mesenteric	0/0	6/6	0/7	1/4	0/6	1/2	0/9	0/2		
Mammary Gland	0/0	0/4	0/5	0/3	0/5	0/2	0/7	0/2		
Ovary	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Pancreas	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Parathyroid Gland	0/0	0/5	0/1	0/2	0/4	0/2	0/6	0/1		
Pituitary Gland	0/0	0/6	0/6	0/4	0/7	0/2	0/9	0/2		
Prostate	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Rectum	0/0	0/5	0/7	0/4	0/7	0/2	0/9	0/2		
Salivary Gland	0/0	0/4	0/7	0/4	0/7	0/2	0/9	0/2		
Sciatic Nerve	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Seminal Vesicle	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Skeletal Muscle	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Skin	0/0	0/6	0/6	0/4	0/7	0/2	0/9	0/2		
Spinal Cord	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Spleen	0/0	3/6	0/7	1/4	0/7	1/2	0/9	0/2		
Sternum	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Stomach	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Testis	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Thymus	0/0	5/6	0/7	1/4	0/7	1/2	0/9	0/2		
Thyroid Gland	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Tongue	0/0	0/6	0/7	0/4	0/7	0/2	0/7	0/2		
Trachea	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Ureter	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Urinary Bladder	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Uterus	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Vagina	0/0	0/6	0/7	0/4	0/7	0/2	0/9	0/2		
Presence of bacteria by culture [1, 2]										
No culture assessment	ND	ND	ND	ND	ND	ND	ND	ND	ND	
ND=Not Done										
[1] All treated animals irrespective of bacteremia status prior to treatment										
[2] Animal was considered positive if at least 1-5 colonies were present on plate										
[3] Histopathology performed at (b) (4)										
[4] Not all animals were assessed microscopically; numbers examined are shown										
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: L20651										
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: L20653, L20602										
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: None										

Comments:

Treatment with ETI-204, 72 hours post-exposure, was effective in improving survival of 64% of the bacteremic animals prior to treatment. Treatment with levofloxacin resulted in 78% survival and treatment with a combination of levofloxacin and ETI-204 resulted in 82% survival. All the untreated control animals succumbed to disease by Day 6. A majority of the animals were febrile, bacteremic, and/or toxemic prior to treatment. Bacteria were observed by microscopic examination of many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation.

Clinical and necropsy findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above.

6.3.1.1.3. Study AR028

This was an exploratory open-label, randomized, controlled, parallel group non-GLP study to assess additive effects of concurrent administration of ETI-204 (16 mg/kg, IV; Baxter) and levofloxacin (6.5 mg/kg for 3 days, oral) under conditions in which levofloxacin is given at lower than humanized dose and in a delayed treatment regimen in rabbit model of inhalational anthrax; and could provide only partial protection in NZW rabbits after challenge with the spores of the Ames strain of *B. anthracis*.⁵³

The objectives of this study was to determine if ETI-204

- could improve the rate of survival when co-administered with a levofloxacin dose that resulted in approximately 50% survival.
- had a negative impact on the rate of survival when co-administered with a levofloxacin dose that resulted in approximately 50% survival.

Study design:

Of the 120 animals that were challenged with *B. anthracis* spores (spore lot no. B39; target 200X LD₅₀) by aerosolization, 84 survived until the time of treatment i.e., approximately 72 hours post-challenge (Table 76). The study design was similar to other studies conducted at [REDACTED] (b)(4) summarized above, except for the following:

- The study was conducted in two phases (60 rabbits in each phase).
- Treatment was initiated at approximately 72 hours post-challenge.
- Tissues were not processed for bacterial culture.
- ECL assay for detecting PA was not performed.
- Anti-PA IgG antibodies were measured prior to challenge and on Day 25 in surviving animals by an ECL assay.
- *In vitro* susceptibility testing to levofloxacin was performed.
- Neuropathological evaluation was performed on selected surviving and non-surviving animals. The goal of evaluation in surviving animals was to examine whether the treatment exacerbates brain lesions during infection and leads to lasting tissue pathology in surviving animals. For this, animals with the highest prior to treatment CFUs were selected as the most

⁵³ [REDACTED] (b)(4) Study Number 2395-100008193: AR028 - An exploratory study to evaluate the effects of ETI-204 when given in combination with levofloxacin on survival in anthrax-challenged NZW rabbits (May 15, 2013).

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likely candidates for the development of pathology. The goal of evaluation in non-surviving animals was to assess the potential adverse effects of the treatment. For this, animals with the longest treatment exposure were selected i.e., animals that had survived for at least 6 hours after treatment). Animals were further selected to achieve comparability between levofloxacin only and combination treatment groups with respect to: (1) duration of exposure, (2) prior to treatment CFU profile, (3) gender, and (4) percent brain bacteremia.

Interim analysis of survival results from Phase I was conducted following completion of Phase I to select the dose for Phase II. A statistically significant difference in the survival rate of animals treated with the combination of ETI-204 + levofloxacin compared to levofloxacin would have resulted in selection of lower dose of ETI-204 in Phase II, to provide information regarding the minimal effective dose of ETI-204 at which an additive effect or interference may be observed. The applicant states that as statistical significance was not achieved based on survival results of Phase I, ETI-204 was administered at 16 mg/kg in Phase II which is same as that in Phase I. Therefore, animals in the groups with the same treatment regimen were combined in a single study population.

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Table 76: Study AR028 – Study design and blood collection schedule for different assays

Study design

Phase	Group	Animals Challenged	Animals Surviving to Randomization	Levofloxacin Dose (mg/kg/day, PO)	ETI-204 Dose (mg/kg, IV)	Antibiotic Regimen	Treatment Initiation (Hours Post-Median Challenge Time)
I	1	60	8 ¹	0 (water)	0 (saline)	Levofloxacin Once Daily X 3	72 (±4)
	2		19	6.5	0 (saline)		
	3		17	6.5	16	ETI-204 Once	
II	4	60	4	0 (water)	0 (saline)	Levofloxacin Once Daily X 3	72 (±4)
	5		19	6.5	0 (saline)		
	6		17	6.5	16	ETI-204 Once	

¹ = In Phase I, 8 rabbits were randomized to control group instead of 4 rabbits as directed by the protocol. This resulted in fewer animals being randomized to treatment groups in Phase I. See Appendix B; DR-12043.

Blood collection and assay schedule

Time-point	Collection Window	Blood Tube type/ Approximate Blood volume	Quantitative Bacteremia	Serum for PA ELISA	Plasma for Levofloxacin Levels	Serum for Anti-PA IgG ELISA	Serum for ETI-204 Levels
Day -7	NA	EDTA ~1.0 mL Chilled EDTA ~1.0 mL SST ~2.5 mL	X	X	X	X	X
24hr PC	± 30 min.	EDTA ~1.0 mL SST ~1.5 mL	X	X			
48hr PC	± 30 min.	EDTA ~1.0 mL SST ~1.5 mL	X	X			
72hr PC	± 30 min.	EDTA ~1.0 mL SST ~1.5 mL	X	X			
1hr PT	± 15 min.	Chilled EDTA ~1.0 mL SST ~ 1.5 mL			X		X
4hr PT	± 30 min.	SST ~1.5 mL					X
8hr PT	± 15 min.	SST ~1.5 mL					X
24hr PT (immediately prior to 2 nd levofloxacin dose)	± 30 min.	EDTA ~1.0 mL Chilled EDTA ~1.0 mL SST ~1.5 mL	X	X	X		X
49hr PT (1hr post last levofloxacin dose)	± 15 min.	EDTA ~1.0 mL SST ~1.5 mL Chilled EDTA ~1.0 mL	X	X	X		X
72hr PT	± 30 min.	EDTA ~1.0 mL SST ~1.5 mL Chilled EDTA ~1.0 mL	X	X	X		X
96hr PT	± 30 min.	EDTA ~1.0 mL SST ~1.5 mL	X	X			
7 days PT	NA	EDTA ~1.0 mL SST ~1.5 mL	X	X			X
14 days PT	NA	EDTA ~1.0 mL SST ~1.5 mL	X	X			X
25 days PT (Day 28)	NA	EDTA ~1.0 mL SST ~2.5 mL	X	X		X	X
Terminal	NA	EDTA ~1.0 mL SST ~2.5 mL	X	X		***	**

PT = post-treatment (based on first treatment); NA=Not applicable, blood draws relative to the day of challenge/first treatment.

** = Serum for ETI-204 levels were collected from animals deceased or sacrificed after treatment initiation.

***= Serum for anti-PA IgG were collected from animals that died or were sacrificed moribund after treatment initiation only if it is possible to obtain sufficient sample volume.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose (mean LD₅₀ 219) were comparable among the three groups; the LD₅₀ was ≥ 200 for approximately 58% of the animals (Table 77). The MMAD for each exposure day was 1.15 µm which is consistent with the particle size range that would reach the alveoli or was consistent with lower respiratory tract deposition.

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Table 77: Study AR028 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 1 Control N = 12	Group 2 Levofloxacin N = 38	Group 3 ETI-204 [‡] + Levofloxacin N = 34
Baseline characteristics and inhaled dose			
Age (months) estimated range [*]	7.6±1.1	7.8±1.1	8.2±0.8
Body weight (kg)	3.2±0.2	3.2±0.1	3.2±0.2
PA-ELISA	0/12	0/38	0/34
Quantitative cultures	0/12	0/38	0/34
Inhaled dose			
cfu x 10 ⁷			(b) (4)
Mean ± SD (Range)			
LD ₅₀ Mean ± SD (Range)	191.8±52.2 (94.7-270.1)	215.8±41 (114.7-316.6)	232.2±40.1 (160.2-326.1)
<200 LD ₅₀ n(%)	7 (58.3)	14 (36.8)	5 (14.7)
≥200 LD ₅₀ n(%)	5 (41.7)**	24 (63.2)	29 (85.3)
Microbial burden prior to treatment (72 hours)			
PA by ELISA			
n (%)	10 (100)	24 (63.2)	23 (67.6)
Log ₁₀ mean±SD (Range) ¹	1.8±0.7 (0.7-3.3)	n = 35 1.4±0.8 (0.7-3.4)	1.6±0.9 (0.7-3.6)
Geometric mean	58.3	27.1	37.3
Quantitative bacteremia			
n (%)	12 (100)	37 (97.4)	34 (100)
Log ₁₀ mean±SD (Range) ²	4.3±0.9 (3.2-6.7)	n=37 3.9±1.2 (1.7-6.9)	3.8±1.5 (0.3-7.5)
Geometric mean (cfu/mL x 10 ⁴)	58.3	27.1	37.3
Time (hours) to PA positive, bacteremia and treatment post-challenge			
Time to first PA⁺ve (ELISA)	n = 10	n = 24	n = 23
Mean±SD	60±12.6 (48-72)	71±4.9 (48-72)	69.9±6.9 (48-72)
Time to bacteremia			
Mean±SD (Range)	48±20.5 (24-72)	n = 37 58.4±19.2 (24-72)	70.6±5.7 (48-72)
Time to treatment	74.8±0.8 (73.2-75.7)	72.5±1.7 (69.2-75.4) [§]	72.3±1.5 (69.8-75.2)
Survivors at the end of study (Day 25)			
Survived to be treated n/N (%)		22/38 (58)	
Bacteremic animals survived to be treated n/N (%)	0/12 (0)	22/37 (59)	23/34 (68)

^{*}Age was not provided for animals in Phase II.

¹For analyses, PA-ELISA of 24 animals was <LLOQ (9.68 ng/ml) and replaced with 4.84 ng/ml. Animals L43139 (Levo, Phase I), L43720 & L43744 (Levo, Phase II) had missing values for PA-ELISA PTT and was excluded from this analyses.

²For analyses, quantitative bacteremia of animal L43132 (ETI-204 & Levo, Phase I) was <LOD (3 cfu/ml) and the value was replaced by 2; six animals were “+” (<LOQ = 100 cfu/ml) and replaced by 50. Animal L43701 (Levo, Phase II) was negative for bacteremia prior or at the treatment initiation and was not included in the analyses for bacteremia.

[§]Animal L43757 in Levo (Phase II) received treatment at 52.1 hours post challenge and had bacteremia of <LLOQ at 24 hours post-challenge.

SD=Standard deviation; PTT=Prior to treatment;

Statistically significance by exact method and Boschloo's one-sided test between Groups 1 and 4; Differences between Groups 2 and 3 not significant (for details see statistics reviews by Drs Xianbin Li and Ling Lan)

Detection of Bacteremia and PA: Of the 84 rabbits, 72 were treated with levofloxacin or ETI-204+levofloxacin and 12 were in the control group. The variability among the results of quantitative culture and the detection of PA by ELISA and ECL assays in 84 rabbits at the time of treatment is shown in Table 78. The samples used for all these assays were collected at the same time points post-challenge. Of the 84 animals, 83 were culture positive and 57 PA positive by ELISA (Table 78). All the PA positive animals were culture positive. PA detection by ELISA was less sensitive than measurement of bacteremia in this study.

Table 78: Study AR028 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture	Qualitative Enriched Culture	Quantitative Culture [†]	Detection Method		Treatment Group				Total (n=84)
			Screening PA (ECL Assay)	Quantitative PA (ELISA Assay) [‡]	Placebo (n=12)	6.5 mg/kg Levo (n=38)	Levo (n=34)		
ND	ND	-	ND	-	0	1	0	1	1
ND	ND	+	ND	-	2	13	11	26	26
ND	ND	+	ND	+	10	24	23	57	57

n = Number of treated animals. Control group was not treated and all control animals are included

Results determined on a per animal basis, not for individual tests. Animal with a positive test from any time prior to treatment is considered as positive.

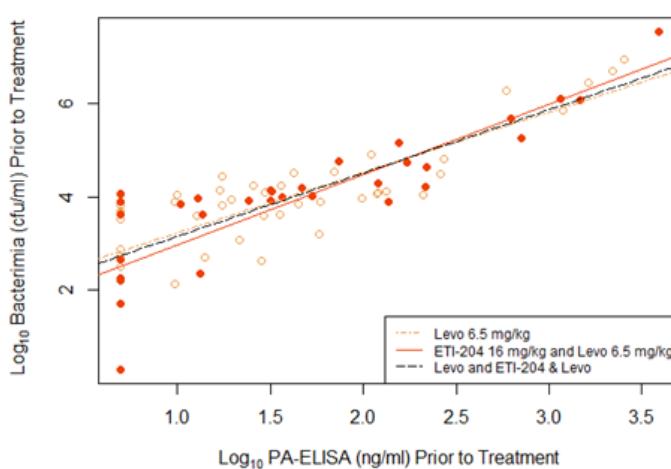
ND = Not Done.

*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40 µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (1 ng/mL); LOD 4 ng/mL

‡LLOQ by free PA ELISA 4.9 ng/mL

Bacteremia and PA levels were positively correlated (Figure 46).

Figure 46: Study AR028 - Correlation between bacteremia and PA-ELISA by group for treated animals



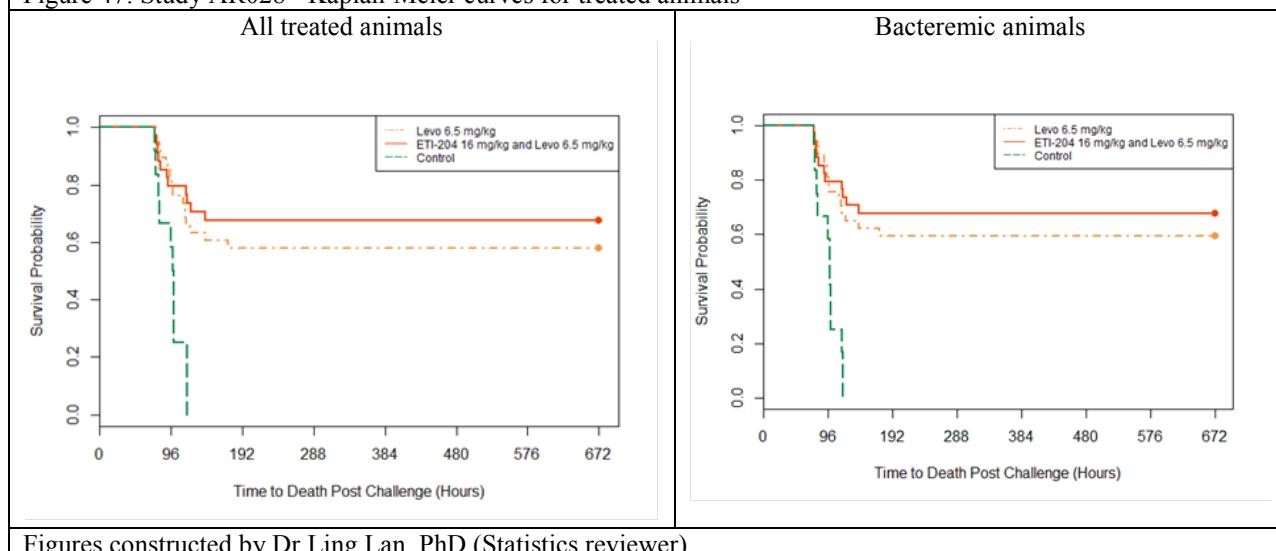
Pearson Correlation between Log10 Bacteremia and Log10 PA-ELISA by group for treated animals

Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on survival: ETI-204 in combination with levofloxacin was effective in improving survival; the survival rate was similar in animals treated with levofloxacin or

levofloxacin + ETI-204. None of the control group animals survived the period of observation (Figure 47 and Table 77).

Figure 47: Study AR028 - Kaplan-Meier curves for treated animals



Figures constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on microbial burden:

Bacteremia: All animals were culture negative by Day 7 post-treatment (Table 79). The changes in bacteremia over time were similar in the ETI-204+levofloxacin or levofloxacin treated animals (Table 79 and Figures 48 and 49). All the control animals remained culture positive until they were found moribund, died, or were necropsied at the end of study.

PA levels: By 24 hours post-treatment, all the animals treated with ETI-204 + levofloxacin were PA negative whereas all the control animals and about 50% of the animals treated with levofloxacin were PA positive (Table 79). All the treated animals were PA negative by 72 hours post-treatment (Figures 48 and 49).

All the control animals remained PA positive until they were found moribund or died.

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Table 79: Study AR028 - Number of animals bacteremic and PA positive by ELISA over time

Study Time	Group 1 Control	Group 2 Levofloxacin	Group 3 ETI-204+Levofloxacin
Number of bacteremic animals			
Day 0	0/12	0/38	0/34
24 hours PC	8/12	16/38	17/34
48 hours PC	12/12	26/38	26/34
72 hours PC	12/12	37/38	33/34
24 hours PT	6/6	20/30	17/27
49 hours PT	NA	14/24	15/25
72 hours PT	NA	10/23	13/23
96 hours PT	NA	11/22	13/23
7 days PT	NA	0/22	0/23
14, 25 days PT	NA	0/22	1/23
Terminal	7/7	11/15	6/10
Number of PA^{+ve} animals			
Day 0	0/12	0/38	0/34
24 hours PC	1/12	0/38	0/34
48 hours PC	6/12	8/38	14/34
72 hours PC	10/12	24/35	23/34
24 hours PT	5/5	14/30	0/27
49 hours PT	NA	4/24	1/25
72 hours PT	NA	0/23	0/23
96 hours PT	NA	0/23	0/23
7 days PT	NA	0/22	0/23
14, 25 days PT	NA	0/22	2/23
Terminal	5/5	6/11	2/9

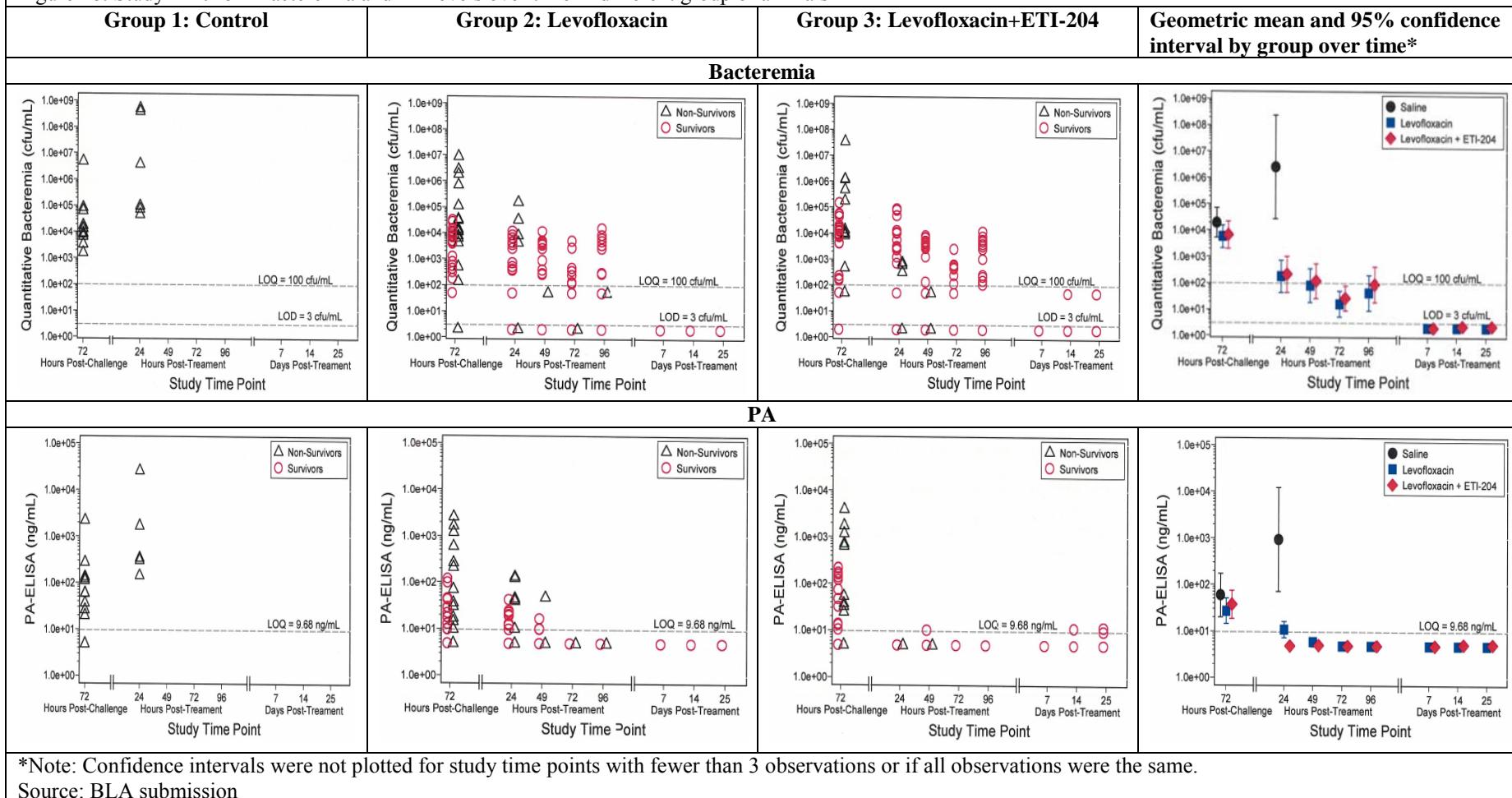
Effect of microbial burden on survival: Increased quantitative bacteremia levels and PA-ELISA levels at the time of or prior to treatment appear to be associated with decreased probabilities of survival (Figure 48).

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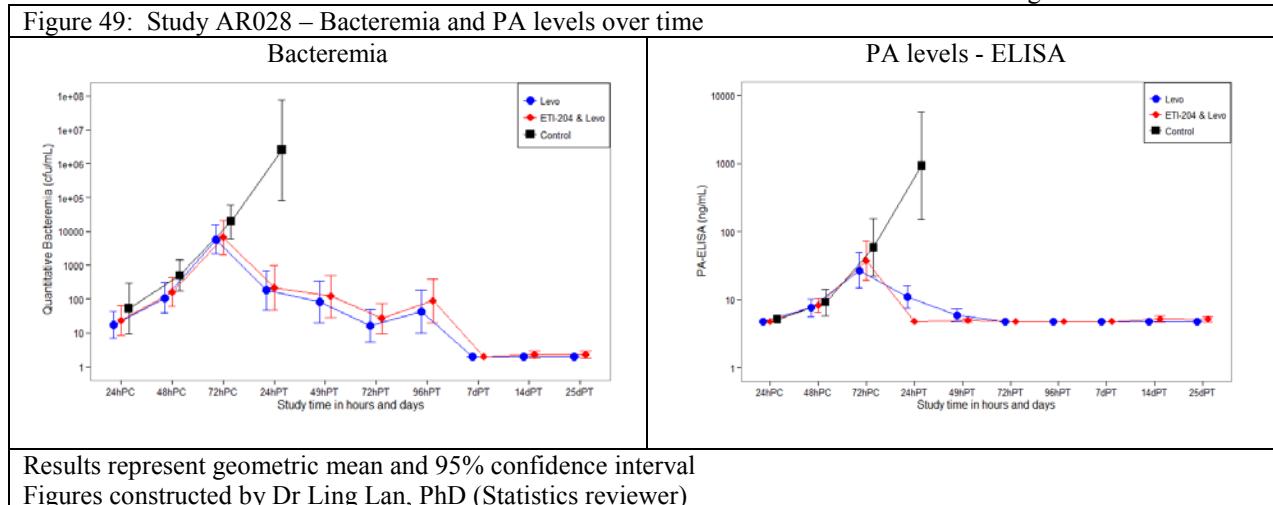
Figure 48: Study AR028 – Bacteremia and PA levels over time in different group of animals



*Note: Confidence intervals were not plotted for study time points with fewer than 3 observations or if all observations were the same.

Source: BLA submission

Figure 49: Study AR028 – Bacteremia and PA levels over time



Results represent geometric mean and 95% confidence interval

Figures constructed by Dr Ling Lan, PhD (Statistics reviewer)

In vitro susceptibility testing: The *in vitro* susceptibility of the isolates to levofloxacin collected at the time animals died or last positive culture prior to death (MICs between 0.25 and 2.0; MIC for one isolate collected from one animal treated with levofloxacin was 2 µg/mL) were similar to challenge strain used (MICs between 0.25 and 0.5 µg/mL) for aerosolization.

Anti-PA IgG antibodies: Anti-PA IgG antibodies were measured prior to challenge and on Day 25 in 45 surviving animals by an ECL assay which targets ETI-204 and any endogenous anti-PA antibody formed. PA was used as a capture reagent and protein A/G as a detection reagent and, therefore, both ETI-204 and endogenous anti-PA IgG antibodies are detected by this assay.

There were 4 animals which were antibody positive prior to challenge. As none of the animals were stated to have ETI-204 at Day 25, the results reflect endogenous anti-PA antibody. The results suggest presence of anti-PA antibodies in all surviving animals (Table 80). Antibody levels in non-surviving animals were not measured.

Table 80: Study AR028 - Anti-PA IgG* in surviving animals at baseline and Day 25 in surviving animals

Study Time	Group 1 Control	Group 2 Levofloxacin	Group 3 ETI-204+Levofloxacin
Proportion of animal anti-PA IgG positive			
Day -7	ND	3/22 (13.6%) [†]	1/23 (4.3%) [†]
Day 25	NA	22/22 (100%)	23/23 (100%)
Anti-PA IgG concentration (ng/mL)* Mean±SD (Range)			
Day -7	ND	282±210 (77-497)	754
Day 25	NA	19080±12.9 (19067-19093)	19041±17.4 (19023-19058)

*Anti-PA IgG by ECL assay - LOD <50 ng/mL at MesoScale discovery at (b) (4); testing performed on animals that were treated

[†]The following animals that were anti-PA IgG positive prior to treatment survived

- 3 animals (L43147, L43157, L43721) treated with levofloxacin, and
- 1 animal (L43160) treated with a combination of ETI-204 + levofloxacin

Although the applicant stated that “Among animals that survived to receive treatment, positive results were obtained in 4/84 animals” at Day -7, however, such information was not available in the datasets. It appears that anti-PA antibodies were not measured in the non-surviving animals.

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Clinical observations: Documentation of reduced food consumption, lethargy, respiratory abnormalities, and reduced amount of stool were most commonly noted. Generally, surviving animals, treated with levofloxacin alone or the combination of levofloxacin and ETI-204, returned to normal between 7 and 10 days post-challenge with the exception of food consumption and scabbing around the port. Animals that succumbed to disease demonstrated a characteristic progression of signs that followed from unremarkable to observations of lethargy, respiratory abnormalities to moribundity and death. These observations are consistent with those observed in other studies summarized above.

Necropsy and Histopathology: Gross lesions examined from animals dying post-challenge included discoloration or foci in the brain and large intestines, enlargement of the mediastinal lymph nodes, and fluid (effusion) in the thymus, and thoracic cavity. These observations are consistent with those observed in other studies summarized above. There were no gross lesions present in any of the animals surviving to study termination on Study Day 25 (Table 81).

No significant microscopic lesions were present in animals that survived treatment; however, microscopic findings consistent with anthrax were present in all animals selected for examination that became moribund prior to the scheduled Study Day 25 necropsies. Lesions typical of anthrax in this study included acute inflammation, necrosis, hemorrhage, fibrin exudation, and lymphoid depletion/necrosis (Table 81).

Neuropathological examination was performed on brains from a subset of surviving and non-surviving animals. Microscopic changes suggesting hemorrhagic meningoencephalitis, a known complication of systemic anthrax were present in all groups including the control group. The incidence and average severity of microscopic changes indicative of hemorrhagic meningoencephalitis (extravascular bacteria, hemorrhage, congestion, inflammation, vasculitis) were comparable in the groups that received levofloxacin or levofloxacin + ETI-204. None of the study survivors had evidence of intravascular or extravascular bacteria or signs or reaction to the bacteria; exception was one animal, treated with a combination of ETI-204 and levofloxacin, with mild hemorrhage in/near meninges – this was interpreted to be unrelated to ETI-204. In the animals that were found dead or were euthanized, none of the morphologic changes were interpreted to be due to the presence of ETI-204 (or levofloxacin), and all of the changes were consistent with anthrax infection.

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Table 81: Study AR028- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	6.5 mg/kg Levo n/N (*)	16 mg/kg ETI-204 +6.5 mg/kg Levo n/N (*)
Brain			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	1/34 (NA)
Microscopic Finding ^{2,3}			
# Necropsied/Total Infected	4/12	12/37	12/34
Brain, Basal Nuclei: Bacteria, Extravascular	0/4	0/12	1/12 (2.00)
Brain, Basal Nuclei: Bacteria, Intravascular	3/4 (2.67)	0/12	0/12
Brain, Basal Nuclei: Inflammation	0/4	0/12	2/12 (3.00)
Brain, Basal Nuclei: Vasculitis	0/4	0/12	2/12 (3.00)
Brain, Cerebellum: Bacteria, Extravascular	0/4	0/12	1/12 (4.00)
Brain, Cerebellum: Bacteria, Intravascular	2/4 (3.00)	0/12	0/12
Brain, Cerebellum: Hemorrhage	0/4	1/12 (3.00)	3/12 (3.67)
Brain, Cerebellum: Inflammation	0/4	0/12	2/12 (2.50)
Brain, Cerebellum: Parenchymal, Necrosis	0/4	0/12	2/12 (3.50)
Brain, Cerebellum: Purkinje Cell Loss/Necrosis	0/4	0/12	3/12 (3.00)
Brain, Cerebellum: Vasculitis	0/4	1/12 (3.00)	1/12 (3.00)
Brain, Cerebral Cortex: Bacteria, Extravascular	1/4 (4.00)	2/12 (2.50)	3/12 (2.67)
Brain, Cerebral Cortex: Bacteria, Intravascular	4/4 (2.75)	0/12	0/12
Brain, Cerebral Cortex: Blood Vessels, Leukocytosis	0/4	1/12 (2.00)	1/12 (3.00)
Brain, Cerebral Cortex: Ghosis, Focal/Multifocal	0/4	1/12 (3.00)	1/12 (3.00)
Brain, Cerebral Cortex: Hemorrhage	1/4 (3.00)	2/12 (2.50)	3/12 (3.33)
Brain, Cerebral Cortex: Inflammation	1/4 (3.00)	1/12 (2.00)	3/12 (2.67)
Brain, Cerebral Cortex: Neuronal Necrosis/Neuron Loss	0/4	0/12	2/12 (3.50)
Brain, Cerebral Cortex: Parenchymal, Necrosis	0/4	0/12	3/12 (3.33)
Brain, Cerebral Cortex: Parenchymal, Vacuolation	0/4	0/12	1/12 (2.00)
Brain, Cerebral Cortex: Perivascular, Microhemorrhage	0/4	1/12 (2.00)	2/12 (2.00)
Brain, Cerebral Cortex: Vasculitis	1/4 (2.00)	3/12 (3.00)	4/12 (2.75)
Brain, Hippocampus: Bacteria, Extravascular	0/4	1/12 (4.00)	1/12 (4.00)
Brain, Hippocampus: Bacteria, Intravascular	2/4 (3.00)	1/12 (3.00)	0/12
Brain, Hippocampus: Hemorrhage	0/4	1/12 (2.00)	1/12 (3.00)
Brain, Hippocampus: Inflammation	0/4	1/12 (3.00)	2/12 (3.00)
Brain, Hippocampus: Vasculitis	0/4	0/12	1/12 (3.00)
Brain, Medulla Oblongata: Bacteria, Intravascular	1/4 (3.00)	0/12	0/12
Brain, Medulla Oblongata: Hemorrhage	1/4 (3.00)	2/12 (3.00)	1/12 (2.00)
Brain, Medulla Oblongata: Vasculitis	0/4	1/12 (3.00)	1/12 (2.00)
Brain, Meninges: Bacteria, Extravascular	1/4 (5.00)	3/12 (3.67)	3/12 (4.33)
Brain, Meninges: Bacteria, Intravascular	3/4 (3.00)	0/12	0/12
Brain, Meninges: Blood Vessels, Leukocytosis	0/4	1/12 (3.00)	2/12 (2.50)
Brain, Meninges: Congestion	1/4 (3.00)	3/12 (2.67)	4/12 (3.00)
Brain, Meninges: Hemorrhage	1/4 (5.00)	2/12 (3.50)	5/12 (3.40)
Brain, Meninges: Inflammation	1/4 (3.00)	3/12 (2.67)	4/12 (3.75)
Brain, Meninges: Vasculitis	1/4 (2.00)	3/12 (2.33)	4/12 (3.00)
Brain, Midbrain: Bacteria, Extravascular	0/4	1/12 (4.00)	1/12 (5.00)
Brain, Midbrain: Bacteria, Intravascular	2/4 (3.00)	1/12 (2.00)	0/12
Brain, Midbrain: Hemorrhage	0/4	1/12 (2.00)	1/12 (4.00)
Brain, Midbrain: Inflammation	0/4	1/12 (3.00)	2/12 (3.00)
Brain, Midbrain: Vasculitis	0/4	1/12 (2.00)	2/12 (2.00)
Brain, Pons Region/Pontine Nuclei: Bacteria, Intravascular	1/4 (3.00)	0/12	0/12
Brain, Thalamus/Hypothalamus: Bacteria, Extravascular	0/4	0/12	1/12 (5.00)
Brain, Thalamus/Hypothalamus: Hemorrhage	1/4 (2.00)	0/12	2/12 (3.00)
Brain, Thalamus/Hypothalamus: Inflammation	0/4	0/12	2/12 (3.00)
Brain, Thalamus/Hypothalamus: Vasculitis	0/4	0/12	2/12 (2.00)
Brain, Ventricular System: Choroid Plexus, Extravascular, Bacteria	0/4	0/12	1/12 (3.00)
Brain, Ventricular System: Hemorrhage	1/4 (4.00)	0/12	0/12
Brain, Ventricular System: Inflammation	0/4	0/12	3/12 (3.00)
Brain, Ventricular System: Periventricular, Edema	0/4	0/12	1/12 (4.00)
Kidney			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	3/6 (2.00)	3/18 (1.33)	2/18 (2.00)
Necrosis	0/6	1/18 (1.00)	0/18

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)

²Neuropathology performed at [REDACTED] (b) (4)

³All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked)⁵Tissues examined microscopically and found unremarkable

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Table 81 (continued): Study 028- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	6.5 mg/kg Levo n/N (*)	16 mg/kg ETI-204 +6.5 mg/kg Levo n/N (*)
Liver			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	2/6 (2.00)	1/18 (2.00)	3/18 (1.67)
Inflammation	0/6	1/18 (1.00)	1/18 (1.00)
Necrosis	2/6 (2.50)	1/18 (3.00)	3/18 (1.33)
Sinusoidal Leukocytosis	5/6 (1.40)	6/18 (1.17)	5/18 (1.60)
Lymph Node, Mediastinal			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	1/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	6/6 (3.00)	5/18 (2.40)	3/18 (3.00)
Edema	6/6 (2.67)	7/18 (2.00)	4/18 (2.50)
Fibrin	6/6 (2.67)	8/18 (2.63)	6/18 (2.83)
Hemorrhage	5/6 (2.20)	7/18 (2.29)	5/18 (1.60)
Inflammation	6/6 (2.83)	8/18 (2.38)	6/18 (2.67)
Lymphocyte Necrosis/Depletion	6/6 (3.17)	8/18 (2.63)	6/18 (3.00)
Lung			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	5/6 (2.00)	2/18 (2.50)	3/18 (2.00)
Edema	4/6 (1.75)	2/18 (1.00)	5/18 (1.40)
Fibrin	5/6 (2.20)	3/18 (1.33)	5/18 (1.40)
Hemorrhage	3/6 (2.33)	1/18 (2.00)	2/18 (2.00)
Inflammation	6/6 (2.17)	6/18 (1.33)	5/18 (1.40)
Thrombosis	0/6	0/18	1/18 (2.00)
Spleen			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	3/6 (2.67)	3/18 (2.00)	3/18 (2.00)
Fibrin	5/6 (2.40)	6/18 (2.00)	5/18 (2.40)
Inflammation	6/6 (2.00)	8/18 (1.38)	5/18 (2.20)
Lymphocyte Necrosis/Depletion	6/6 (3.17)	8/18 (2.25)	6/18 (2.33)
Cavity, Thoracic			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	1/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	6/12	18/37	18/34

NA, not applicable

* Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²Neuropathology performed at (b) (4)

³All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score: 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)

⁴All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked) ⁵Tissues examined microscopically and found unremarkable

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Table 81 (continued): Study 028- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group		
	Placebo n/N (*)	6.5 mg/kg Levo n/N (*)	16 mg/kg ETI-204 +6.5 mg/kg Levo n/N (*)
Intestine, Large			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	1/12 (NA)	1/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4}			
# Necropsied/Total Infected	6/12	18/37	18/34
Bacteria	0/ 6	1/18 (3.00)	0/18
Fibrin	0/ 6	1/18 (2.00)	0/18
Hemorrhage	0/ 6	1/18 (3.00)	0/18
Inflammation	0/ 6	1/18 (3.00)	0/18
Lymphocyte Necrosis/Depletion	0/ 6	1/18 (2.00)	0/18
Gross lesions			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	6/12	18/37	18/34
Thymus			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	1/12 (NA)	0/37 (NA)	0/34 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	6/12	18/37	18/34
Skin			
Macroscopic Finding ¹			
# Necropsied/Total Infected	12/12	37/37	34/34
Gross Lesions	0/12 (NA)	0/37 (NA)	1/34 (NA)
Microscopic Finding ^{1,4,5}			
# Necropsied/Total Infected	6/12	18/37	18/34
NA, not applicable			
* Mean severity of lesion			
Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, enriched, quantitative).			
¹ Gross necropsy and histopathology pathology performed at [REDACTED] (b) (4)			
² Neuropathology performed at [REDACTED] (b) (4)			
³ All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (slight); 2 (minimal); 3 (mild); 4 (moderate); 5 (severe)			
⁴ All microscopic findings were graded at [REDACTED] (b) (4) according to the following scale, with the associated numerical score: 1 (minimal); 2 (mild); 3 (moderate); 4 (marked) ⁵ Tissues examined microscopically and found unremarkable			

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the surviving animals irrespective of the treatment. However, bacteria were observed in almost all the tissues from all the animals in the control group and some of the tissues from animals treated with levofloxacin or a combination of ETI-204+levofloxacin that died (Table 82).

Cultures: Some of the tissues from the animals that died were processed for culture. Almost all the tissues (brain, liver, lymph nodes and spleen) from control animals were culture positive. Of the 16 non-surviving animals treated with levofloxacin, 13 were culture positive in at least one tissue; whereas 6 of the 11 animals that died after treatment with levofloxacin + ETI-204 were culture positive in at least one tissue (Table 82). Tissues from surviving animals were not processed for culture.

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Table 82: Study AR028-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		Levo 6.5mg/kg		ETI-204 16mg/kg+Levo 6.5mg/kg	
	Survivors (N=0)	Non Survivors (N=12)	Survivors (N=22)	Non Survivors (N=16)	Survivors (N=23)	Non Survivors (N=11)
Presence of bacteria by microscopy [1, 5]						
Brain: Total [3, 4]	0/0	4/4	0/4	3/8	0/4	3/8
Brain, Basal Nuclei: Extravascular	0/0	0/4	0/4	0/8	0/4	1/8
Brain, Basal Nuclei: Intravascular	0/0	3/4	0/4	0/8	0/4	0/8
Brain, Cerebellum: Extravascular	0/0	0/4	0/4	0/8	0/4	1/8
Brain, Cerebellum: Intravascular	0/0	2/4	0/4	0/8	0/4	0/8
Brain, Cerebral Cortex: Extravascular	0/0	1/4	0/4	2/8	0/4	3/8
Brain, Cerebral Cortex: Intravascular	0/0	4/4	0/4	0/8	0/4	0/8
Brain, Hippocampus: Extravascular	0/0	0/4	0/4	1/8	0/4	1/8
Brain, Hippocampus: Intravascular	0/0	2/4	0/4	1/8	0/4	0/8
Brain, Medulla Oblongata: Intravascular	0/0	1/4	0/4	0/8	0/4	0/8
Brain, Meninges: Extravascular	0/0	1/4	0/4	3/8	0/4	3/8
Brain, Meninges: Intravascular	0/0	3/4	0/4	0/8	0/4	0/8
Brain, Midbrain: Extravascular	0/0	0/4	0/4	1/8	0/4	1/8
Brain, Midbrain: Intravascular	0/0	2/4	0/4	1/8	0/4	0/8
Brain, Pons/Pontine Nuclei: Intravascular	0/0	1/4	0/4	0/8	0/4	0/8
Brain, Thalamus/Hypothalamus: Extravascular	0/0	0/4	0/4	0/8	0/4	1/8
Brain, Ventricular System: Plexus	0/0	0/4	0/4	0/8	0/4	1/8
Kidney	0/0	3/6	0/10	3/8	0/12	2/6
Liver	0/0	2/6	0/10	1/8	0/12	3/6
Lymph Node, Mediastinal	0/0	6/6	0/10	5/8	0/12	3/6
Lung	0/0	5/6	0/10	2/8	0/12	3/6
Spleen	0/0	3/6	0/10	3/8	0/12	3/6
Cavity, Thoracic	0/0	0/6	0/10	0/8	0/12	0/6
Intestine, Large	0/0	0/6	0/10	1/8	0/12	0/6
Gross lesions	0/0	0/6	0/10	0/8	0/12	0/6
Thymus	0/0	0/6	0/10	0/8	0/12	0/6
Skin	0/0	0/6	0/10	0/8	0/12	0/6
Presence of bacteria by culture [1, 2]						
Brain	ND	11/12	ND	10/16	ND	6/11
Liver	ND	10/12	ND	8/16	ND	3/11
Lymph Node, Mediastinal	ND	12/12	ND	8/16	ND	4/11
Spleen	ND	12/12	ND	11/16	ND	6/11

ND=Not Done

- [1] All treated animals irrespective of bacteremia status prior to treatment
[2] Animal was considered positive if at least 1-5 colonies were present on plate

[3] Neuropathology performed at (b) (4)

[4] Animals that was positive in at least one area for either extra or intra-vascular bacteria

[5] Histopathology performed at (b) (4) with exception of brain; Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: L43701

Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: L43144, L43145, L43107, L43146, L43147, L43155, L43166, L43717, L43720, L43721, L43722, L43753, L43139, L43705, L43744, L43103, L43132, L43151, L43160, L43707, L43708, L43752, L43755, L43760, L43117, L43142

Comments:

The study showed that levofloxacin or a combination of ETI-204 and levofloxacin, administered at approximately 72 hours post-challenge, were effective in improving survival in 58% and 69% of the rabbits, respectively; however, the survival was not significantly different between the two groups. A majority (99%) of the animals were bacteremic at the time of treatment whereas 57 of 84 animals were PA positive. All the PA positive animals were culture positive. PA detection by ELISA was less sensitive than measurement of bacteremia in this study. However, there was a positive correlation between bacterial load and PA levels.

By 24 hours post-treatment, all the animals treated with ETI-204+ levofloxacin were PA negative whereas all the control animals and a majority of the animals treated with levofloxacin were PA positive; all the treated animals were PA negative by 72 hours post-treatment. All the animals treated with a combination of ETI-204+levofloxacin or levofloxacin were culture negative by Day 7 post-treatment. The changes in bacteremia over time were similar in the ETI-204+levofloxacin or levofloxacin treated animals. All the control animals remained culture and PA positive until they were found moribund or died. Increased quantitative bacteremia levels and increased PA levels prior to or at the time of treatment, were both associated with decreased probabilities of survival.

Anti-PA IgG antibodies were measured prior to challenge and on Day 25 by an ECL assay which detects ETI-204 and any endogenous anti-PA antibodies formed as the method uses PA as a capture reagent and protein A/G as a detection reagent. As none of the animals were stated to have ETI-204 at Day 25, the results reflect endogenous anti-PA antibody. The results suggest presence of anti-PA antibodies in all surviving animals. Antibody levels in non-surviving animals were not measured.

Bacteria were observed by histological examination in the brain, liver, lymph nodes, lung, and spleen of the majority of the animals in the control group and in some of the non-surviving animals treated with levofloxacin or levofloxacin + ETI-204; however, no bacteria were observed in the tissues from all the surviving treated animals. Some of the tissues from the animals that died were processed for culture. Almost all the tissues (brain, liver, lymph nodes and spleen) from control animals as well as the treated animals that died were culture positive.

Clinical and necropsy findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above. Neuropathological examination was performed in select survivors and non-survivors. The neuropathologist concluded those animals treated with levofloxacin with or without ETI-204 and succumbed to the anthrax infection were more likely to mount an inflammatory response as compared to the untreated animals. However, survivors did not have microscopic changes with the exception of one combination treated animal with mild hemorrhage in/near meninges interpreted to be unrelated to ETI-204. Overall, it was concluded that study survivors did not have any CNS lesions (morphologic changes) due to ETI-204.

6.3.1.1.4. Study AR034

This was an open-label, randomized, controlled, factorial design non-GLP study to assess the efficacy of 16 mg/kg ETI-204 (IV; Lonza product) and levofloxacin (50 mg for 3 days, oral) in NZW rabbits after primary and secondary challenge with the spores of the Ames strain of *B. anthracis*.⁵⁴

The **primary objective** was to demonstrate that ETI-204 administered IV alone or in combination with antibiotics following primary challenge with spores of *B. anthracis* results in development of protective immunity as measured by increased survival in the absence of treatment following secondary challenge.

⁵⁴ (b) (4) Study Number 2637-100012211: AR034 - Re-challenge of rabbits treated previously for inhalational anthrax with intravenous ETI-204 to assess protective immunity (March 18, 2014).

The **secondary objectives** were to determine whether rabbits treated with ETI-204 or ETI-204 in combination with levofloxacin following primary challenge

- were more likely to survive a secondary challenge with spores of *B. anthracis* as compared to rabbits treated with antibiotics alone,
- demonstrated longer time to death following secondary challenge with spores of *B. anthracis* as compared to rabbits treated with antibiotics alone, and
- have significantly higher levels of circulating anti-PA IgG antibodies at the time of secondary challenge as compared to rabbits treated with antibiotics alone.

Study design:

There were two phases for this study.

Phase I: Animals weighing 2.8 to 4.2 kg were randomized to four groups (Table 83). The animals were treated with ETI-204, levofloxacin or a combination of ETI-204+levofloxacin at 30 hours post-challenge. The aerosolized procedure, the targeted 200X LD₅₀ (spore lot no. B39), maintenance and follow-up of the animals such as measurement of survival, time to death, microbial burden (bacteremia by qualitative and quantitative cultures and PA by ELISA), anti-PA IgG antibodies by ELISA, and TNA were similar to those summarized above for rabbit studies conducted at [REDACTED]^{(b)(4)}. Animals were followed for up to 6 months post-challenge in phase I (Table 83).

Phase II: The surviving treated rabbits from Groups 1, 2, and 3 (average body weight 3.9 kg) from Phase I were included in Phase II of the study; in addition, 12 experimentally naïve rabbits that age matched the survivors of Phase I study were included.

The time of secondary challenge (C2) was determined by the Study Director in consultation with the Applicant after observing the profiles of circulating endogenous anti-PA IgG antibodies analyzed throughout the first month of primary challenge (C1; phase I) and monthly thereafter through 6 months post-primary challenge (PC1). It was decided that a secondary challenge 9 months after primary challenge would be suitable to assess the objectives of this study.

The targeted LD₅₀ and other study procedures were same as that for the Phase I study except that no treatment was administered in phase 2 and animals were followed up to Day 21 post-second challenge (PC2). The follow-up measurements were same as for phase I (Table 84).

The phase I part of the study was not blinded whereas the phase II was blinded.

At the time of gross necropsy of animals, a small tissue section of brain, spleen, liver, and bronchial lymph nodes were collected and processed for bacterial cultures.

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Table 83: Study AR034 - Study design

Group	Number of Animals	ETI-204 Dose (mg/kg, IV)	Levofloxacin Dose (mg/kg/day) x 3 days	Time of Dosing (hours post-mean challenge)
Phase I				
1	20	16	0 (vehicle)	30
2	20	0 (saline)	50	30
3	20	16	50	30
4	8	0 (saline)	0 (vehicle)	30
Phase II				
1	Survivors Group 1	None	None	N/A
2	Survivors Group 2	None	None	N/A
3	Survivors Group 3	None	None	N/A
4	12 Naïve	None	None	N/A

Challenge with *B. anthracis*

Timeline: C1d0, C1d7, C1d14, C1d21 (Phase I); C2d0, C2d7, C2d21 (Phase II)

Re-challenge with *B. anthracis*

Note: Group 3 animals were administered ETI-204 and within 10 minutes levofloxacin was administered

C1= Primary challenge; C2= Secondary challenge; d=Day

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Table 84: Study AR034 - Blood collection assay schedule

Time-Point	Time Window	Blood Tube Type/Approx. Volume	Quantitative Bacteremia	Serum for PA ELISA	Serum for ETI-204 Levels	Plasma for Levofloxacin Levels	CBC	Serum for Anti-PA IgG ELISA	Serum for TNA	PBMC ^b
Phase I										
Day -2 C1	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL Chilled EDTA ~1 mL Heparin ~ 8 mL	X*	X	X	X	X	X	X	X
PTT ^a	Within 30 minutes of treatment	EDTA ~ 1.0 mL SST ~ 1.0 mL	X	X						
1hr PFT	± 5 min	SST ~1.5 mL Chilled EDTA ~1 mL			X	X				
7 days PC1	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X	X			X		
14 days PC1	Relative to Challenge Day	EDTA ~1.0 mL SST ~ 3.0 mL	X	X	X			X		
28 days PC1	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~3.0 mL	X	X	X			X	X	
2 month PC1	± 4 days	SST ~ 1.5 mL						X	X	
3 month PC1	± 4 days	SST ~ 1.5 mL Heparin ~ 8 mL						X	X	X
4 month PC1	± 4 days	SST ~ 1.5 mL						X	X	
5 month PC1	± 4 days	SST ~ 1.5 mL						X	X	
6 month PC1	± 4 days	SST ~ 1.5 mL CPT ~ 8 mL						X	X	X
Phase II										
Day -7 C2	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL Heparin~ 8 mL	X*	X	X	X	X	X	X	X
24hr PC2	± 1 hour	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X		
72 hr PC2	± 1 hour	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X		
120 hr PC2	± 1 hour	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X		
7 days PC2	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X		
14 days PC2	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X		
21 days PC2	Relative to Challenge Day	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				X	X	
Terminal ^c (Phase I and Phase II)	N/A	EDTA ~ 1.0 mL SST ~ 3.0 mL	X	X				**	**	

C1= primary challenge; C2=secondary challenge; PC1=post-primary challenge ; PC2=post-secondary challenge; PFT=post-first treatment

^a Prior to treatment (within 30 minutes prior to treatment)

^b. Sterility of PBMCs was confirmed following collection

^c Terminal samples were collected from animals that were found dead or euthanized, if possible

* Qualitative bacteremia only

** Serum for anti-PA IgG ELISA/TNA was collected from non-surviving animals during Phase II only (if possible)

N/A = Not applicable

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose (Phase I: mean LD₅₀ was 218.5; Phase II: mean LD₅₀ was 301. These LD₅₀s were comparable among the animals in the four groups; the LD₅₀ was ≥ 200 for approximately 65% of the animals in Phase I and 100% animals in Phase II (Table 85). The MMAD for each exposure day was between 1.11 – 1.22 μm which is consistent with the particle size range that would reach the alveoli.

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Phase I: All animals were culture negative on Day -2 i.e., prior to primary challenge. All animals except one (L40854 in Group 3 – PA concentration 29.3 ng/mL) were PA negative prior to challenge (Table 85). This could be due to past exposure, or may be a false positive finding due to cross-reactivity with other organisms (for details see microbiology review by Dr Berkeley).

All animals except one animal (L40845 in Group 3 – anti-PA IgG 57.4 ng/mL) were anti-PA IgG antibody negative; this could be due to past exposure, or may be a false positive finding due to cross-reactivity with other organisms. All the treated animals (Groups 1, 2, and 3) were TNA negative prior to challenge (Table 85).

Time between challenge and treatment initiation was between 26 and 30 hours.

Phase II: Prior to secondary challenge (Day -7), the mean body weights for animals in all four groups were similar (Table 85). All animals were culture negative prior to second challenge. Except for one animal (L40836 – PA positive in Group 1), all animals were PA negative. The anti-PA IgG antibodies by ELISA were reported in all animals except 2 (L40845 and L40866) in Group 3. A majority of the treated animals in Groups 1, 2 and 3 were TNA positive; 3 animals [one in Group 2 (L40821) and 2 in Group 3 (L40845 and L40866)] were TNA negative.

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Table 85: Study AR034 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Phase I				Phase II			
	Group 4 Control N = 8	Group 1 ETI-204 [‡] N = 20	Group 2 Levofloxacin [§] N = 20	Group 3 ETI-204 [‡] + Levofloxacin [§] N = 20	Group 4 Experimentally naïve control N = 12	Group 1 ETI-204 [‡] N = 13	Group 2 Levofloxacin [§] N = 20	Group 3 ETI-204 [‡] + Levofloxacin [§] N = 19
Baseline characteristics prior (Day -2) to first challenge				Baseline characteristics prior (Day -7) to second challenge				
Age (months) estimated	8.0±0	8.0±0	8.0±0	8.0±0	11.3±0.98	NA	NA	NA
Body weight (kg) Mean±SD	3.2±0.3	3.3±0.3	3.3±0.3	3.3±0.3	3.93±0.12	3.85±0.43	3.91±0.41	3.93±0.39
PA ELISA n (%)	<LLOQ	<LLOQ	<LLOQ	1 (5)	<LLOQ	1 (5)	<LLOQ	<LLOQ
Bacteremia (Qualitative)	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Anti-PA IgG -ELISA n (%)	<LLOQ	<LLOQ	<LLOQ	1 (5)	<LLOQ	13 (100)	19 (95.0)	17 (89.5)
TNA ED ₅₀	ND	<LLOQ	<LLLQ	<LLLQ	ND	13 (100)	19 (95.0)	17 (89.5)
TNA NF ₅₀	ND	<LLOQ	<LLOQ	<LLOQ	ND	13 (100)	19 (95.0)	17 (89.5)
Inhaled dose								
Total Inhaled Dose: cfu x 10 ⁷ Mean ± SD (Range)								
LD ₅₀ Mean ± SD (Range)	221.9±47 (150-279)	238.1±58.6 (136-367)	209.2±41 (128-320)	207.1±37.4 (149-297)	316.3±69.2 (238-421)	314.5±87.3 (220-520)	286.9±59.6 (196-413)	298.1±64.7 (204-404)
<200 LD ₅₀ n (%)	3 (37.5)	4 (20.0)	8 (40.0)	9 (45.0)	0	0	0	0
≥200 LD ₅₀ n (%)	5 (62.5)	16 (80.0)	12 (60.0)	11 (55.0)	12 (100)	13 (100)	20 (100)	19 (100)
Time (hours) to treatment and bacteremia post challenge								
Time to treatment Mean ± SD (Range)	27.4±1.3 (25.8-29.4)	28±1.4 (26-30.3)	27.9±1.5 (25.7-30.2)	27.5±1.2 (25.5-29.5)	NA ¹	NA ¹	NA ¹	NA ¹
Time to bacteremia Mean±SD (Range)	62.8±46.8 (26-139)	28.1±1.3 (26-30)	27.8±1.5 (26-30)	27.4±1.1 (26-30)	44.2±31.7 (22.9-118.2)	71.2 (n=1)	95.7±35.5 (71-121) (n=2)	83.9±43.9 (26-119) (n=4)
Survivors at the end of Phase I				Survivors at the end of Phase II				
Survival	0/8 (0)	13/20 (65.0)*	20/20 (100)	19/20 (95.0)	0/12	13/13 (100)	19/20 (95.0)	17/19 (89.5)
Survival in animals bacteremic PTT	0/4	10/17 (59.0)*	18/18 (100)	16/17 (94.1)	NA ¹	NA ¹	NA ¹	NA ¹

SD=Standard deviation; PTT=Prior to treatment; [‡]ETI-204 single dose (16 mg/kg); [§]Levofloxacin dose and duration (50 mg/kg for 3 days)

¹Not applicable as no treatment was administered in phase II.

Qualitative culture LOD =25 cfu/mL; quantitative culture LLOQ 100 cfu/mL; PA ELISA LLOQ=9.68 ng/mL. PA ECL positive control 2 ng/mL LOD=4 ng/mL

*Statistically significance by exact method and Boschloo's one-sided test between Groups 1 and 4; Differences between Groups 2 and 3 not significant (for details see statistics reviews by Drs Xianbin Li and Ling Lan)

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Microbial burden and antibody response of the animals at the time of or prior to treatment:

Phase I: At the time of treatment, the PA levels were below the LLOQ for a majority of the animals (56/68, 82%) in all four groups; the bacteremia levels were similar among the animals in Groups 1-3, and lower in the control Group 4 animals (Table 86).

Table 86: Study AR034 (Phase 1) – Number of animals PA positive and bacteremic prior to treatment

Parameters	Group 4 Control N = 8	Group 1 ETI-204 (16 mg/kg) N = 20	Group 2 Levo (50 mg/kg) N = 20	Group 3 ETI-204 (16 mg/kg) + Levo (50 mg/kg) N = 20
PA - ELISA				
n (%)	0 (0)	4/19 (21.1)	1 (5)	4/19 (21.1)
Log ₁₀ (ng/mL) mean±SD (Range)	<LOD	0.83±0.31 (<LOD-4.1)	(<LOD-52.1)	(<LOD-28.5)
Quantitative Bacteremia				
n (%)	4 (50)	17 (85)	18 (90)	17 (85)
cfu/mL	1.4±1.3	2.8±1.5	2.5±1.2	2.8±1.6
Log ₁₀ Mean±SD	(0.3-3.7)	(0.3-5.2)	(0.3-4.1)	(0.3-5.3)
Geometric mean (cfu/mL)	24.4	594.7	338.0	588.0
Time (hours) to treatment post-challenge				
Mean ± SD	27.4±1.3 (25.8-29.4)	28±1.4 (26-30.3)	27.9±1.5 (25.7-30.2)	27.5±1.2 (25.5-29.5)
SD=Standard deviation; PTT=Prior to treatment; Qualitative culture LOD =25 cfu/mL; quantitative culture LLOQ 100 cfu/mL; PA ELISA LLOQ=9.68 ng/mL.				

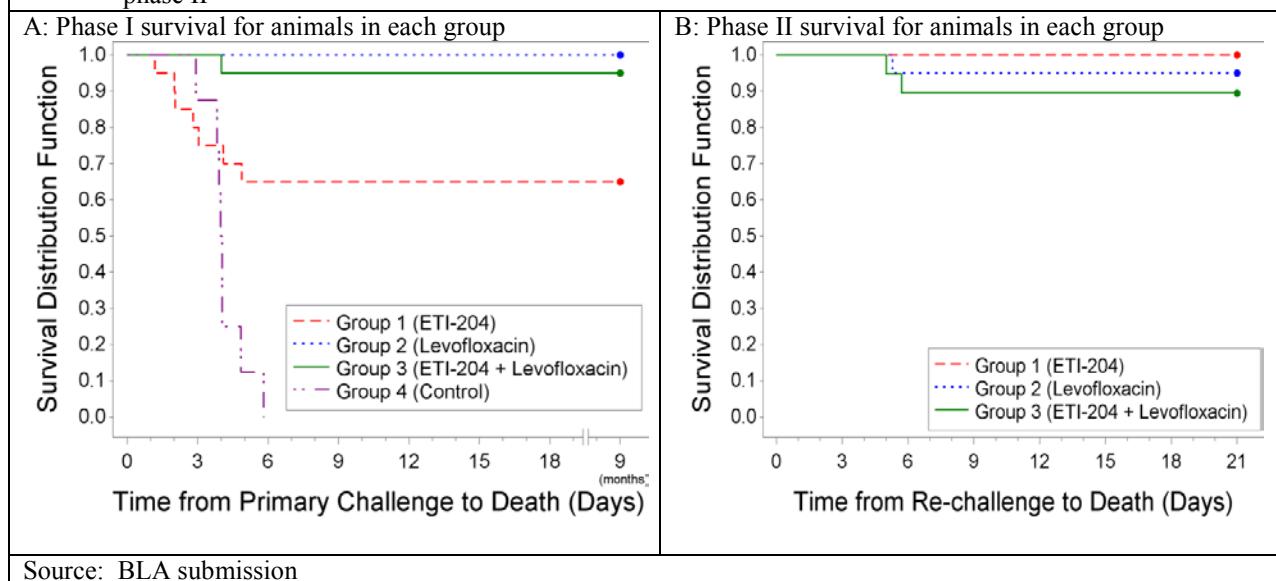
Phase II: No treatment was administered immediately prior to or after second challenge. A majority of the animals were culture negative and PA negative prior to second challenge (Table 85). However, a majority of the animals were anti-PA IgG and TNA positive 7 days prior to challenge (Table 85).

Effect of treatment on survival:

Phase I: ETI-204 was effective in improving survival in 65% of the animals; 7 animals died by Day 5. Levofloxacin or levofloxacin + ETI-204 were more effective in improving survival (Table 85 and Figure 50A). All the animals in the control untreated group died by Day 6.

Phase II: All the experimentally naïve control animals died by Day 7. A majority of the animals treated with ETI-204, levofloxacin, or a combination of ETI-204 and levofloxacin survived until Day 21 post second challenge (Figure 50B and Table 85).

Figure 50: Study AR034 - Kaplan-Meier curves representing time-to-death and survival by group in phase I and phase II



Source: BLA submission

Effect of treatment on bacteremia and PA levels at different time points:

Phase I:

Bacteremia: Complete resolution of bacteremia occurred by Day 7 after the first challenge (Table 87 and Figure 51). All terminal specimens (blood collected prior to euthanasia or after an animal was found dead) were culture positive, except for animal L40832 (Group 1, ETI-204 treatment). This animal was bacteremic prior to treatment, but had a negative terminal blood culture on Day 5 PC1. However, the brain tissue from this animal was positive for *B. anthracis*.

PA: A majority of the animals were PA negative by Day 7 post challenge (Table 87 and Figure 51). All terminal specimens (blood collected prior to euthanasia or after an animal was found dead) from control animals that were assessed ($n=4$) had a quantifiable level of free PA. However, all terminal specimens assessed from animals treated with ETI-204 alone were below the LLOQ ($n=6$).

One animal (#L40854), treated with ETI-204 + levofloxacin had a positive baseline PA-ELISA result at Phase I baseline i.e., prior to primary challenge; the animal survived to the end of study in Phase II.

Phase II:

Bacteremia: All animals in Groups 1 through 3 that were exposed to second challenge had no detectable bacteremia at nearly all time points; bacteremia in survivors was transient and present in low numbers (Table 87 and Figure 51). However, the three animals that succumbed to disease after the second challenge were bacteremic. In addition, all naïve control animals that were challenged during Phase II were bacteremic; the bacteremia levels at 24, 72, and 120 hours post-secondary challenge were greater than the animals in either of the treatment groups.

PA: All animals, except one, in Groups 1, 2 and 3 were PA negative (Table 87 and Figure 51); one animal in Group 1 (ETI-204 treatment; animal # L40836) that was PA positive prior to

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second challenge remained PA positive at scheduled sacrifice on Day 21 after re-challenge. It appears that the detection of PA in the Phase II samples for this animal was not related to an assay conduct issue (QCs, curve, and samples ran as expected on the plate), but was most likely related to something specific for this animal (i.e., interference, cross-reactivity) (for details see Dr Berkeley's review). Two Group 2 animals (#L40821 and #L40833) treated with levofloxacin were PA positive at Day 5 post second challenge time point; animal L40821 survived to the scheduled sacrifice, while animal L40833 died shortly after the Day 5 of second challenge time point and also had detectable free PA levels in the terminal sample. Two of the Group 3 animals (L40829 and L40859) treated with ETI-204 and levofloxacin were PA positive at 72 hours post second challenge; animal L40829 continued to have a quantifiable free PA level until the scheduled sacrifice, while animal L40859 died shortly after the Day 5 of second challenge time point even though the PA was below LLOQ at Day 5.

The Phase II naïve control animals had detectable free PA levels as early as 24 hours of challenge. At Days 3 and 5, the geometric mean free PA level in the Phase II control group was significantly greater than those in Groups 1-3 (Figure 51).

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Table 87: Study AR034 - Number of animals bacteremic and PA positive at different time points

Time points	Phase I				Phase II			
	Group 4 Control	Group 1 ETI-204 [‡]	Group 2 Levofloxacin [§]	Group 3 ETI-204 [‡] + Levofloxacin [§]	Group 4 Experimentally naïve control	Group 1 ETI-204 [‡]	Group 2 Levofloxacin [§]	Group 3 ETI-204 [‡] + Levofloxacin [§]
Bacteremia n/N (Range - cfu x 10³/mL)								
Prior to challenge (qualitative)	0/8	0/20	0/20	0/20	0/12	0/13	0/20	0/19
24 hours PC	ND	ND	ND	ND	8/12 (0.001-65.3)	0/13	0/20	1/19
PTT (~30 hours)	4/8 (0.001-5.3)	17/20 (0.001-158)	18/20 (0.001-10.3)	17/20 (0.001-4130)	NA	NA	NA	NA
Day 3	ND	ND	ND	ND	6/7 (0.001-34700)	1/13 (0.1)	1/20 (1)	1/19 (0.001)
Day 5	ND	ND	ND	ND	2/2 (0.2-7.6)	0/13	2/20 (1)	1/19 (0.001)
Day 7	NA	0/13	0/20	0/19	NA	0/13	0/19	1/18 (0.001)
Day 14	NA	0/13	0/20	0/19	NA	0/13	0/19	0/18
Day 21	NA	ND	ND	0/19	NA	0/13	0/19	0/18
Day 28	NA	0/13	0/20	0/19	NA	ND	ND	ND
Unscheduled terminal	7/7 (0.001-523000)	7/7 (0.001-165)	NA	1/1 (3.1)	9/12 (0.001-62300)	NA	1/1 (177)	2/2 (0.4-3120)
Free PA ELISA n/N (Range - ng/mL)								
Prior to challenge	0/8	0/20	0/20	1/20 (29.3)	0/12	1/13 (80)	0/20	0/19
24 hours PC	ND	ND	ND	ND	2/12 (10-34)	1/13 (32.4)	0/20	0/19
PTT (~30 hours)	0/8	4/19 (14.6-42.1)	1/20 (52.1)	4/19 (12.9-28.5)	NA	NA	NA	NA
Day 3	NA	ND	ND	ND	5/7 (25-15400)	1/13 (108)	0/20	2/19 (10.1-26.6)
Day 5	NA	ND	ND	ND	2/2 (54.8-99.2)	1/13 (212)	2/20 (24.7-84.7)	1/18 (15.3)
Day 7	NA	0/13	0/20	2/19 (11.2-12.3)	NA	1/13 (205)	0/19	1/17 (10.6)
Day 14	NA	1/13 (13)	1/20 (15.6)	2/19 (11.0-11.2)	NA	1/13 (119)	0/19	1/17 (36.3)
Day 21	ND	ND	ND	ND	NA	1/13 (47.2)	0/19	1/17 (17.3)
Day 28	ND	1/13 (10.9)	1/20 (16.0)	0/19	NA	ND	NA	ND
Unscheduled terminal	4/4 (2400-18500)	0/6	NA	NA	5/5 (682-17000)	NA	1/1 (181)	ND

[‡]ETI-204 single dose (16 mg/kg); [§]Levofloxacin dose and duration (50 mg/kg for 3 days)

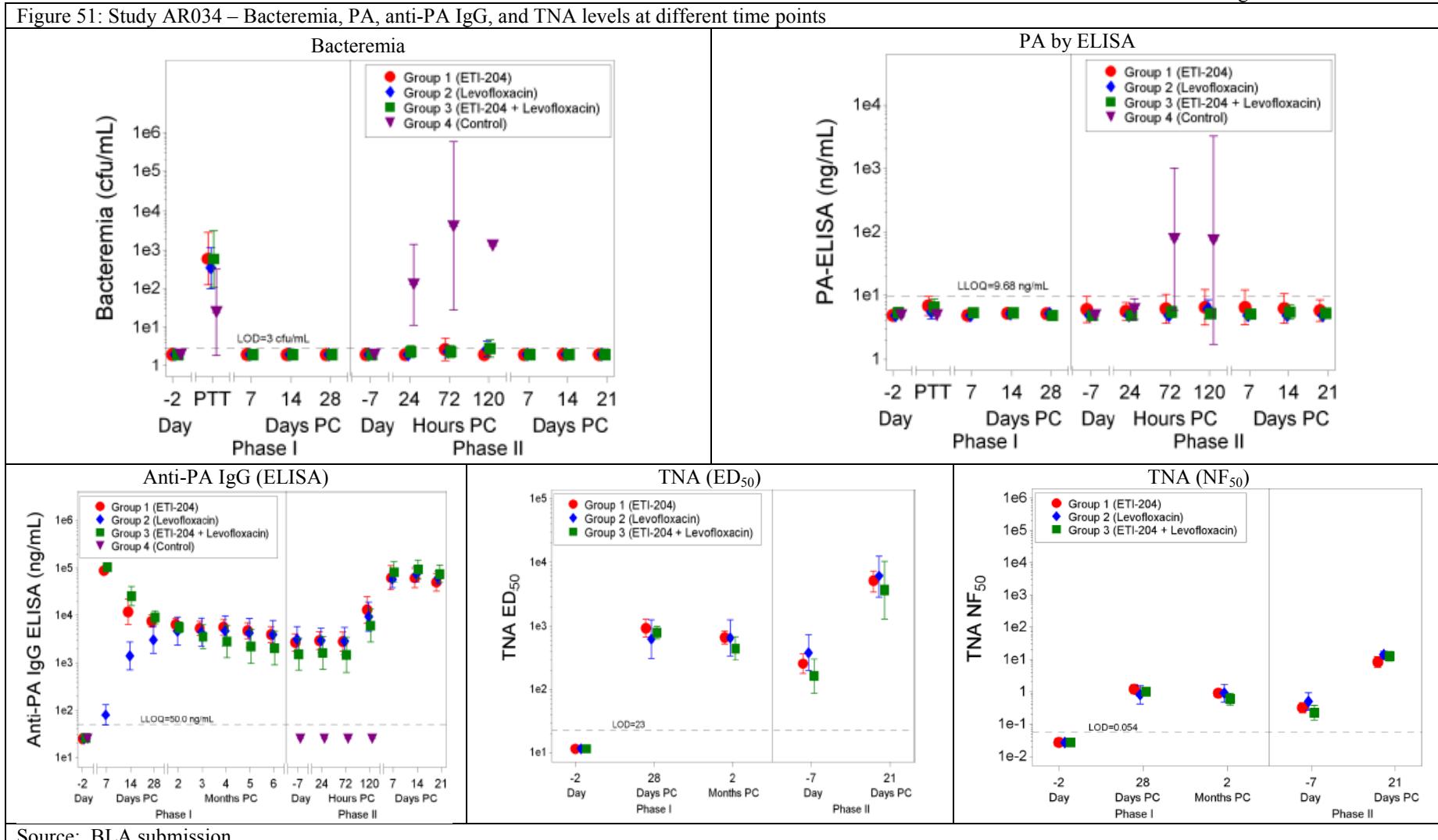
PC=post-challenge
PTT=prior to treatment

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Figure 51: Study AR034 – Bacteremia, PA, anti-PA IgG, and TNA levels at different time points



Source: BLA submission

Anti-PA IgG and toxin neutralizing antibody response:

Anti-PA IgG levels:

Phase I: A majority of the animals were anti-PA IgG antibody positive between Day 7 and Month 7 post-challenge. At Days 7, 14, and 28 post-challenge, the anti-PA IgG levels in animals treated with levofloxacin (Group 2) were lower than animals treated with ETI-204 (Group 1) or ETI-204 + levofloxacin (Group 3). As ETI-204 is also detected in the anti-PA IgG ELISA, the high levels of anti-PA IgG antibodies in animals administered ETI-204 (Groups 1 and 3) may be a reflection of endogenous IgG +ETI-204; the anti-PA IgG in animals treated with levofloxacin (Group 2) is a reflection of endogenous anti-PA IgG (Table 88). However, by Day 28 PC1, the ETI-204 levels were either BLOQ or reduced compared to previous time points. Therefore, antibody levels at Day 28 and after may reflect anti-PA IgG levels.

Phase II: A majority of the treated animals were anti-PA IgG positive at all the time points tested until Day 21 post-challenge (Table 88 and Figure 51); antibody levels increased at Day 7 after second challenge and there were no significant differences among the groups for anti-PA IgG levels at any of these time points. All treated animals (Groups 1, 2 and 3) that survived the primary challenge were anti-PA IgG positive prior to the secondary challenge except animals L40845 and L40866 (Group 3; ETI-204 and levofloxacin treatment); animal L40845 had an anti-PA IgG titer that was below the LLOQ by 4 months post-primary challenge. However, there was a quantifiable titer for this animal starting on Day 7 PC2 until the scheduled sacrifice (Day 21 post-challenge). The anti-PA IgG level for animal L40866 was below the LLOQ by Month 5 post-challenge. The antibody titer remained below the LLOQ through the 72 hour PC2 time point and this animal died prior to the next time point to be assessed for antibody levels.

The anti-PA IgG level for animal L40821 (Group 2; levofloxacin treatment) was below the LLOQ at all the time points through 6 months PC1, but this animal had a quantifiable level on Day -7 C2. This titer was quantifiable 24 hours PC2, and from Day 5 PC2 through the scheduled sacrifice.

Based on applicant analysis (logistic regression models fitted to the Phase II survival data from treated animals to examine the effects of treatment in Phase I and Phase II Day -7 anti-PA IgG levels on survival), the effect of Day -7 prior to second challenge anti-PA IgG levels on survival was significant, suggesting a probability of survival increased as Day -7 C2 anti-PA IgG levels increased. The relationship between the Day -7 prior to second challenge anti-PA IgG level and survival was not significantly different between the groups. The applicant states that these model results should be interpreted with caution since they included only three animals that died in Groups 2 and 3.

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Table 88: Study AR034 - Number of animals bacteremic and PA positive at different time points

Time points	Phase I				Phase II			
	Group 4 Control	Group 1 ETI-204 [‡]	Group 2 Levofloxacin [§]	Group 3 ETI-204 [‡] + Levofloxacin [§]	Group 4 Experimentally naïve control	Group 1 ETI-204 [‡]	Group 2 Levofloxacin [§]	Group 3 ETI-204 [‡] + Levofloxacin [§]
Anti-PA IgG ELISA n/N (μg/mL)								
Prior to challenge	0/8	0/20	0/20	1/20 (0.06)	0/12	13/13 (0.7-7)	20/20 (0.05-21.5)	17/19 (0.4-6.5)
24 hours PC	ND	ND	ND	ND	0/12	13/13 (0.8-8.3)	20/20 (0.06-20.6)	17/19 (0.4-7.0)
Day 3	ND	ND	ND	ND	0/7	13/13 (0.7-9.6)	19/20 (0.7-21.6)	17/19 (0.08-8.4)
Day 5	ND	ND	ND	ND	0/2	13/13 (2.3-46.9)	20/20 (0.2-28.7)	18/19 (1.7-31.1)
Day 7	NA	13/13 (49.3-128)	12/20 (0.06-0.3)	19/19 (58.9-158)	NA	13/13 (11.9-250)	19/19 (5.6-190)	17/17 (9.0-177)
Day 14	NA	13/13 (2-53.4)	19/20 (0.2-37.4)	19/19 (1.7-82.1)	NA	13/13 (20.3-158)	19/19 (14.5-400)	17/17 (11.3-275)
Day 21	NA	ND	ND	ND	NA	13/13 (12.7-147)	19/19 (13.5-129)	17/17 (22.9-252)
Day 28	NA	13/13 (3.7-19.2)	19/20 (0.9-44.3)	19/19 (3.3-30.1)	NA	NA	NA	NA
Month 2	NA	13/13 (2.2-13.4)	19/20 (1.3-24.2)	19/19 (1.4-11.4)	NA	NA	NA	NA
Month 3	NA	13/13 (1.6-11.6)	19/20 (1.3-24.9)	19/19 (0.1-11.3)	NA	NA	NA	NA
Month 4	NA	13/13 (1.8-15.4)	19/20 (12.5-35.8)	18/19 (0.06-11.5)	NA	NA	NA	NA
Month 5	NA	13/13 (1.5-13.8)	19/20 (1.2-26.5)	17/19 (0.8-9.1)	NA	NA	NA	NA
Month 6	NA	13/13 (1.3-9.5)	19/20 (1.5-21.8)	17/19 (0.6-8.4)	NA	NA	NA	NA
Unscheduled terminal	ND				0/6	NA	1/1 (0.01)	NA
TNA (ED₅₀)								
Prior to challenge	ND	0/13	0/20	0/19	ND	13/13 (95-658)	19/20 (44-4373)	17/19 (22-612)
Day 21	NA	ND	ND	ND	ND	13/13 (1369-12194)	19/20 (4314-16347)	17/17 (1304-16824)
Day 28	NA	13/13 (392-3203)	19/20 (94-6951)	19/19 (306-1421)	ND	NA	NA	NA
Month 2	NA	13/13 (356-1108)	19/20 (110-4579)	19/19 (35-1282)	ND	NA	NA	NA
TNA (NF₅₀)								
Prior to challenge	ND	0/13	0/20	0/19	ND	13/13 (0.1-0.9)	19/20 (0.05-5.5)	17/19 (0.03-1.1)
Day 21	NA	ND	ND	ND	ND	13/13 (3.6-22.0)	19/20 (3.2-42.1)	17/17 (2.2-32.0)
Day 28	NA	13/13 (0.5-3.7)	19/20 (0.1-3.8)	19/19 (0.5-1.8)	ND	NA	NA	NA
Month 2	NA	13/13 (0.4-1.7)	19/20 (0.1-6.8)	19/19 (0.05-1.5)	ND	NA	NA	NA

ETI-204 single dose (16 mg/kg); [§]Levofloxacin dose and duration (50 mg/kg for 3 days)

Toxin neutralization antibody:

The primary endpoints of the TNA assay are the ED₅₀⁴⁷ and the NF₅₀⁵¹.

Phase I: A majority of the surviving animals in Groups 1, 2, and 3 were TNA (ED₅₀ and NF₅₀) positive at Day 28 and Month 2 post-challenge. One animal (L40821 in Group 2) treated with levofloxacin survived but was TNA negative; this animal did not have a detectable ED₅₀ or NF₅₀ titer at Day 28 or 2 months post-challenge, which was consistent with the lack of an anti-PA IgG level in this animal through 6 months after primary challenge. There were no significant differences among the treatment groups for TNA at any time point during Phase I (Table 88). The TNA levels were not measured in non-surviving animals.

Phase II: A majority of the surviving animals in Groups 1, 2, and 3 were TNA (ED₅₀ and NF₅₀) positive at Day 21 post second challenge (Table 88). Three animals [L40833 (Group 2; levofloxacin treatment), L40866 (Group 3; ETI-204 and levofloxacin treatment), and L40845 (Group 3; ETI-204 and levofloxacin treatment)] that did not survive were TNA negative on Day -7 prior to second challenge; the TNA titer at Day 21 was not measured. As discussed above, animal L40821 was TNA (ED₅₀ or NF₅₀) negative at the time points assessed after the primary challenge; however, this animal did survive to the scheduled sacrifice and was TNA (ED₅₀ and NF₅₀) positive at Day 21 after second challenge.

TNA titer increased at Day 21 compared to Day-7 of second challenge (Figure 51). There were no significant differences in TNA positive response among the animals in different treatment groups for TNA ED₅₀ and NF₅₀ values either before or after secondary challenge.

Based on applicant analysis (logistic regression models fitted to the Phase II survival data from treated animals) the effects of treatment during Phase I and Day -7 prior to second challenge TNA ED₅₀ or NF₅₀ titers on survival was examined. Like for the anti-PA IgG antibody response by ELISA, the effect of Day -7 prior to second challenge, TNA ED₅₀ or NF₅₀ values on survival was significant. Overall, the results suggest that the probability of survival increased as the Day -7 second challenge TNA ED₅₀ or NF₅₀ levels increased after adjusting for group differences. These model results should be interpreted with caution since they included only three animals that died.

In vitro susceptibility testing: Not done.

Clinical Observations:

Phase I: Following challenge, the majority of animals exhibited abnormal outward clinical signs consistent with inhalational anthrax; these include lethargy, reduced food consumption, lacrimation, stool abnormalities, and respiratory abnormalities were most commonly noted. Animals that succumbed to disease demonstrated a characteristic progression of signs that followed from unremarkable to observations of lethargy, respiratory abnormalities, and finally moribundity. Animals were observed as normal during one month PC1 until secondary challenge (9 months PC1), except for occasional stool abnormalities and a clinical observation for animal L40823.

Phase II: Following secondary challenge through Day 7 post-challenge, animals in Groups 1-3 were documented as lethargic (5/49 survivors; 2/3 non-survivors) with signs of respiratory abnormalities (2/49 survivors; 2/3 non-survivors), stool abnormalities (17/49 survivors; 1/3 non-

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survivors), and reduced food consumption (24/49 survivors; 3/3 non-survivors). The animals returned to normal observations generally between 7-11 days PC2 with occasional stool abnormalities or reduced food consumption noted.

The body weights taken during quarantine, Phase I Day 0, monthly starting approximately 2 months PC1 through approximately 9 months PC1 and Phase II Day 0 were recorded. Animals that survived scheduled sacrifice increased in weight compared to their quarantine weights.

Necropsy and Histopathology: No gross lesions were reported in rabbits that died or became moribund following challenge in Phase I. In Phase II, only one animal (L64197; Group 4 control) that died had gross lesions as reflected by red foci in the large intestinal sacculus rotundus and appendix, which are lesions typical of anthrax in rabbits. Histopathology was not performed.

Assessment of bacteria in tissues:

Histology: Microscopic examination of tissues was not performed.

Culture:

Phase I: Bronchial lymph nodes, brain, liver and spleen from 8 treated animals that died during phase I of the study were processed for bacterial cultures (Table 89). Of the seven animals treated with ETI-204 that died, at least one of the tissues was culture positive in six animals. All the tissues from two animals (L40872 treated with ETI-204 and L40824 treated with levofloxacin + ETI-204) were culture negative; however blood cultures collected at terminal time point were positive.

Phase II: All of the animals that died during phase II had a positive tissue culture result for at least three of the tissues tested. All animals that survived the re-challenge did not have a positive culture from the tissues assessed (Table 89).

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Table 89: Study AR034 - Number of animals culture positive for *B. anthracis* in tissues

Phase I								
Tissue	Placebo		Levo 50mg/kg		ETI-204 16mg/kg		ETI-204 16mg/kg + Levo 50mg/kg	
	Survivors (N=0)	Non Survivors (N=8)	Survivors (N=20)	Non Survivors (N=0)	Survivors (N=13)	Non Survivors (N=7)	Survivors (N=19)	Non Survivors (N=1)
Presence of bacteria by microscopy [1]								
No microscopic bacterial assessment	ND	ND	ND	ND	ND	ND	ND	ND
Presence of bacteria by culture [1, 2]								
Bronchial Lymph Node	ND	8/ 8	ND	0/ 0	ND	4/ 7	ND	0/ 1
Brain	ND	8/ 8	ND	0/ 0	ND	5/ 7	ND	0/ 1
Liver	ND	8/ 8	ND	0/ 0	ND	4/ 7	ND	0/ 1
Spleen	ND	8/ 8	ND	0/ 0	ND	5/ 7	ND	0/ 1
Phase II								
Tissue	Untreated Control		Levo 50mg/kg		ETI-204 16mg/kg		ETI-204 16mg/kg + Levo 50mg/kg	
	Survivors (N=0)	Non Survivors (N=12)	Survivors (N=19)	Non Survivors (N=1)	Survivors (N=13)	Non Survivors (N=0)	Survivors (N=17)	Non Survivors (N=2)
Presence of bacteria by microscopy [1]								
No microscopic bacterial assessment	ND	ND	ND	ND	ND	ND	ND	ND
Presence of bacteria by culture [1, 2]								
Bronchial Lymph Node	0/ 0	12/12	0/19	1/ 1	0/13	0/ 0	0/17	2/ 2
Brain	0/ 0	12/12	0/19	0/ 1	0/13	0/ 0	0/17	2/ 2
Liver	0/ 0	12/12	0/19	1/ 1	0/13	0/ 0	0/17	2/ 2
Spleen	0/ 0	12/12	0/19	1/ 1	0/13	0/ 0	0/17	2/ 2

ND=Not Done

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate

Comments:

The study was performed in two phases to evaluate the effect of re-challenge with *B. anthracis* spores. In phase I, rabbits were treated with either ETI-204, levofloxacin, or a combination of ETI-204 + levofloxacin at approximately 30 hours post-exposure and followed for up to 9 months post-exposure. The results show that levofloxacin (20/20) or a combination of ETI-204 + levofloxacin (19/20) were more effective in improving survival compared to ETI-204 (13/20) monotherapy. All the control animals succumbed to *B. anthracis* infection. All the animals that died were bacteremic and PA positive whereas all the animals that survived were culture and PA negative by Day 7 post-challenge. Phase I part of the study was not blinded.

The 52 survivors from Phase I and 12 experimentally naïve animals (approximate age- and weight-matched) were challenged with an average dose of 301 (± 69) LD₅₀ equivalents of *B. anthracis* Ames spores via aerosol exposure nine months after the primary challenge; the LD₅₀ was 200 or above in all animals in second exposure compared to primary challenge (65% of the animals). All animals were culture negative prior to re-challenge; only one animal was PA positive prior to re-challenge. Phase II part of the study was blinded to prevent potential bias in the study conduct and outcome assessment.

Bronchial lymph nodes, brain, liver and spleen from 8 treated animals that died during phase I of the study were processed for bacterial cultures; seven of the 8 animals treated with ETI-204 that died, at least one of the tissues was culture positive in seven animals. All the tissues from two animals were culture negative; however blood cultures collected at terminal time point were

positive. No gross lesions were observed in rabbits that died or became moribund following B. anthracis challenge in Phase I.

No treatment was administered during Phase II. Animals were followed for up to 21 days after second challenge. A majority of the re-challenged animals survived until Day 21 (13/13 treated with ETI-204; 19/20 treated with levofloxacin and 17 of 19 treated with the combination of ETI-204 + levofloxacin) and were culture and PA negative; bacteremia and PA positive findings in survivors was transient and present in small number (10%) of the animals. All animals that survived the re-challenge did not have a positive culture from the tissues assessed; however, all of the animals that died during phase II had a positive B. anthracis tissue culture result for at least three of the tissues tested. All naïve control animals that were re-challenged were bacteremic and PA positive; the bacteremia and PA levels at 24, 72, and 120 hours post-secondary challenge were greater than the animals in either of the treatment groups. Only one animal (control) that died post-challenge had gross lesions in Phase II; lesions were consistent with acute B. anthracis infection.

A majority of the animals were anti-PA IgG positive between Day 7 and Month 7 post-challenge as well as at all-time points tested after re-challenge. In phase II, there were no significant differences among the groups for anti-PA IgG levels at any of these time points; however in phase I, the anti-PA IgG levels in animals treated with levofloxacin (Group 2) were lower than those in animals treated with ETI-204 (Group 1) or ETI-204 + levofloxacin (group 3) at Days 7, 14, and 28 post-challenge. This could be due to the detection of ETI-204 in anti-PA IgG ELISA assay used. The TNAs at Day 28 after primary challenge as well as Day-7 and Day 21 after second exposure were detected in surviving animals. TNA titer increased at Day 21 compared to Day-7 second challenge. There was no difference in antibody levels among the animals in the three treatment groups. Overall, the study suggests development of immunity in animals surviving after primary challenge. One of the limitations of the study is that the anti-PA IgG levels and TNA were not measured in non-surviving animals (both treated and control).

6.3.1.1.5. Study AR007

This was an open-label, controlled, factorial design GLP study with ETI-204 and levofloxacin in 57 NZW rabbits challenged with the spores (spore lot no. B24) of the Ames strain of *B. anthracis* weighing 2.2 to 2.7 kg, conducted at [REDACTED] ^{(b)(4)}⁵⁵. The primary objective was to demonstrate that post-exposure administration of ETI-204 leads to increased survival above that of levofloxacin (50 mg/kg).

Study design:

The study design was similar to the rabbit studies summarized above except that animals were divided in to six groups; treatment with ETI-204 ([REDACTED] ^{(b)(4)} monoclonal antibody) was administered IV or IM 9±3 hours post-exposure alone or in combination with human equivalent dose of levofloxacin by gavage (Table 90). Body temperature was not measured. Blood samples were collected in EDTA to determine bacteremia (probably qualitative) in moribund rabbits, rabbits found dead, and from the survivors on Study Day 34 (end of study). PA was not

⁵⁵ [REDACTED] ^{(b)(4)} Study Number 538-G005372: AR007 - Test of ETI-204 in rabbit spore challenge model post-exposure with/without levofloxacin (June 27, 2006).

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measured. Gross necropsies were performed on rabbits found dead or moribund euthanized to confirm death or illness due to anthrax infection; tissues such as brain, liver, spleen, kidney, lung, mediastinal lymph node and gross lesions were collected and processed for histopathology.

Table 90: Study AR007 – Study design

Group	Treatment	ETI-204 Dose	Levo Dose	Treatment Initiation	Therapy Duration (Route)	No. of Animals
1	Control (PBS*)	N/A**	N/A	9±3 hours	Single dose (IV)	9
2	Levo + PBS	N/A	50 mg/kg	9±3 hours	5 days (oral)	12
3	ETI-204 + Levo control	10 mg (4 mg/kg)	N/A	9±3 hours	Single dose (IV)	9
4	ETI-204 + Levo	10 mg (4 mg/kg)	50 mg/kg	9±3 hours	Single dose (ETI-204; IV) + 5 days (Levo; oral)	9
5	ETI-204 + Levo control	20 mg (8 mg/kg)	N/A	9±3 hours	Single dose (IM)	9
6	ETI-204 + Levo	20 mg (8 mg/kg)	50 mg/kg	9±3 hours	Single dose (ETI-204; IM) + 5 days (Levo; oral)	9

ETI-204 dose 10 mg (4 mg/kg) or 20 mg (8 mg/kg) single dose; Levo=levofloxacin 50 mg/kg for 5 days
 * ETI-204 control used phosphate buffered saline (PBS) IV
 ** N/A – Not applicable

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose (mean LD₅₀ 274±51; (b) (4) cfu) were comparable among different groups; the LD₅₀ dose was ≥ 200 for approximately 91% of the animals (Table 91). The MMAD for each exposure day was 1.15 µm which is consistent with the particle size range that would reach the alveoli.

Time between challenge and treatment initiation was similar among the animals in different study groups.

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Table 91: Study AR007 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 1 Control N = 9	Group 2 Levo [§] N = 12	Group 3 ETI-204 (10 mg IV) [‡] N = 9	Group 4 ETI-204 (10 mg IV) [‡] + Levo [§] N = 9	Group 5 ETI-204 (20 mg IM) [‡] N = 9	Group 6 ETI-204 (20 mg IM) [‡] + Levo [§] N = 9
Baseline characteristics						
Age (years) estimated	4.0±0	4.0±0	4.0±0	4.0±0	4.0±0	4.0±0
Body weight (kg)	2.5±0.1	2.5±0.1	2.5±0.1	2.5±0.2	2.5±0.1	2.5±0.1
Inhaled challenged dose						
Total Inhaled Dose: cfu x 10 ⁷	(b) (4)					
Mean ± SD (Range)						
LD ₅₀	268.6±47.5	297.3±55.2	287.8±69.5	262.3±40.8	270.4±38.4	252.6±41.7
Mean ± SD (Range)	(153-304)	(222-396)	(158-400)	(191-320)	(201-317)	(199-299)
<200 LD ₅₀ n(%)	1 (11.1)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	2 (22.2)
≥200 LD ₅₀ n(%)	8 (88.9)	12 (100.0)	8 (88.9)	8 (88.9)	9 (100.0)	7 (77.8)
Time (hours) between challenge and treatment						
Time to treatment; Mean ± SD ^b	7.9±1	8.7±1	8.4±0.9	8.2±0.7	8.4±1.2	9.1±1.1
Survivors and number of bacteremic animals at the end of study (Day 34)						
n (%)	0 (0) [§]	4 (33)**	9 (100)**	8 (88.9)**	9 (100)**	9 (100)**
Time to death (hours) Range	2.4-5	11.9-34	34	16.8-34	34	34
Bacteremia ^a n (%)	8 (88.9) [§]	8 (66.7)	0 (0)	1 (11.1)	0 (0)	0 (0)

[‡]ETI-204 dose was a fixed dose of 10 mg or 20 mg represent 4 mg/kg and 8 mg/kg, respectively.

[§]One animal in the control group was found moribund and sacrificed on Day 5; this animal was culture negative and no bacteria found in spleen.

SD=Standard deviation; PTT=Prior to treatment; Levo=levofloxacin.

^aPresence of bacteria by qualitative culture in blood and spleen at terminal time point (day of death, moribund, or terminal sacrifice); LOD =not specified

^bThe time to treatment was defined as the time from challenge to treatment.

**Statistically significance by one-sided Fisher's exact test; this varies with the test used (for details see statistics reviews by Drs Xianbin Li and Ling Lan)

Detection of bacteremia and PA: Not applicable as bacteremia and PA levels were not measured at different time points.

Effect of treatment on survival: ETI-204 administered IV (10 mg i.e., ~4 mg/kg) or IM (20 mg i.e., ~8 mg/kg) was effective in improving survival in all the animals whereas levofloxacin at a dose of 50 mg/kg improved survival in 33% of the animals (Table 91 and Figure 52) i.e., about 67% of the animals in the levofloxacin treated group died after cessation of treatment. Although the reason for lower survival (33%) in animals treated with levofloxacin is unclear, it is possible that this could be due to shorter duration of treatment with the antibacterial drug alone. It would have been useful if bacteremia and PA levels were measured in the animals at different time points.

All animals treated with ETI-204 IM (20 mg) in combination with levofloxacin survived the period of observation. However, one animal treated with ETI-204 (10 mg) died on Day 17. Of the 9 untreated control group animals, 8 died within 4 days of exposure; one animal was found moribund and sacrificed on Day 5 and no bacteria were found in blood or spleen. All animals that died were bacteremic. All the rabbits that survived were culture negative.

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Figure 52: Study AR007 - Kaplan-Meier curves representing time-to-death and survival by group

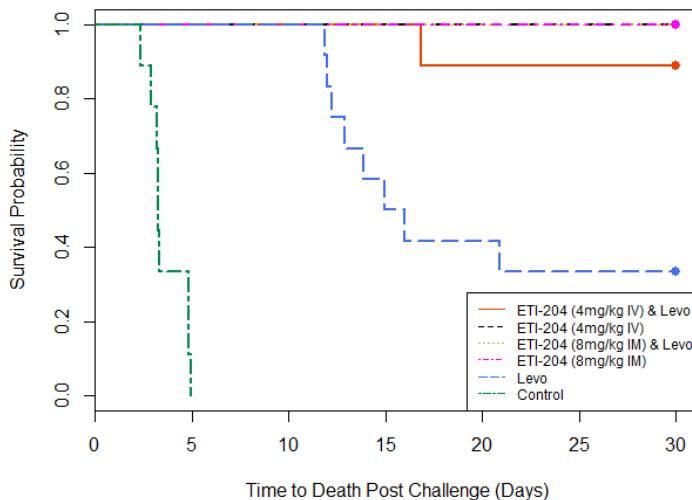


Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

Clinical observations: Following challenge, the majority of animals that died were lethargic, uncoordinated and showed stool abnormalities as well as ulceration (skin). A majority of the animals that died and some of the surviving rabbits exhibited inappetance; such findings were not reported in a majority of the animals that survived (Table 92).

Table 92: Study AR007 - Summary of clinical observations

Clinical Observation	Number of animals in each group that demonstrated the clinical observation at least once during the observation period					
	Group 1 (n=9)	Group 2 (n=12)	Group 3 (n=9)	Group 4 (n=9)	Group 5 (n=9)	Group 6 (n=9)
dead/euthanized ^a	9	8	0	1	0	0
facial swelling	0	3	0	0	0	1
iatrogenic trauma ^b	0	0	0	0	0	1
lethargic	4	6	0	0	0	0
lacrimation	0	1	0	0	0	0
moribund	1	1	0	0	0	0
normal	9	12	9	9	9	9
no stool	2	5	0	0	0	0
not eating	9	11	2	3	1	3
rhinorrhea	1	1	0	0	0	0
reduced stool	1	0	0	0	0	0
seizure	1	0	0	0	0	0
soft stool	1	0	0	0	0	0
ulceration (skin)	0	0	0	0	0	1
uncoordinated	0	0	0	0	0	0

^a Does not include animals that were euthanized on Study Day 34.

^b Self trauma is occasionally seen after tranquilizers or anesthetics are administered and are unrelated to the anthrax challenge.

Necropsy and Histopathology: Gross lesions reported include discolorations (hemorrhage) in the brain and intestine (cecum); fluid (transudate) in the thoracic cavity; enlargement of mediastinal lymph nodes; and edema of skin (eyelid or ventral neck).

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Tissue bacterial assessments:

Histology: No bacteria were observed in one non-surviving animal in the control group and one surviving animal treated with 20 mg ETI-204 (IM) + levofloxacin (Table 93). Other tissues from surviving and surviving animals were not examined histologically.

Culture: The results showed absence of bacteria in spleen tissue from animals that survived the period of observation; spleen from most of the animals that died were culture positive (Table 93). Tissues other than spleen were not evaluated.

Table 93: Study AR007-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		Levo 50mg/kg		ETI-204 ~4mg/kg IV		ETI-204 ~4mg/kg IV + Levo 50mg/kg		ETI-204 ~8mg/kg IM		ETI-204 ~8mg/kg IM + Levo 50mg/kg	
	Non		Non		Non		Non		Non		Non	
	Survivors (N=0)	Non (N=9)	Survivors (N=4)	Non (N=8)	Survivors (N=9)	Non (N=0)	Survivors (N=8)	Non (N=1)	Survivors (N=9)	Non (N=0)	Survivors (N=9)	Non (N=0)
Presence of bacteria by microscopy [1, 2]												
Brain	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Kidney	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Liver	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Lung	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Mediastinal	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Lymph Node												
Mediastinum	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Skin	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Spleen	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
Thymus	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Presence of bacteria by culture [1]												
Spleen	0/0	7/9	0/4	7/8	0/9	0/0	0/8	1/1	0/9	0/0	0/9	0/0

[1] All treated animals irrespective of bacteremia status prior to treatment
[2] Histopathology performed at ^{(b) (4)} Not all animal were assessed microscopically; numbers examined are shown
Qualitative bacteremia was performed only at end of study; PA (ECL and ELISA) analysis was not performed

Comments:

The study showed that treatment with ETI-204 IV (10 mg) or IM (20 mg) monotherapy or in combination with levofloxacin, approximately 9 hours post-exposure was more effective than levofloxacin monotherapy. Lower survival rate in animals treated with levofloxacin could be due to administration of treatment before germination of spores to the vegetative form commences as well as shorter duration of treatment; germination of spores can take at least 12 hours.⁶ Antibacterial treatment early after spore challenge can lead to persistence of spores and disease development following cessation of therapy.¹

A majority of the animals that survived were culture negative and no bacteria, by culture, were reported in the spleen. However, most of the animals that died were culture positive in blood and spleen. Tissues other than spleen were not evaluated by culture. Tissues from all animals were not examined for the presence of bacteria by histology.

Lethargy and not-eating were the most common abnormal observations among the rabbits that died. Gross pathology lesions observed were typical of inhalation anthrax in rabbits and included discolorations (hemorrhage) in the brain and intestine (cecum); fluid (transudate) in the thoracic cavity; enlargement of mediastinal lymph nodes; and edema of skin (eyelid or ventral neck).

6.3.1.2. ETI-204 in combination with doxycycline

6.3.1.2.1. Study AP-10-055

This was an open-label, non-randomized, controlled, factorial design non-GLP study to assess the efficacy of ETI-204 (IV; Baxter product) and low dose of doxycycline (IV, 2 mg/kg for 3 days, administered by electric infusion pump over 5 minutes) in 24 NZW rabbits (b)(4)

(b)(4) with the spores of the Ames strain of *B. anthracis*, conducted at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).⁵⁶ The Ames strain was a primary subculture obtained by USAMRIID (February 2002; source not specified). The objective was to determine the added benefit of adjunct therapy in combination with a low dose of doxycycline in comparison to the antibiotic alone.

Study design:

Animals were divided into 3 groups (Table 94). Animals were infected by aerosolized route by placing in individual cat restraint bags in preparation for whole-body head-out plethysmography. Animals were exposed to 200 LD₅₀ spores in a muzzle-only aerosol chamber within a class III biological safety cabinet for a time-calculated aerosol exposure. Aerosols were generated by a three-jet Collison nebulizer. The presented dose was calculated for each NZW rabbit by multiplying the total volume (V_t) of experimental atmosphere inhaled ($V_t = V_m \times \text{length of exposure}$) by the aerosol concentration (C_e) ("presented dose" $C_e \times V_t$). This equation assumed constant minute volume and constant aerosol concentration over time with complete (100%) respiratory deposition. Aerosol concentration was calculated by:

$$(C_{\text{sampled}} \times V_{\text{sampled}})/(Q_{\text{sampled}} \times t_{\text{sampled}})$$

where C_{sampled} = the titrated concentration of the sampler, V_{sampled} = the volume of the collection media in the sampler, Q_{sampled} = the flow rate through the sampler, and t_{sampled} = the total time the sample was taken.

Treatment with doxycycline (2 mg/kg, b.i.d. for 3 days⁵⁷), doxycycline + ETI-204 (8 mg/kg), or saline were administered based on PA positive findings by the ECL assay or approximately 30 hours post-exposure which ever occurred first. Doxycycline was administered every 12 hours for 3 days. Animals were followed at different time intervals for microbiological evaluations and blood cell count, until Day 21 post-challenge (Table 94); survival was measured until Day 30 post-challenge. Animals with a score indicative of a very poor health status (i.e., score ≥ 7) during the monitoring period were euthanized. Animals were necropsied and ear with tattoo, thoracic pluck (lungs, heart, trachea, esophagus, with attached mediastinum), spleen, kidneys, liver and brain were collected and processed for histological evaluations.

Microbiological measurements:

Bacteremia: Bacteremia was measured by culturing 0.3 to 0.5 mL blood with SPS (yellow top tubes) on sheep blood agar plates and incubated for 18 to 24 hours at 37°C.

⁵⁶ U.S. Army Medical Research Institute of Infectious Diseases (Sponsor NIAID) Study Number AP-10-055: Efficacy of Anthim® ETI-204 monoclonal antibody as an adjunct therapy in a New Zealand White rabbit partial survival model for inhalational anthrax (Aug 23, 2013).

⁵⁷ The doxycycline dose of 2 mg/kg (twice daily x 3 days IV) was based on the results of previous studies conducted at USAMRIID in which this dose resulted in 40-60% survival. 2 mg/kg dose=0.6 mg/kg or 36 mg human dose based on body surface area.

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ECL assay: PA was measured in whole blood (50 µL) by ECL assay using three PA-specific monoclonal antibodies (Critical Reagents Program, Aberdeen Proving Grounds Edgewood Area, Chestertown, MD) as a capture-antibody mix, and a polyclonal rabbit serum made against PA was used as a detector antibody to measure PA. Purified antibodies were labeled with biotin using standard coupling methods and biotinylated antibodies pre-bound to streptavidin-coated paramagnetic beads. Two negative controls and two levels of positive controls (whole blood spiked with PA) were included. All samples were tested in duplicate and read using the M1M analyzer. Samples were considered positive if the ECL signal to noise (background) was ≥1.2 times the average of the negative control or > three standard deviations of the negative controls, which ever was greater. Antigenemia results were determined within one hour of blood collection.

Table 94: Study AP-10-055 - Study design and blood collection schedule

Group	Number of animals	Treatment assignment [‡]
1	4	Vehicle (saline)
2	10	Doxycycline (2 mg/kg, b.i.d. for 3 days) [‡]
3	10	Doxycycline (2 mg/kg, b.i.d. for 3 days) + ETI-204 (8 mg/kg, single dose IV)*

*In Group 3, ETI-204 treatment occurred immediately after initiation of antibiotic alone treatment.

[‡]Administered by electric infusion pump via IV

Blood collection schedule

Day	Description	Collection Volume		Cumulative Total	
		ml	% blood vol	ml	% blood vol
-7	PRE-EXPOSURE (VAP Surgery)	2	1.2	2	1.2
-2	Before being moved to BSL3	1	0.6	3	1.8
0	POST-EXPOSURE (At 15, 18, 21 and 24 Hrs)	4	2.4	7	4.2
1	POST EXPOSURE (At 27 and 30 Hrs)	2	1.2	9	5.4
	POST-ANTIBIOTIC				
1	10 mins After treatment	1	0.6	10	6.0
2	24±8 Hrs ("Day 1")	1	0.6	11	6.6
3	24±8 Hrs ("Day 2")	1	0.6	12	7.2
4	24±8 Hrs ("Day 3")	1	0.6	13	7.8
5	24±8 Hrs ("Day 4")	1	0.6	14	8.4
6	24±8 Hrs ("Day 5")	1	0.6	15	9.0
7	24±8 Hrs ("Day 6")	1	0.6	16	9.6
8	24±8 Hrs ("Day 7")	1	0.6	17	10.2
2-8	Evaluate Antibiotic	3	1.8	20	12.0
15	7±2 Days ("Day 14")	1	0.6	21	12.6
22	7±2 Days ("Day 21")	1	0.6	22	13.2

Results:

Baseline characteristics: The age of the animals at the time of challenge and their body weights were not specified. The animals were stated to be adults with body weight ranging between 3

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and 5 kg. It appears that one animal (#42 treated with ETI-204 + doxycycline) was PA positive by ECL prior to exposure; all animals were culture negative.

The LD₅₀ was highly variable and ranged from 34X to 773X LD₅₀ with a mean of 379 (Table 95); 71% of the rabbits were exposed to LD₅₀ of ≥200. The LD₅₀ was <200 in 3 of the 4 animals in the control group (Table 95).

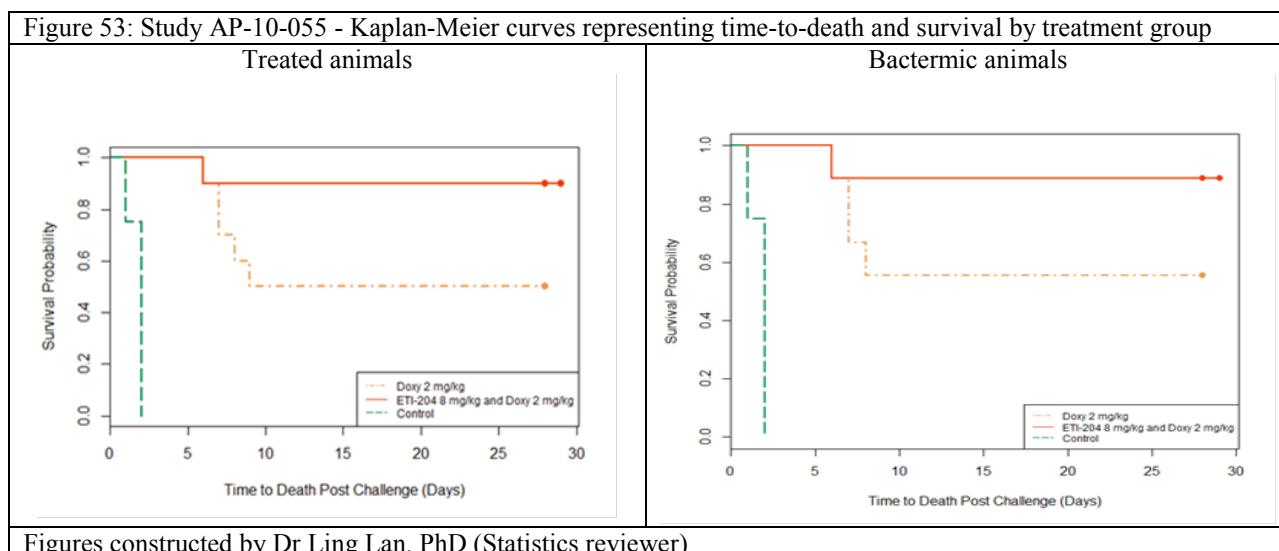
A majority of the animals were PA positive by the ECL assay and bacteremic at the time of treatment; one animal treated with doxycycline and one treated with ETI-204+doxycycline were PA and culture negative at the time of treatment (Table 95).

Table 95: Study AP-10-055 - Baseline characteristics, inhaled dose of <i>B. anthracis</i> , time to treatment and survival			
Parameters	Placebo (Group 1) N=4	Doxycycline (2 mg/kg b.i.d for 3 days) (Group 2) N=10	Doxycycline (2 mg/kg b.i.d for 3 days) + ETI-204 (16 mg/kg) (Group 3) N=10
Baseline characteristics and inhaled dose			
Age (years) estimated range	NA	NA	NA
Body weight (kg)	3 - 5	3 - 5	3 - 5
PA-ECL	0/4	0/10	1/10
Inhaled dose			
Total Inhaled Dose: cfu x 10 ⁷	(b) (4)		
Mean ± SD (Range)			
LD ₅₀ dose	173.5±198.3	381.03±239.8	458.6±182.2
Mean ± SD (Range)	(50.4-467.4)	(33.6-708.3)	(209.7-773.3)
<200 LD ₅₀ dose n(%)	3 (75.0)	4 (40.0)	0
≥200 LD ₅₀ dose n(%)	1 (25.0)	6 (60.0)	10 (100)
Trigger for Treatment (PA by ECL)^a and bacteremia prior to treatment			
Positive Screening PA/ECL Assay n (%)	4 (100)	9 (90)	9 (90)
Bacteremia n (%)	4 (100)	9 (90)	9 (90)
Survivors at the end of study (Day 28)			
Survivors – all animals	0 (0)	5 (50.0)	9 (90.0)
Survivor - bacteremic animals	NA	5/9 (55.6)	8/9 (88.9)
SD=Standard deviation PTT Prior to treatment; Quantitative bacteremia LOD Limit of detection=Not specified; PA ELISA LLOQ Lower limit of quantification=Not specified.			
The doxycycline dose of 2 mg/kg (twice daily x 3 days IV) was based on the results of previous studies conducted at USAMRIID in which this dose resulted in 40-60% survival.			
^a The trigger for treatment was defined as the time from challenge to a positive PA-ECL post-challenge. Statistically significant by Fisher's exact test and Boschloo's one-sided test; no difference between the animals treated with the doxycycline or doxycycline+ETI-204			

Effect of treatment on survival: The results showed that 90% of the animals treated with the combination of ETI-204 and doxycycline survived the 28 day period of observation; doxycycline was less effective (Table 95 and Figure 53). All the treated non-surviving animals died between Day 6 and 9; the animals in the control group died between Days 1 and 2.

Of the two rabbits that never tested positive for the presence of PA in whole blood or became bacteremic, only one (#47) survived, while the other (#45), succumbed to disease at 7 days post-exposure.

Effect of treatment on microbial burden: All surviving animals became culture negative and PA negative. Animals that succumbed to disease were culture positive and PA positive.



Clinical observations: Animals were monitored at least two times a day. The scoring parameters were:

- appearance - 0: normal (coat smooth, eyes/nose clear, nose twitching); 1: increased respirations/dyspnea or rough coat; 2: hunched up, listless or agitated, abdominal breathing, open mouth breathing, head not erect, recumbent.
- natural behavior - 0: normal; 1: minor changes, teeth grinding, decreased food consumption; 2: decreased mobility and alertness, aggression; 3: absence of eating/drinking, vocalization, decreased mobility).
- provoked behavior - (0: normal (will hop when touched, may tense/resist handling); 1: subdued but normal only when touched/provoked, 2: subdued even when stimulated, 4: unresponsive when stimulated, weak, precomatose.
- neurological behavior (yes/no).
- over grooming/alopecia.
- hyperactivity.
- spinning in cage.
- dilated or constricted pupils.

The rabbits scoring between 4-6 were monitored with increased frequency (three times/day), with the endpoint criteria for humane euthanasia, indicative of very poor health status, being a score of ≥ 7 .

The intervention scores were within normal limits (0-3) up until time to-death. The single rabbit that was euthanized, exhibited the highest score (9); the scores ranged from normal to elevated (4-6) in rabbits that were found dead. It is possible that in those animals that exhibited scores within normal limits and were found dead was due to the rapid progression of disease. The

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scores in all surviving animals were within normal limits. Although the clinical observations were not presented as scores in the studies performed at [REDACTED] (b) (4) clinical observations were consistent with those reported for [REDACTED] (b) (4) studies in rabbits summarized above.

Necropsy and Histopathology: Gross changes in most animals that succumbed included non-collapsing lungs that were sharply mottled red; an enlarged friable spleen and liver; hemorrhage, congestion, and/or edema in multiple tissues; and pleural effusion. Additionally, three animals, [#43 and #60 (treated with doxycycline); #55 (treated with doxycycline + ETI-204)], had lesions consistent with meningitis. In animal #43 and #55, bacilli were observed within the meninges. Two animals (#59 and #62) treated with doxycycline and eight animals treated with ETI-204+doxycyclcine), had mild to moderate aspiration pneumonia.

All control group animals succumbed to disease within 2 days post-challenge with typical anthrax lesions. In addition, two animals (#39 and #37), had mild aspiration pneumonia.

Histological changes included necrotizing splenitis; pneumonia (due to infection with *B. anthracis*); adrenal gland necrosis and hemorrhage; degeneration and necrosis of renal tubules; mediastinitis, meningitis; and bacilli noted within blood vessels of multiple tissues and organs. These are consistent with reported lesions in other studies summarized above. Atypical histologic findings included aspiration pneumonia (noted in twelve animals) and meningitis noted in three animals: #43, #60, and #55.

Tissue bacterial assessments:

Histology: Histological examination showed presence of bacteria in the tissues from all animals that succumbed to death. Presence of bacteria was not reported in any animal that survived the period of observation.

Culture: Tissues were not processed for culture.

Comments:

The results showed that a majority (90%) of the animals treated with the combination of ETI-204 and doxycycline survived the 28 day period of observation; doxycycline was less effective at the dose (2 mg/kg for 3 days) tested. All the treated non-surviving animals died between Day 6 and 9; the animals in the control group died between Day 1 and 2. Histological examination showed presence of bacteria in the tissues in all animals that succumbed to death. Presence of bacteria was not reported in any animal that survived the period of observation.

Clinical and necropsy findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above.

6.3.2. Cynomolgus monkeys

The applicant conducted two studies (NIAID 1056 and NIAID 2469) in cynomolgus monkeys to evaluate the efficacy of ETI-204 8 mg/kg in combination with a low dose (10 mg/kg) of ciprofloxacin.

6.3.2.1. Study 1056

This was a randomized, controlled, open-label factorial design, non-GLP study with ETI-204 and ciprofloxacin in 48 cynomolgus monkeys challenged with the spores (spore lot no. B35) of the Ames strain of *B. anthracis* weighing 2.2 to 4.1 kg, conducted at [REDACTED] ^{(b) (4)} ⁵⁸. The trigger for treatment was PA positive finding, in serum, by the ECL assay.

Primary Objective

To assess the efficacy of a 8 mg/kg dose of ETI-204 administered IV in combination with a low dose of ciprofloxacin (10 mg/kg⁵⁹) compared to ciprofloxacin monotherapy on survival rate, when administered in delayed fashion (24±12 hours after the first positive PA result by ECL) following challenge.

Primary Endpoints

Survival rate by Day 28 post-challenge.

Secondary Objectives

Secondary objectives were to

- evaluate efficacy of ETI-204 when used after detection of positive PA by ECL.
- evaluate all untreated controls until death or euthanasia to provide data on disease progression in *B. anthracis* aerosol challenged cynomolgus monkeys.

Study design:

This study was not a GLP-compliant study but was conducted in compliance with the study protocol. The study measurements (such as prescreening of animals, aerosolization, and trigger for treatment) were similar to that for the monotherapy studies except for the following:

- Oral and rectal swabs were taken from each animal prior to challenge and tested by the PCR assay for the presence of *Klebsiella* by [REDACTED] ^{(b) (4)}. The applicant states that all oral and rectal swab samples from the animals were negative for *K. pneumoniae* and the associated virulence factors (magA and rmpA). However, three animals (A07043, A08105, A07507) were listed, in the medical records, as *Klebsiella* positive and included in the study.
- During the pre-challenge period, blood was collected from each animal and tested for neutralizing antibodies by the TNA and anti-PA IgG antibodies by ELISA. All animals were negative for toxin neutralizing properties and a humoral immune response to anthrax PA by TNA and IgG ELISA, respectively, prior to challenge. Antibody response post-treatment was not measured.
- Bacteremia was measured by qualitative (direct streaking of about 40 µL blood over blood agar plates) and quantitative methods.
- Ciprofloxacin MICs were determined on positive cultures from terminal blood samples by the broth dilution method using Mueller Hinton Broth (MHB) and cultures incubated for 16-20 hours.
- Animals were randomized to 4 groups (Table 96).

⁵⁸ [REDACTED] ^{(b) (4)} Study Number 1056-G607605: Efficacy of monoclonal antibody given in combination with ciprofloxacin in the cynomolgus macaques therapeutic model of inhalational. March 20, 2012.

⁵⁹ Ciprofloxacin humanized dose in non-human primates is approximately 75 mg based on its exposure equivalent to 500 mg; therefore, based on a body weight of 2.9 kg in Study AP1056, 75 mg fixed dose will be 25.9 mg/kg.

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Table 96: Study 1056 - Study groups and blood collection/assay schedule

Study groups

Group	Treatment	No. of Animals	Antibody Dose	Ciprofloxacin Dose	Treatment Initiation	Therapy Duration
1	Antibody	8	8 mg/kg	N/A	ECL Positive	Single dose (IV bolus)
2	Ciprofloxacin	16	N/A	10 mg/kg	ECL Positive + 24 hr ± 12 hr	4 days (oral gavage)
3	Antibody + Ciprofloxacin	16	8 mg/kg	10 mg/kg	ECL Positive + 24 hr ± 12 hr	Single dose (IV bolus; antibody) + 4 days (ciprofloxacin; oral gavage)
4	Untreated Control	8	N/A	N/A	N/A	N/A

ECL positive = positive protective antigen result determined by electrochemiluminescence

hr = hours

IV = intravenous

N/A = not applicable

Group 3 animals were administered ciprofloxacin followed by ETI-204

Blood collection and assay schedule

Group	Blood collections (Bacteremia culture, Hematology, ECL, and PA-ELISA)
1	Day -7^; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post-median challenge time; Days 5*, 8*, 14*, 28*, PTT, 5±5 min Post-treatment^, Terminal [#]
2	Day -7^; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post-median challenge time; PTT, Days 5*, 8*, 14*, 28*, Terminal [#]
3	Day -7^; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post-median challenge time; PTT, Days 5*, 8*, 14*, 28*, 5±5 min Post-treatment^, Terminal [#]
4	Day -7^; hours 24, 30, 36, 42, 48, 54, 60, 66, 72 post-median challenge time; Days 5*, 8*, 14*, 28*, Terminal [#]

* = No Electrochemiluminescence (ECL) analysis was performed at these time points.

PTT = Prior to treatment, a blood sample collected within 30 minutes of treatment administration.

[#] = Terminal samples were subject to bacteremia culture and PA-ELISA.

[^] = Approximately 1.0 mL blood was collected into an SST tube for serum isolation. Samples were frozen and will be shipped to a Sponsor-directed facility or discarded.

Blood collected in EDTA tubes for bacterial cultures

Of the 48 monkeys randomized to the four study groups, 6 died before treatment initiation in Groups 2 and 3 (Table 97); this was perhaps due to a delay in treatment after PA positive findings. Animals in Group 1 were treated with ETI-204 within three hours of obtaining a positive PA-ECL finding. However, treatment of animals in Groups 2 and 3 was delayed; in Group 2 animals, ciprofloxacin (10 mg/kg) was initiated by gavage 24±12 hours after ECL positive findings, for 4 days. The animals in Group 3 were administered ciprofloxacin as for Group 2 animals. Additionally, ETI-204 was administered after the first dose of ciprofloxacin.

Table 97: Study 1056: Animal disposition before treatment initiation

	Group 4 Control	Group 1 [‡] ETI-204 (8 mg/kg)	Group 2 [‡] Cipro (10 mg/kg)	Group 3 [‡] Cipro (10 mg/kg) + ETI-204 (8 mg/kg)	Total
Animals challenged	8	8	16	16	48
Animals who died before treatment	0 [‡]	0	3	3	6/40 (15%)
Animals who survived to be treated*	NA [‡]	8	13 [§]	13 [§]	34/40 (85%)

*Represent modified intent to treat (mITT)

[‡]No vehicle was administered to control group animals.

[§] Group 2: ciprofloxacin (10 mg/kg) treatment initiated by gavage 24±12 hours after PA-ECL positive findings, for 4 days.

Group 3: ciprofloxacin as for Group 2 animals + ETI-204 administered after the first dose of ciprofloxacin.

Cipro=ciprofloxacin

Results:

All animals were PA negative (by both ELISA and ECL assay), culture negative as well as antibody negative (anti-PA IgG and TNA) on Day-7 i.e., prior to challenge. Age, gender, body weight, and challenge dose (mean LD₅₀ 185±67) were comparable among the animals in different groups; the LD₅₀ dose was ≥ 200 for approximately 29% of the animals (Table 98). The MMAD for each exposure day was 1.13 µm which is consistent with the particle size range that would reach the alveoli.

The mean time to trigger ranged from 30 to 34 hours among the three groups. All the animals were PA positive between 21 to 44 hours. The mean time to treatment was approximately, 36 hours for animals treated with ETI-204; however, the mean treatment time was approximately 49 hours for animals treated with ciprofloxacin alone or ciprofloxacin + ETI-204 (Table 98).

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Table 98: Study AP1056 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 4 [†] Control N = 8	Group 1 ETI-204 (8 mg/kg) N = 8	Group 2 Cipro (10 mg/kg) N = 16	Group 3 ETI-204 (8 mg/kg) + Cipro (10 mg/kg) N = 16
Baseline characteristics				
Age (years) estimated range	3.0±0.5	3.0±0	3.0±0.4	2.9±0.3
Body weight (kg)	2.9±0.4	2.9±0.4	2.9±0.3	2.9±0.4
PA (ECL and ELISA)	0/8	0/8	0/16	0/16
Anti-PA IgG	0/8	0/8	0/16	0/16
TNA	0/8	0/8	0/16	0/16
Inhaled dose				
Total inhaled dose: cfux10 ⁷ Mean ± SD (Range)	(b) (4)			
LD ₅₀ dose Mean ± SD (Range)	187±28 (146-218)	202±84 (83-360)	178±82 (112-377)	182±58 (127-315)
<200 LD ₅₀ dose n(%)	4(50)	5 (62.5)	14 (87.5)	11(68.8)
≥200 LD ₅₀ dose n(%)	4(50)	3 (37.5)	2 (12.5)	5 (31.2)
Trigger for Treatment (PA by ECL)^a and microbial burden prior to treatment				
Positive Screening PA/ECL Assay n (%)	8 (100%) [§]	8 (100%)	13 (81.3%)	13 (81.3%)
Bacteremia				
Qualitative bacteremia n (%)	8 (100) [§]	8 (100%)	13 (81.3%)	13 (81.3%)
Quantitative bacteremia n (%)	8 (100) [§]	8 (100%)	13 (81.3%)	13 (81.3%)
Log ₁₀ Mean±SD (Range) cfu/mL	5.7±1.9 [§] (4.1-8.5)	4.5±0.7 (3.8-5.8)	4.8±1.3 (2.9-8.1)	4.3±1.4 (3.2-8.7)
Geometric mean cfu/mL x 10 ⁴	48.3 [§]	3.3	5.7	1.8
PA levels by ELISA (ng/mL)				
Log ₁₀ ± SD [‡]	2.8±0.6 [§]	1.4±0.8	2.5±0.4	2.4±0.6
Mean (Range)	1798.4±3619.5 [§] (224.5-9974.1)	78.6±122.7 (1.2-371.1)	734.1±1614 (113.4-6083)	1002.9±2721.4 (31.5-10000)
Time (hours) between challenge, trigger, and treatment				
Time to trigger/PA^{+ve} (ECL) post-challenge^a Mean±SD (Range)	34.8±4.4 [§] (24.2-39)	31.9±5 (24.7-37.5)	32.4±6.5 (21.2-44)	34.3±2 (30.8-37.9)
Time to bacteremia Mean±SD (Range)	33.2±4.8 [§] (24.2-39.0)	30.4±5.1 (24.7-37.5)	30.1±4.5 (21.2-36.8)	33.6±3.5 (26.8-40.7)
Time to treatment post challenge; Mean ± SD ^b	NA	35.8±5.0	48.7±1.6	49.3±3.3
Time from trigger to treatment Mean	NA	3.9	16.3	15
Survivors at the end of study (Day 28)				
mITT animals (Survived to be treated)	0/8	4/8 (50)**	2/13 (15.4%)	8/13 (61.5)**

[‡] PA PTT for monkey A07179 (Group 1) was below the limit of detection (<LOD) of 2.4 ng/mL and replaced with 1.2 ng/mL for the analysis; for animals A07895 (Group 3) PA >10000 and replaced with 10000 for the analysis.

[§]All animals in the control group were PA positive and bacteremic by 36 hours. All animals were bacteremic by quantitative culture at 48 hours.

[†]No vehicle was administered.

SD=Standard deviation; PTT=Prior to treatment;

Qualitative culture LOD =25 cfu/mL; quantitative culture LLOQ 250 cfu/mL; Total PA ELISA LLOQ=2.4 ng/mL. PA ECL the results had to be greater than the positive control (2 ng/mL); LOD 4 ng/mL

^aThe trigger for treatment was defined as the time from challenge to a positive PA-ECL

^bThe time to treatment was defined as the time from challenge to treatment.

**Statistically significance by exact method and Boschloo's one-sided test; this varies with the test used (for details see statistics reviews by Drs Xianbin Li and Ling Lan).

Detection of Bacteremia and PA: The variability among the results of two bacterial culture methods and the detection of PA by ELISA and ECL assays is shown in Table 99. The samples used for all these assays were collected at the same time points post-challenge. All animals (n=42) were positive for bacteremia by both the culture methods (Table 99). Of the 34 monkeys in Groups 1, 2 and 3, only 1 one animal was PA negative by ELISA. All the 8 animals in the control untreated group became PA positive by ELISA and ECL methods within 36 hours post-challenge; these animals were also bacteremic within 36 hours by qualitative method and 48 hours by quantitative method. Both qualitative and quantitative culture methods for measuring bacteremia as well as ECL assay for measuring PA were equally sensitive in this study.

Table 99: Study 1056 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Detection Method					Treatment Group				Total (n=42)
Qualitative Culture*	Qualitative Enriched Culture	Quantitative Culture†	Screening PA (ECL Assay)*	Quantitative PA (ELISA Assay)‡	Untreated Control (n=8)	8 mg/kg ETI-204 (n=8)	10 mg/kg Cipro (n=13)	+ 10 mg/kg Cipro (n=13)	
+	ND	+	+	-	NA	1	0	0	1
+	ND	+	+	+	NA	7	13	13	33

n = Number of treated animals. Control group was not treated and all control animals are not included

Results determined on a per animal basis, not for individual tests.

Animal with a positive test from any time prior to treatment is considered as positive.

ND = Not Done. NA=Not applicable

*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40 µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL)

‡LLOQ by quantitative culture 250 cfu/mL; LLOQ by free PA ELISA 2.4 ng/mL

Based on applicant's analysis, there was a significant positive correlation between log-transformed quantitative bacteremia values and log-transformed PA-ELISA values at 24 hours post-median challenge and at the prior to treatment time point (Pearson correlation).

Effect of treatment on survival: Treatment with ETI-204 (8 mg/kg) or ETI-204 + ciprofloxacin was effective in improving survival compared to the untreated group or ciprofloxacin (Figure 54 and Table 98). All the treated non-surviving animals died within 8 days of challenge. All the animals in the control group died within 5 days.

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Figure 54: Study AP1056 - Kaplan-Meier curves representing time-to-death and survival by group

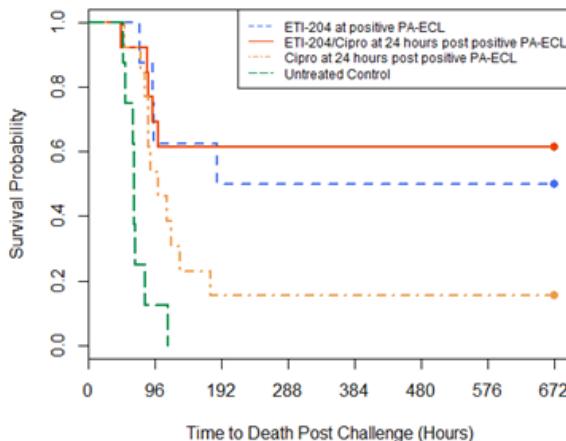
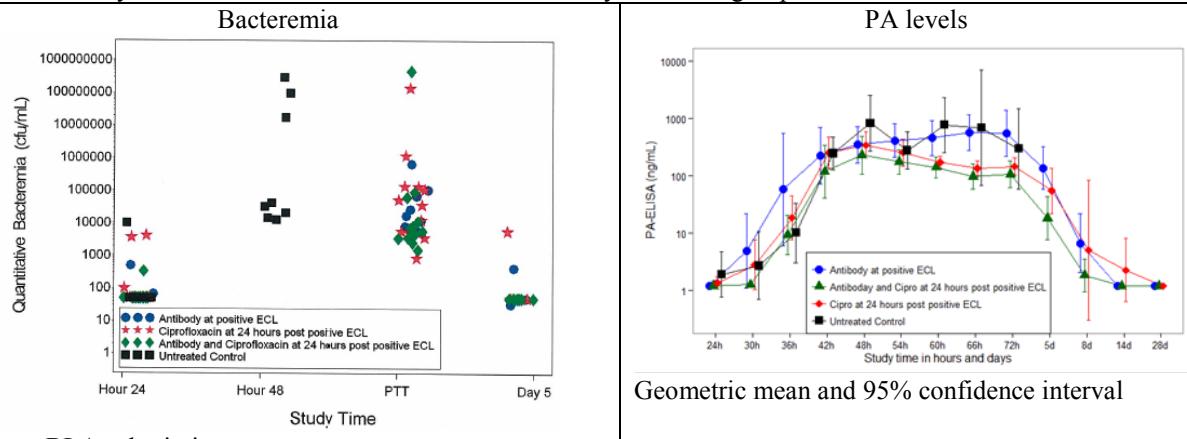


Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on bacteremia: Bacteremia levels were measured at Days 1, 2, and 5 post-challenge time points. The results showed an increase in bacteremia until Day 2 followed by a decrease at Day 5 (Figure 55). The mean time to resolution of bacteremia was shorter in animals treated with ciprofloxacin + ETI-204 (9.4 hours) or ciprofloxacin (8.2 hours; only 2 animals survived) compared to those treated with ETI-204 (36.7 hours) (Table 100). A majority [33/34 (97%)] of the terminal samples from animals that did not survive until Day 28 were bacteremic.

Figure 55: Study 1056: PA levels and bacteremia over time by treatment group



Source: BLA submission

Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

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Table 100: Study 1056-Proportion of bacteremic (qualitative and quantitative cultures) animals at each time point

Group	Study time point	Bacteremia		Group	Study time point	Bacteremia	
		Qualitative n/N	Quantitative n/N (CFU/mL range)			Qualitative n/N	Quantitative n/N (CFU/mL range)
ETI-204	Day-7	0/8	NA	Cipro	Day-7	0/16	NA
	Hour 24	1/8	2/8 (66.7 - 500)		Hour 24	3/16	3/16 (100 - 4130)
	Hour 30	5/8	NA		Hour 30	12/16	NA
	Hour 36	8/8	NA		Hour 36	16/16	NA
	PTT	8/8	8/8 (5730 - 6.7x10 ⁵)		Hour 42	13/13	NA
	Hour 42	8/8	NA		Hour 48	13/13	NA
	Hour 48	8/8	NA		PTT	13/13	13/13 (867 - 1.4x10 ⁸)
	Hour 54	7/8	NA		Hour 54	11/13	NA
	Hour 60	7/8	NA		Hour 60	5/12	NA
	Hour 66	6/8	NA		Hour 66	2/12	NA
	Hour 72	5/8	NA		Hour 72	3/12	NA
	Day 5	1/5	2/5 (33.3 - 433)		Day 5	1/4	1/4 (5.8x10 ³)
	Day 8, 14, 28	0/5	NA		Day 8, 14, 28	0/2	NA
ETI-204 + Cipro	Day-7	0/16	NA	Control	Day-7	0/8	NA
	Hour 24	1/16	1/16 (333)		Hour 24	1/8	1/8 (1x10 ⁴)
	Hour 30	7/16	NA		Hour 30	3/8	NA
	Hour 36	15/16	NA		Hour 36	8/8	NA
	Hour 42	16/16	NA		Hour 42	8/8	NA
	Hour 48	14/14	NA		Hour 48	8/8	8/8 (1.3x10 ⁴ - 3x10 ⁸)
	PTT	13/13	8/8 (1.5x10 ³ -4.6x10 ⁸)		Hour 54	6/6	NA
	Hour 54	9/12	NA		Hour 60	6/6	NA
	Hour 60	2/12	NA		Hour 66	3/3	NA
	Hour 66	2/12	NA		Hour 72	2/2	NA
	Hour 72	2/12	NA		PTT	NA	NA
	Day 5	0/8	0/8		Day 5	0/0	NA
	Day 8, 14, 28	0/8	NA		Day 8, 14, 28	0/0	NA

Cipro=ciprofloxacin

Effect of treatment on PA: All animals treated with ETI-204 following a positive ECL result (Group 1) were negative for PA by 66 hours post-median challenge. Similarly, all animals treated with ciprofloxacin and ETI-204 approximately 24 hours after a positive PA-ECL result (Group 3) were negative for PA by ECL by 60 hours post-median challenge. However, all animals that were treated with only ciprofloxacin approximately 24 hours after a positive PA-ECL result (Group 2) remained PA positive by ECL at 72 hours post-median challenge (Table 101).

PA levels were measured at different time points by ELISA until Day 28 in all surviving animals. Challenged animals exhibited peaks in circulating PA levels around Day 2 that decreased around Day 5 and were resolved in survivors following treatment (Figure 55).

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Table 101: Study 1056-Proportion of animals positive for PA-ECL at each time point

Group	Study Time Point	Ratio [#]	Proportion (95% Confidence Interval)	Group	Study Time Point	Ratio [#]	Proportion (95% Confidence Interval)
1 (Antibody @ ECL)	Day -7	0/8	0.00 (0.00, 0.37)	2 (Ciprofloxacin @ ECL + ~24 hr)	Day -7	0/16	0.00 (0.00, 0.21)
	Hour 24	1/8	0.13 (0.00, 0.53)		Hour 24	3/16	0.19 (0.04, 0.46)
	Hour 30	3/6	0.50 (0.12, 0.88)		Hour 30	6/10	0.60 (0.26, 0.88)
	Hour 36	7/8	0.88 (0.47, 1.00)		Hour 36	14/16	0.88 (0.62, 0.98)
	Hour 42	0/7	0.00 (0.00, 0.41)		Hour 42	14/14	1.00 (0.77, 1.00)
	Hour 48	0/8	0.00 (0.00, 0.37)		Hour 48	13/13	1.00 (0.75, 1.00)
	Hour 54	0/7	0.00 (0.00, 0.41)		Hour 54	13/13	1.00 (0.75, 1.00)
	Hour 60	2/8	0.25 (0.03, 0.65)		Hour 60	12/12	1.00 (0.74, 1.00)
	Hour 66	0/8	0.00 (0.00, 0.37)		Hour 66	12/12	1.00 (0.74, 1.00)
	Hour 72	0/8	0.00 (0.00, 0.37)		Hour 72	12/12	1.00 (0.74, 1.00)
	PTT	8/8	1.00 (0.63, 1.00)		PTT	13/13	1.00 (0.75, 1.00)
3 (Ciprofloxacin + Antibody @ ECL + ~24 hr)	Day -7	0/16	0.00 (0.00, 0.21)	4 (Untreated control)	Day -7	0/8	0.00 (0.00, 0.37)
	Hour 24	0/16	0.00 (0.00, 0.21)		Hour 24	1/8	0.13 (0.00, 0.53)
	Hour 30	5/10	0.50 (0.19, 0.81)		Hour 30	1/6	0.17 (0.00, 0.64)
	Hour 36	16/16	1.00 (0.79, 1.00)		Hour 36	8/8	1.00 (0.63, 1.00)
	Hour 42	16/16	1.00 (0.79, 1.00)		Hour 42	8/8	1.00 (0.63, 1.00)
	Hour 48	14/14	1.00 (0.77, 1.00)		Hour 48	8/8	1.00 (0.63, 1.00)
	Hour 54	1/12	0.08 (0.00, 0.38)		Hour 54	6/6	1.00 (0.54, 1.00)
	Hour 60	0/12	0.00 (0.00, 0.26)		Hour 60	6/6	1.00 (0.54, 1.00)
	Hour 66	0/12	0.00 (0.00, 0.26)		Hour 66	3/3	1.00 (0.29, 1.00)
	Hour 72	0/12	0.00 (0.00, 0.26)		Hour 72	2/2	1.00 (0.16, 1.00)
	PTT	13/13	1.00 (0.75, 1.00)		PTT	/	/

= Number positive for PA by ECL/Number in Group with a sample result at the indicated sample time point;
ECL = positive protective antigen result determined by electrochemiluminescence; hr = hours;
PA = protective antigen; PTT = prior to treatment.

Antibody=ETI-204

Source: BLA submission

Effect of microbial burden on survival: Based on applicant's analysis (fitted logistic regression model), the PA levels detected by ELISA or bacteremia levels measured by quantitative cultures prior to treatment time point was not a significant predictor of survival ($p = 0.1794$ and $p = 0.7810$, respectively) in this study. However, based on FDA analysis (for details see Dr Ling Lan's statistics review), an increase in PA levels at the time of treatment were associated with decreased survival time in animals treated with ETI-204 in combination with ciprofloxacin. These analyses are limited by a small sample size.

In vitro susceptibility testing: The *in vitro* susceptibility of the isolates to ciprofloxacin (MICs 0.25 to 0.5 μ g/mL) collected at the time animals died or last positive culture prior to death were similar to the challenge strain used (spore lot no. B35 - MIC 0.5 μ g/mL) for aerosolization. The ciprofloxacin MIC against another spore lot (no. B34) was stated to be 0.25 μ g/mL.

Clinical observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these observations are consistent with those reported in the natural history studies and other studies summarized above. In treated animals that survived to the end of the study, most of these abnormal observations were not observed.

The body weights of animals post-challenge or post-treatment were not specified.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included fluid within the thoracic cavity, skin and thymus; discoloration (hemorrhage) of the adrenal glands, meninges, lungs, duodenum, jejunum, and colon; and enlargement (hemorrhage) of the bronchial lymph nodes. These lesions corresponded microscopically to tissue necrosis, hemorrhage, edema, and inflammation in affected tissues.

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There were no anthrax infection-associated gross findings in animals that survived to scheduled euthanasia on Day 28 post-challenge (Table 102). These observations are consistent with those reported in the natural history studies and other studies summarized above.

Microscopic lesions such as tissue necrosis, hemorrhage, edema, and inflammation were observed in animals that died or were found moribund (Table 102). Findings within the brain/meninges tended to predominate in animals treated with ciprofloxacin and/or ETI-204 (similar incidence and severity among treatment groups). Widespread intravascular bacteria were observed more often in untreated animals. Although some of the brain findings were observed in untreated animals that succumbed to infection, they were less severe. There were no anthrax-related microscopic findings in animals that survived until the end of the study.

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Table 102: Study 1056- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	10 mg/kg Cipro n/N (*)	8 mg/kg ETI-204 + 10 mg/kg Cipro n/N (*)
Adrenal Gland				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	1/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	5/ 8 (1.00)	0/ 8	0/13	1/13 (1.00)
Hemorrhage	0/ 8	0/ 8	1/13 (3.00)	0/13
Tissue is unremarkable	0/ 8	0/ 8	1/13 (NA)	1/13 (NA)
Aorta				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bone Marrow				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Brain				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	1/ 8 (NA)	3/ 8 (NA)	8/13 (NA)	4/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	7/ 8 (1.29)	3/ 8 (3.33)	11/13 (2.82)	5/13 (2.40)
Hemorrhage	1/ 8 (3.00)	3/ 8 (2.67)	10/13 (2.40)	4/13 (2.75)
Meningeal inflammation	1/ 8 (2.00)	3/ 8 (2.67)	10/13 (2.10)	4/13 (1.75)
Vascular necrosis	0/ 8	2/ 8 (3.00)	1/13 (2.00)	1/13 (3.00)
Cavity, Thoracic				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	1/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Cecum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	1/ 8 (1.00)	0/13	1/13 (1.00)
Cervix				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	3/ 8 (1.00)	0/ 8	0/13	0/13
Colon				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	1/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	0/ 8	0/13	1/13 (1.00)
Inflammation	0/ 8	1/ 8 (3.00)	0/13	0/13
Necrosis	0/ 8	1/ 8 (3.00)	0/13	0/13
Duodenum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	1/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	5/ 8 (1.00)	0/ 8	1/13 (1.00)	1/13 (1.00)
Tissue is unremarkable	0/ 8	1/ 8 (NA)	0/13	0/13

NA, not applicable.

*Mean severity of lesion

Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 102 (continued): Study 1056- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (%)	8 mg/kg ETI-204 n/N (%)	10 mg/kg Cipro n/N (%)	8 mg/kg ETI-204 + 10 mg/kg Cipro n/N (%)
Epididymis				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	3/ 8 (1.00)	0/ 8	1/13 (1.00)	1/13 (1.00)
Esophagus				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	2/ 8 (1.00)	0/13	1/13 (1.00)
Eye				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	7/ 8 (1.00)	2/ 8 (2.00)	6/13 (1.83)	5/13 (1.20)
Meningeal hemorrhage (optic nerve)	0/ 8	1/ 8 (1.00)	2/13 (2.50)	1/13 (1.00)
Meningeal inflammation (optic nerve)	0/ 8	2/ 8 (2.00)	5/13 (1.40)	2/13 (1.50)
Femur				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	12/13
Gallbladder				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Heart				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	1/ 8 (1.00)	1/13 (1.00)	1/13 (1.00)
Mixed cell infiltrate	0/ 8	0/ 8	0/13	1/13 (2.00)
Ileum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	0/ 8	0/13	1/13 (1.00)
Jejunum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	0/ 8	0/13	1/13 (1.00)
Kidney				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	1/ 8 (1.00)	1/13 (1.00)	1/13 (1.00)
Liver				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	1/ 8 (1.00)	0/13	1/13 (1.00)
Hepatocellular necrosis	0/ 8	2/ 8 (1.50)	2/13 (1.50)	0/13
Mixed cell infiltrate	0/ 8	0/ 8	1/13 (2.00)	0/13
Sinusoidal leukocytosis	2/ 8 (1.00)	2/ 8 (2.50)	3/13 (1.33)	0/13

NA, not applicable. *Mean severity of lesion

Animals' bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 102 (continued): Study 1056-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	8 mg/kg ETI-204 n/N (*)	10 mg/kg Cipro n/N (*)	+ 10 mg/kg Cipro n/N (*)
Lung				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	3/ 8 (NA)	0/ 8 (NA)	1/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	7/ 8 (1.43)	2/ 8 (1.00)	0/13	1/13 (2.00)
Fibrous exudation	1/ 8 (4.00)	0/ 8	1/13 (3.00)	0/13
Hemorrhage	3/ 8 (2.33)	0/ 8	1/13 (2.00)	0/13
Tissue is unremarkable	0/ 8	0/ 8	1/13 (NA)	0/13
Lymph Node, Bronchial				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	1/ 8 (NA)	0/ 8 (NA)	4/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	5/ 8 (1.60)	2/ 8 (1.50)	1/13 (1.00)	1/13 (3.00)
Edema	2/ 8 (1.50)	0/ 8	2/13 (1.00)	1/13 (2.00)
Hemorrhage	2/ 8 (1.50)	0/ 8	4/13 (2.50)	2/13 (2.50)
Inflammation	0/ 8	0/ 8	1/13 (1.00)	1/13 (2.00)
Lymphoid necrosis/depletion	3/ 8 (1.33)	0/ 8	5/13 (1.80)	3/13 (1.33)
Lymph Node, Mandibular				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	7/ 8 (1.14)	2/ 8 (1.00)	1/13 (1.00)	1/13 (1.00)
Hemorrhage	1/ 8 (4.00)	0/ 8	0/13	0/13
Lymphoid necrosis/depletion	2/ 8 (2.50)	0/ 8	1/13 (1.00)	1/13 (1.00)
Lymph Node, Mesenteric				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	7/ 8 (1.43)	0/ 8	1/13 (1.00)	1/13 (1.00)
Hemorrhage	1/ 8 (2.00)	0/ 8	0/13	0/13
Lymphoid necrosis/depletion	3/ 8 (1.33)	0/ 8	0/13	1/13 (2.00)
Lymph Node, Other				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	1/ 8 (3.00)	0/ 8	0/13	0/13
Hemorrhage	1/ 8 (3.00)	0/ 8	0/13	0/13
Lymphoid necrosis/depletion	1/ 8 (2.00)	0/ 8	0/13	0/13
Mammary Gland				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	12/13
Ovary				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	1/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	3/ 8 (1.00)	1/ 8 (1.00)	0/13	0/13
Cyst	0/ 8	0/ 8	1/13 (NA)	0/13
Pancreas				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Gross Lesions	0/ 8 (NA)	0/ 8 (NA)	0/13 (NA)	0/13 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	8/ 8	13/13	13/13
Bacteria	6/ 8 (1.00)	0/ 8	1/13 (1.00)	1/13 (1.00)

NA, not applicable.

*Mean severity of lesion

Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

Tissue bacterial assessments:

Histology: The results showed absence of bacteria in the tissues from animals that survived the period of observation. However, bacteria were observed in many of the tissues from the animals that died or were found moribund; there appear to be more tissues from animals from the control group with bacteria compared to the ciprofloxacin treated animals (Table 103).

Culture: Tissues were not processed for bacterial cultures.

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Table 103: Study 1056 - Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Untreated Control		ETI-204 8mg/kg		Cipro 10mg/kg		ETI-204 8mg/kg+Cipro 10mg/kg	
	Non Survivors (N=0)		Non Survivors (N=4)		Non Survivors (N=2)		Non Survivors (N=8) Non Survivors (N=5)	
	Survivors (N=0)	Survivors (N=8)	Survivors (N=4)	Survivors (N=4)	Survivors (N=2)	Survivors (N=11)	Survivors (N=8)	Survivors (N=5)
Presence of bacteria by microscopy [1, 3, 4]								
Adrenal Gland	0/0	5/8	0/4	0/4	0/2	0/11	0/8	1/5
Aorta	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/5
Bone Marrow	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/5
Brain	0/0	7/8	0/4	3/4	0/2	11/11	0/8	5/5
Cavity, Thoracic	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/5
Cecum	0/0	6/8	0/4	1/4	0/2	0/11	0/8	1/5
Cervix	0/0	3/8	0/4	0/4	0/2	0/11	0/8	0/5
Colon	0/0	6/8	0/4	0/4	0/2	0/11	0/8	1/5
Duodenum	0/0	5/8	0/4	0/4	0/2	1/11	0/8	1/5
Epididymis	0/0	3/8	0/4	0/4	0/2	1/11	0/8	1/5
Esophagus	0/0	6/8	0/4	2/4	0/2	0/11	0/8	1/5
Eye	0/0	7/8	0/4	2/4	0/2	6/11	0/8	5/5
Femur	0/0	0/8	0/4	0/4	0/2	0/11	0/7	0/5
Gallbladder	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/5
Heart	0/0	6/8	0/4	1/4	0/2	1/11	0/8	1/5
Ileum	0/0	6/8	0/4	0/4	0/2	0/11	0/8	1/5
Jejunum	0/0	6/8	0/4	0/4	0/2	0/11	0/8	1/5
Kidney	0/0	6/8	0/4	1/4	0/2	1/11	0/8	1/5
Liver	0/0	6/8	0/4	1/4	0/2	0/11	0/8	1/5
Lung	0/0	7/8	0/4	2/4	0/2	0/11	0/8	1/5
Lymph Node, Bronchial	0/0	5/8	0/4	2/4	0/2	1/11	0/8	1/5
Lymph Node, Mandibular	0/0	7/8	0/4	2/4	0/2	1/11	0/8	1/5
Lymph Node, Mesenteric	0/0	7/8	0/4	0/4	0/2	1/11	0/8	1/5
Lymph Node, Other	0/0	1/8	0/4	0/4	0/2	0/11	0/8	0/5
Mammary Gland	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/4
Ovary	0/0	3/8	0/4	1/4	0/2	0/11	0/8	0/5
Pancreas	0/0	6/8	0/4	0/4	0/2	1/11	0/8	1/5
Parathyroid Gland	0/0	2/6	0/3	0/3	0/2	0/8	0/7	0/2
Pituitary Gland	0/0	6/8	0/3	3/4	0/2	4/11	0/8	1/5
Prostate	0/0	3/8	0/4	0/4	0/2	1/10	0/8	1/5
Rectum	0/0	7/8	0/4	0/4	0/2	0/11	0/8	1/5
Salivary Gland	0/0	6/8	0/4	1/4	0/2	0/11	0/8	1/5
Sciatic Nerve	0/0	4/8	0/4	0/4	0/2	0/11	0/8	0/5
Seminal Vesicle	0/0	3/8	0/4	0/4	0/2	1/11	0/8	1/5
Skeletal Muscle	0/0	5/8	0/4	0/4	0/2	1/11	0/8	1/5
Skin	0/0	5/8	0/4	0/4	0/2	0/11	0/8	1/5
Spinal Cord	0/0	3/8	0/4	0/4	0/2	0/11	0/8	1/5
Spleen	0/0	6/8	0/4	1/4	0/2	1/11	0/8	1/5
Sternum	0/0	0/8	0/4	0/4	0/2	0/11	0/8	0/5
Stomach	0/0	7/8	0/4	0/4	0/2	0/11	0/8	1/5
Testis	0/0	3/8	0/4	0/4	0/2	1/11	0/8	1/5
Thymus	0/0	8/8	0/4	1/4	0/2	1/11	0/8	1/5
Thyroid Gland	0/0	6/8	0/4	1/4	0/2	1/11	0/8	1/5
Tongue	0/0	5/8	0/4	0/4	0/2	0/11	0/8	1/5
Trachea	0/0	5/8	0/4	2/4	0/2	1/11	0/8	1/5
Ureter	0/0	0/7	0/4	0/4	0/2	0/10	0/8	0/5
Urinary Bladder	0/0	6/8	0/4	2/4	0/2	0/11	0/8	1/5
Uterus	0/0	3/8	0/4	1/4	0/2	0/11	0/8	0/5
Vagina	0/0	3/8	0/4	1/4	0/2	0/11	0/8	0/5
Presence of bacteria by culture [1, 2]								
No culture assessment	ND	ND	ND	ND	ND	ND	ND	ND

ND=Not Done

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate

[3] Histopathology performed at (b) (4)

[4] Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: None

Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: None

Comments:

Treatment with ETI-204 at the time of PA positive finding resulted in 50% (4/8) survival, while a delay in ciprofloxacin treatment until 24 hours (± 12 hours) following a positive PA-ECL resulted in 15% (2/13) survival. Of the 13 animals treated with ETI-204 + ciprofloxacin 24 hours (± 12 hours) after a positive PA positive finding, 8 (62%) survived. The survival result of the combination therapy group was statistically significant from the survival result of the ciprofloxacin treated group suggesting an added-benefit of the combination treatment. All of the untreated animals succumbed to disease within Day 5 of challenge. Overall, the study suggests that delayed treatment with ETI-204+ciprofloxacin is more effective than ciprofloxacin. Such a combination therapy was more effective than ETI-204 administered immediately after PA positive findings.

Bacteremia levels increased until Day 2 post-challenge followed by a decrease at Day 5 i.e., within 24 hours of treatment; the mean time to resolution of bacteremia was shorter in animals treated with ciprofloxacin + ETI-204 (9.4 hours) or ciprofloxacin (8.2 hours; only 2 animals survived) compared to those treated with ETI-204 (36.7 hours). A majority of the terminal samples from animals that did not survive until Day 28 were bacteremic.

PA was not detectable in any of the animals treated with ETI-204 or ETI-204 + ciprofloxacin by 66 and 60 hours, respectively, post-median challenge. However, all animals that were treated with only ciprofloxacin approximately 24 hours after a positive PA-ECL result remained PA positive by ECL at 72 hours post-median challenge. By ELISA, however, PA levels that peaked around Day 2 post-challenge were shown to resolve in surviving animals following treatment between Days 8 and 28.

Based on applicant's analysis (fitted logistic regression model), the PA levels detected by ELISA or bacteremia levels measured by quantitative cultures prior to treatment time point was not a significant predictor of survival ($p = 0.1794$ and $p = 0.7810$, respectively) in this study. However, based on FDA analysis, an increase in PA levels at the time of treatment were associated with decreased survival time.

Bacteria were observed by microscopic examination of many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation. Culture of tissues was not performed.

Clinical and necropsy findings were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above.

6.3.2.2. Study 2469

This was a randomized, controlled, open-label, non-GLP study with ETI-204 and ciprofloxacin in 56 cynomolgus monkeys challenged with the spores (spore lot no. B36) of the Ames strain of *B. anthracis* weighing 2.5 to 5.5 kg, conducted at [REDACTED] ^{(b)(4)}⁶⁰ The trigger for treatment was PA positive finding, in serum, by the ECL assay.

⁶⁰ [REDACTED] (b)(4) Study Number 2469-G937901: Efficacy of ciprofloxacin in an aerosol challenge model of *B. anthracis* (Ames strain) with and without adjunctive therapy in cynomolgus macaques: Study 2 (February 25, 2014).

Primary Objective

The primary objective was to assess the efficacy of ETI-204 (8 mg/kg) administered alone and in combination with ciprofloxacin (10 mg/kg) when administered in a delayed fashion (24±12 hours after the first positive PA-ECL) following inhalational exposure to *B. anthracis*.

Secondary Objectives

Secondary objectives were to

- evaluate the efficacy of two doses of ciprofloxacin (10 mg/kg and 26 mg/kg) when used in a similar delayed fashion as primary objective (there was no group of 26 mg/kg ciprofloxacin in combination with ETI-204).
- evaluate untreated controls until death or euthanasia to provide data on disease progression in *B. anthracis* aerosol challenged cynomolgus monkeys.

Study design:

The study design was similar to that of the Study 1056 (summarized above) except that

- during the pre-challenge period, blood was collected from each animal and assayed by TNA and anti-PA IgG ELISA to determine the presence of protective antibodies against anthrax (Table 104). All animals were negative by TNA and approximately 20% (15/72) of animals were anti-PA IgG positive (0.145 to 2.386 µg/mL) prior to randomization; of these 12 animals were included in the study.
- animals were randomized by gender, weight, *Klebsiella* status⁶¹, and anti-PA IgG ELISA status into four groups of 16 animals each and one group of eight animals. Although, animals were randomized to 5 groups; however, information for one of the group was redacted as another anti-PA agent (not specified) was administered.
- The PA ELISA used in this study was performed at [REDACTED] (b) (4) and was different from that used in the Study 1056. Total PA was measured using a purified polyclonal anti-PA IgG (rabbit anti-PA IgG); the LLOD 4.2 ng/mL, and the upper limit of detection (ULOD) was 24,000 ng/mL (for details see Dr Lynette Berkeley's microbiology review).

⁶¹ On page 9 of the report, the applicant states that "Animals were tested for *Klebsiella* prior to shipment to [REDACTED] (b) (4). Based on testing results provided from [REDACTED] (b) (4) 11% (8/72) of the animals were positive for *Klebsiella* prior to shipment" These animals were listed as *Klebsiella* positive on medical records. The method used for identification of *Klebsiella* was not specified. Six of the *Klebsiella* positive animals were included in the study.

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Table 104: Study 2469 - Study groups and blood collection/assay schedule

Study design

Group	Treatment	ETI-204 Dose	Ciprofloxacin Dose	Treatment Initiation	Therapy Duration	No. of Animals
1	Ciprofloxacin	N/A	10 mg/kg	ECL Positive + 24±12 hours	4 days (oral)	16
2	Ciprofloxacin	N/A	26 mg/kg	ECL Positive + 24±12 hours	4 days (oral)	16
3	ETI-204+ Ciprofloxacin	8 mg/kg	10 mg/kg	ECL Positive + 24±12 hours	Single dose (ETI-204; IV) + 4 days (Cipro; oral)	16
4	Control	N/A	N/A	N/A	N/A	8

That applicant redacted information for one of the groups (Group 3). Therefore, Groups 4 and 5 in applicant's submission represented as Groups 3 and 4, respectively in this review

Applicant's group numbers -1: Cipro (10 mg/kg); 2: Cipro (26 mg/kg); 4: ETI-204 (8 mg/kg) + Cipro (10 mg/kg); 5: untreated control

Blood collection schedule

Time Point*	Quantitative Bacteremia	Qualitative Bacteremia	Hematology	CRP	PA-ELISA	ECL	Serum for future PK analysis
							EDTA ~ 1.0-1.5 mL
Day -7		X	X	X	X	X	X ^c
24 hours	Groups 1-5	X	X	X	X	X	
30 hours		X	X	X	X	X	
36 hours		X	X	X	X	X	
42 hours		X	X	X	X	X	
48 hours	Group 5	X	X	X	X	X	
54 hours		X	X	X	X	X	
60 hours		X	X	X	X	X	
66 hours		X	X	X	X	X	
72 hours		X	X	X	X	X	
Day 5	Groups 1-5	X	X	X	X		
Day 8		X	X	X	X		
Day 14/15		X	X	X	X		
Day 28		X	X	X	X		
Terminal ^b		X		X	X		
PTT ^b	Groups 1-4	X	X	X	X	X	
5 min ± 5 min post-treatment ^c							X

* = Challenge Day D animals had samples collected on Day 15 post-challenge per DR-12264.

^a = Samples were collected from terminal samples and processed to serum when possible. CRP analysis was performed on moribund animals

^b PTT = blood sample collected immediately prior to treatment (within 30 minutes)

^c = Sample only collected from Group 3 and Group 4 animals (reference DR-12344)

NA=not applicable. Cipro=ciprofloxacin

Of the 56 monkeys randomized to the four study groups, 7 died before treatment initiation in Groups 1, 2 and 3 (Table 105); this was perhaps due to a delay in treatment after PA positive findings. Treatment of animals in Groups 1, 2 and 3 was delayed; ciprofloxacin (10 mg/kg or 26 mg/kg) treatment was initiated by gavage 24±12 hours after ECL positive findings, for 4 days.

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Table 105: Study 2469 - Animal disposition before treatment initiation

	Group 4[‡] Control	Group 1[§] Cipro (10 mg/kg)	Group 2[§] Cipro (26 mg/kg)	Group 3[§] ETI-204+ Cipro	Total
Animals challenged	8	16	16	16	56
Anti-PA IgG positive pre-challenge	1	3	4	4	12 (21.4%)
TNA	0	0	0	0	0 (0%)
Klebsiella	1	1	2	2	6 (10.7%)
PA positive by ECL or ELISA	0	0	0	0	0 (0%)
Culture positive	0	0	0	0	0 (0%)
Animals who died before treatment	0 [†]	3	2	2	7/48 (15%)
Animals who survived to be treated*		13	14	14	41/48 (85%)

*Represent modified intent to treat (mITT)

[‡]No vehicle was administered to control group animals.

[§]Ciprofloxacin (10 mg/kg or 26 mg/kg) treatment initiated by gavage 24±12 hours after ECL positive findings, for 4 days.

Group 1: ciprofloxacin as for Group 2 animals + ETI-204 administered after the first dose of ciprofloxacin.
Cipro=ciprofloxacin

Results:

All animals were PA negative (by both ELISA and ECL assay) as well as culture negative on Day-7 i.e., prior to challenge. Age, gender, body weight, and challenge dose (mean LD₅₀ 223.4±46.5) were comparable among three groups; however, the LD₅₀ dose was ≥ 200 for approximately 67% of the animals (Table 106). The MMAD for each exposure day ranged from 1.19 – 1.22 µm which is consistent with the particle size range that would reach the alveoli.

The mean time to trigger ranged from 24 to 54 hours post-exposure among the three treatment groups. The time to treatment was between 45 and 73 hours post-exposure (Table 106).

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Table 106: Study 2469 - Baseline characteristics, inhaled dose of *B. anthracis*, time to treatment and survival

Parameters	Group 4 [§] Control N = 8	Group 1 Cipro (10 mg/kg) N = 16	Group 2 Cipro (26 mg/kg) N = 16	Group 3 ETI-204 (8 mg/kg) + Cipro (10 mg/kg) N = 16
Baseline characteristics				
Age (months) estimated range	52.5±7.9	49.4±5.3	48.9±4.8	47.3±2.6
Body weight (kg)	3.8±0.8	3.7±0.7	3.7±0.6	3.6±0.8
Inhaled dose				
Total inhaled dose (cfu x 10 ⁷) Mean ± SD (Range)	(b) (4)			
LD ₅₀ Mean ± SD (Range)	228.1±42.1 (187, 307)	219.4±31.8 (163-266)	227.1±65.4 (156-374)	203.2±38.7 (156-298)
<200 LD ₅₀ n(%)	1 (12.5)	4 (25.0)	7 (43.8)	9 (56.2)
≥200 LD ₅₀ n(%)	7 (87.5)	12 (75.0)	9 (56.2)	7 (43.8)
Trigger for Treatment (PA by ECL)^a and microbial burden prior to treatment				
Positive Screening PA/ECL Assay n/N (%)	8/8 (100) [§]	14/14 (100)	13/13 (100)	14/14 (100)
Bacteremia (cfu/mL)				
Qualitative bacteremia n (%)	8/8 (100) [§]	13/13 (100)	14/14 (100)	14/14 (100)
Quantitative bacteremia				
Log ₁₀ Mean±SD (cfu/mL)*	4.0±1.1 [§]	4.9±1.7	4.1±1.6	3.9±1.7
Geometric mean (cfu/mL x 10 ⁴)	1.0 [§]	8.8	1.4	0.8
PA levels by ELISA (ng/mL)				
Log ₁₀ ± SD (n)*	2.0±0.5 [§] (8)	2.6±0.9 (11) [†]	2.6±0.5 (14)	2.5±0.5 (14)
Mean± SD (Range)	151.3±110.1 [§] (12.1-365)	2611.4±7104.6 (6.7-24000)	857.8±937.3 (63.8-2680)	632.9±778.2 (99.6-2190)
Time (hours) between challenge, trigger, and treatment				
Time to trigger/PA ^{+ve} (ECL) post challenge ^a Mean±SD (Range)	37.9±7.4 (27.9-45.9)	37.4±7 (24.1-54.4)	36.6±4.7 (28.5-43.0)	35.0±4.4 (24.5-43.5)
Time to PA ^{+ve} (ELISA) post challenge ^a Mean±SD (Range)	35.8±3.6 (29.6-40.8)	35.9±8.5 (23.6-60.6)	35.1±4 (28.5-43.0)	32.5±4.6 (24.5-42.7)
Time to bacteremia Mean±SD (Range)	34.3±7.6 [§] (24.1-45.6)	34.7±11.2 (24.1-72.0)	33.6±5.8 (22.4-43.0)	29.8±5.7 (22.7-37.5)
Time to treatment post challenge; Mean ± SD ^b	NA	53.4±10.4 (45.2-72.0)	56.4±11.7 (46.1-73.0)	50.4±6.7 (46.0-73.1)
Time from trigger to treatment; Mean	NA	16	19.8	15.4
Survivors at the end of study (Day 28)				
mITT animals (Survived to be treated) n/N (%)	2/8 (25.0)	4/13 (30.8)	7/14 (50)	8/14 (57.1)

[§] No vehicle was administered. All animals in the control group were PA^{+ve} and bacteremic by 48 hours; however, there is no PTT.

*The result for animal A11973 treated with Cipro 10 mg/kg is greater than the ULOD (24000 ng/mL), replaced with 24000 ng/mL for the statistical analysis.

SD=Standard deviation; PTT=Prior to treatment;

Qualitative culture LOD =25 cfu/mL; quantitative culture LLOQ 250 cfu/mL; PA ELISA LLOQ=0.87 ng/mL. PA ECL positive control - 2 ng/mL; LOD 4 ng/mL.

^aThe trigger for treatment was defined as the time from challenge to a positive PA-ECL

^bThe time to treatment was defined as the time from challenge to treatment.

**Statistically significance by exact method and Boschloo's one-sided test; this varies with the test used (for details see statistics review by Ling Lan)

Detection of Bacteremia and PA: The variability among the results of two bacterial culture methods and the detection of PA by ELISA and ECL assays is shown in Table 107. The samples used for all these assays were collected at the same times post-challenge. As stated above, all

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animals were positive for bacteremia by the quantitative culture method and PA positive by ECL and ELISA (Table 107). One animal in the ciprofloxacin 10 mg/kg group was culture negative by the qualitative culture method. All 8 animals in the control untreated group, became PA positive by ELISA and ECL methods within 42 hours and 48 hours, respectively, post-challenge; these animals were also bacteremic within 48 hours by both qualitative method and quantitative method. Both the culture methods for measuring bacteremia and ECL and ELISA for detecting PA were equally sensitive in this study.

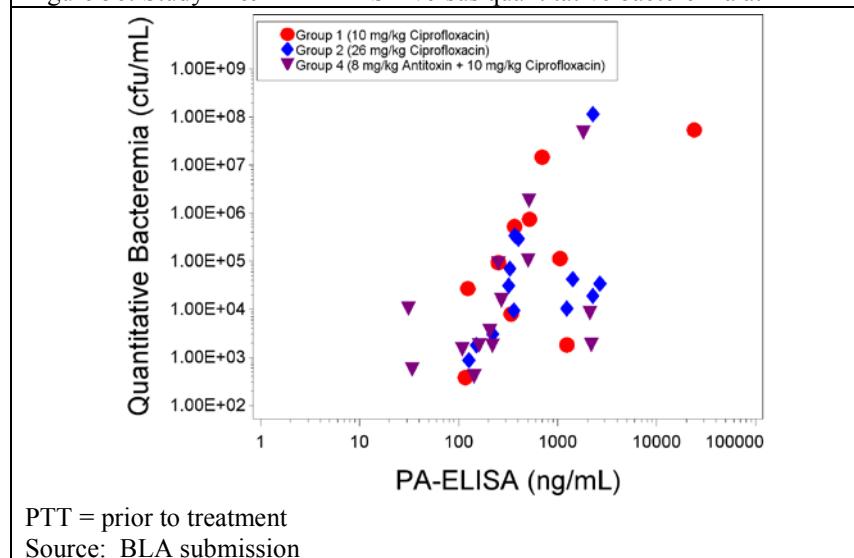
Table 107: Study 2469 - Agreement among detection methods of bacteremia and serum PA any time prior to treatment of the animals

Qualitative Culture*	Detection Method				Treatment Group				Total (n=49)	
	Qualitative Enriched Culture	Quantitative Culture†	Quantitative PA Screening PA (ELISA Assay)		Untreated Control (n=8)	10 mg/kg Cipro (n=13)	26 mg/kg Cipro (n=14)	8 mg/kg ETI-204 + 10 mg/kg Cipro (n=14)		
			PA	Screening PA (ELISA Assay)						
-	ND	+	+	+	NA	1	0	0	1	
+	ND	+	+	+	NA	12	14	14	40	

n = Number of treated animals. Control group was not treated and all control animals are not included.
Results determined on a per animal basis, not for individual tests.
Animal with a positive test from any time prior to treatment is considered as positive.
ND = Not Done. NA=Not applicable
*Qualitative methods were used on occasions when only positive/negative result was desired and are not quantitative. Approximately 40 µL of whole blood was directly plated on solid medium for qualitative culture assessment and LOD by qualitative culture can be estimated as 25 cfu/mL; PA ECL is a qualitative assay, for a sample to be reported as positive, the results had to be greater than the positive control (2 ng/mL)
†LLOQ by quantitative culture 250 cfu/mL; LLOQ by free PA ELISA 4.2 ng/mL

The animals with lower quantitative bacteremia counts exhibited lower PA levels and animals with higher quantitative culture counts exhibited higher PA concentrations. Based on the applicant's analysis, there was a positive correlation between log-transformed quantitative bacteremia and log-transformed PA-ELISA values at 24 hours post-median challenge and at the prior to treatment time point (Figure 56).

Figure 56: Study 2469 - PA-ELISA versus quantitative bacteremia at PTT



Effect of treatment on survival:

Treatment with ETI-204 (8 mg/kg) + ciprofloxacin (10 mg/kg) or ciprofloxacin (26 mg/kg) was effective in improving survival compared to the untreated group or ciprofloxacin at a dose of 10 mg/kg (Figure 57 and Table 106); however such differences were not statistically significant. Two of the 8 untreated control animals survived the period of observation; the reason for the survival of two untreated animals is unclear. All the non-surviving animals died within 8 days of challenge.

Figure 57: Study 2469 - Kaplan-Meier curves representing time-to-death and survival by group

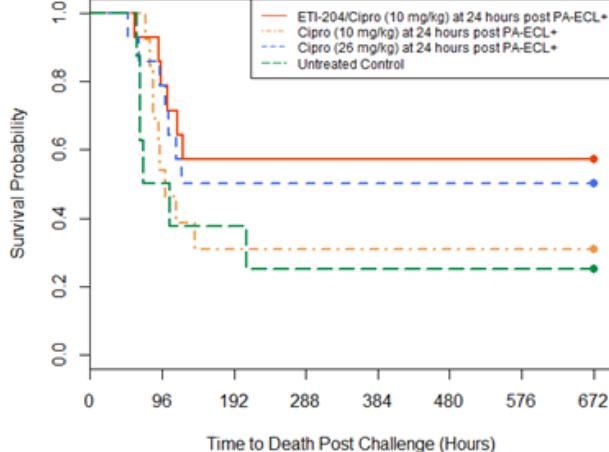


Figure constructed by Dr Ling Lan, PhD (Statistics reviewer)

Effect of treatment on bacteremia: A majority of the animals became culture negative by Day 5 post-challenge. Quantitative bacteremia was measured at Days 1, 2, and 5 post-challenge time points. The results showed an increase in bacteremia until Day 2 followed by a decrease at Day 5 (Figure 58). A majority of the animals that died or found moribund were bacteremic (Table 108).

Effect of treatment on PA: A majority of the animals treated with ETI-204 + ciprofloxacin approximately 24 hours after a positive PA-ECL result were PA negative by 60 hours post-median challenge. However, all animals that were treated with only ciprofloxacin (10 mg/kg or 26 mg/kg) approximately 24 hours after a positive PA-ECL result remained PA positive by ECL until 72 hours post-median challenge (Table 109).

An increase in PA levels, by ELISA, was observed post-challenge that peaked by 48 hours (Figure 58). The PA levels decreased following treatment in all groups and resolved in survivors following treatment, which is consistent with previous studies.

All the surviving monkeys treated with ciprofloxacin or ciprofloxacin + ETI-204 became culture negative and PA negative.

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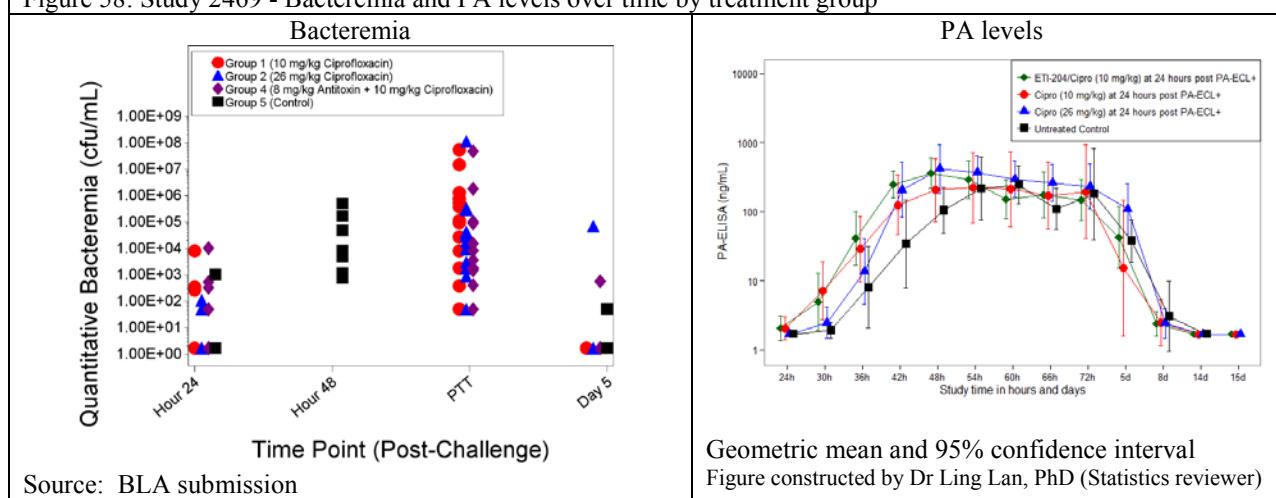
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Table 108: Study 2469-Proportion of animals bacteremic (qualitative and quantitative culture) at each time point

Group	Study time point	Bacteremia		Group	Study time point	Bacteremia	
		Qualitative n/N	Quantitative n/N (CFU/mL range)			Qualitative n/N	Quantitative n/N (CFU/mL range)
Cipro 10 mg/kg	Day-7	0/16	ND	Cipro 26 mg/kg	Day-7	0/16	ND
	Hour 24	3/16	3/16 (2.7×10^2 - 8×10^3)		Hour 24	1/16	$2/16 (3.3-1.1 \times 10^2)$
	Hour 30	7/16			Hour 30	6/16	
	Hour 36	14/16			Hour 36	14/16	
	Hour 42	15/16			Hour 42	14/14	
	Hour 48	14/15			Hour 48	12/14	
	Hour 54	11/14			Hour 54	8/13	
	Hour 60	5/14			Hour 60	7/13	
	Hour 66	5/14			Hour 66	5/12	
	Hour 72	5/14			Hour 72	5/12	
	Day 5	0/5	0/5		Day 5	1/8	0/5
	PTT	12/13	13/13 ($87-5.4 \times 10^7$)		PTT	13/14	$14/14 (3.33-1.1 \times 10^8)$
	Day 8, 14, 28	0/4			Day 8, 14, 28	1/7	
	Unscheduled terminal	9/9			Unscheduled terminal	6/7	
ETI-204 + Cipro 10 mg/kg	Day-7	0/16	ND	Control	Day-7	0/8	ND
	Hour 24	4/16	$6/14 (6.7-1 \times 10^4)$		Hour 24	1/8	$1/8 (1.0 \times 10^3)$
	Hour 30	10/16			Hour 30	4/8	
	Hour 36	16/16			Hour 36	5/8	
	Hour 42	14/15			Hour 42	7/8	
	Hour 48	14/15			Hour 48	8/8	
	Hour 54	9/15			Hour 54	8/8	
	Hour 60	5/14			Hour 60	7/8	
	Hour 66	2/14			Hour 66	5/6	
	Hour 72	1/13			Hour 72	3/5	
	Day 5	1/9	$1/9 (5.6 \times 10^3)$		Day 5	1/3	$1/3 (20)$
	PTT	13/14	$14/14 (23-4.8 \times 10^7)$		PTT	NA	NA
	Day 8, 14, 28	0/8			Day 8	1/3	
	Unscheduled terminal	5/6			Day 14, 28	0/2	
	Cipro=ciprofloxacin				Unscheduled terminal	6/6	

Figure 58: Study 2469 - Bacteremia and PA levels over time by treatment group



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Table 109: Study 2469 - Proportion of animals positive for PA-ECL at each time point

Group	Time Point	PA-ECL		Group	Time Point	PA-ECL	
		Number Abnormal /N	Proportion (Clopper-Pearson 95% Confidence Interval)			Number Abnormal /N	Proportion (Clopper-Pearson 95% Confidence Interval)
1	Hour 24	1/16	0.06 (0.00, 0.30)	2	Hour 24	0/16	0.00 (0.00, 0.21)
	Hour 30	3/16	0.19 (0.04, 0.46)		Hour 30	3/16	0.19 (0.04, 0.46)
	Hour 36	11/16	0.69 (0.41, 0.89)		Hour 36	11/16	0.69 (0.41, 0.89)
	Hour 42	14/16	0.88 (0.62, 0.98)		Hour 42	16/16	1.00 (0.79, 1.00)
	Hour 48	14/14	1.00 (0.77, 1.00)		Hour 48	14/14	1.00 (0.77, 1.00)
	Hour 54	14/14	1.00 (0.77, 1.00)		Hour 54	13/13	1.00 (0.75, 1.00)
	Hour 60	13/13	1.00 (0.75, 1.00)		Hour 60	13/13	1.00 (0.75, 1.00)
	Hour 66	13/13	1.00 (0.75, 1.00)		Hour 66	12/12	1.00 (0.74, 1.00)
	Hour 72	13/13	1.00 (0.75, 1.00)		Hour 72	12/12	1.00 (0.74, 1.00)
	PTT	13/13	1.00 (0.75, 1.00)		PTT	14/14	1.00 (0.77, 1.00)
4	Hour 24	1/16	0.06 (0.00, 0.30)	5	Hour 24	0/8	0.00 (0.00, 0.37)
	Hour 30	3/16	0.19 (0.04, 0.46)		Hour 30	3/8	0.38 (0.09, 0.76)
	Hour 36	14/16	0.88 (0.62, 0.98)		Hour 36	3/8	0.38 (0.09, 0.76)
	Hour 42	15/15	1.00 (0.78, 1.00)		Hour 42	6/8	0.75 (0.35, 0.97)
	Hour 48	14/15	0.93 (0.68, 1.00)		Hour 48	8/8	1.00 (0.63, 1.00)
	Hour 54	4/15	0.27 (0.08, 0.55)		Hour 54	8/8	1.00 (0.63, 1.00)
	Hour 60	2/14	0.14 (0.02, 0.43)		Hour 60	8/8	1.00 (0.63, 1.00)
	Hour 66	1/13	0.08 (0.00, 0.36)		Hour 66	6/6	1.00 (0.54, 1.00)
	Hour 72	3/13	0.23 (0.05, 0.54)		Hour 72	5/5	1.00 (0.48, 1.00)
	PTT	14/14	1.00 (0.77, 1.00)				

1: Ciprofloxacin (10 mg/kg); 2: Ciprofloxacin (26 mg/kg); 4: ETI-204 (8 mg/kg) + Ciprofloxacin (10 mg/kg); 5: untreated control

Source: BLA submission

Effect of microbial burden on survival: Higher bacteremia and PA levels at the time of trigger were associated with a decreased probability of survival.

Anti-PA antibodies: As stated above, all animals included in the study were tested for anti-PA IgG antibodies by ELISA and TNA. Of all the animals included in the study, 12 were anti-PA IgG antibody positive by ELISA but none of the animals were TNA positive. There does not appear to be any correlation between presence of anti-PA IgG antibodies and response to challenge or treatment (Table 110). For example, three control animals were antibody positive and only one of them survived; of the two control animals that survived, only one animal tested positive for anti-PA IgG (Animal A12335); nine anti-PA IgG positive animals that were randomized to treatment groups, three succumbed to challenge. The reason for anti-PA IgG positive findings in experimentally naïve animals is unclear. The possibility of cross-reactivity or false positive findings by the anti-PA IgG assay was not examined and cannot be ruled out (for details see microbiology review by Dr Lynette Berkeley).

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Table 110: Study 2469 - Survival status of animals positive for anti-PA IgG prior to challenge

Group	Animal ID	Survived
Untreated control	A07577	No
	A07712	No
	A12335	Yes
Ciprofloxacin 10 mg/kg	A07681	No
	A11047	Yes
	A11436	No
Ciprofloxacin 26 mg/kg	A10641	Yes
	A12134	No
	A11039	No
ETI-204 8 mg/kg + Ciprofloxacin 10 mg/kg	A11978	No
	A12170	No
	A12050	Yes

In vitro susceptibility testing: The *in vitro* susceptibility of the isolates to ciprofloxacin collected at the time animals died or last positive culture prior to death (MICs 0.25 to 0.5 µg/mL; MIC for the isolate from one animal A10650 treated with ciprofloxacin 10 mg/kg+ETI-204 was 1 µg/mL) were similar to that of the challenge strain used (spore lot no. B36; MIC between 0.125 and 0.25 µg/mL) for aerosolization. The MIC against another spore lot (no. B35) was stated to be 0.25 µg/mL. Treatment with 10 mg/kg or 26 mg/kg of ciprofloxacin for four days did not promote the growth of ciprofloxacin resistant bacteria.

Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these observations were consistent with those reported in the natural history studies summarized above. In treated animals that survived to the end of the study, most of these abnormal observations were not observed.

The body weights of animals post-challenge or post-treatment were not specified.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included fluid within the abdominal and thoracic cavities, thymus, and skeletal muscle; discoloration (hemorrhage) of the meninges, lungs, liver; and enlargement (hemorrhage) of the bronchial and mediastinal lymph nodes. There were no anthrax infection-associated gross findings in animals that survived to scheduled euthanasia on Day 28 post-challenge (Table 111). These observations are consistent with those summarized above in the natural history and treatment studies summarized above.

Microscopic lesions such as tissue necrosis, hemorrhage, edema, and inflammation were consistent with anthrax infection and those observed in the natural history studies and treatment studies summarized above. Large rod-shaped bacteria consistent with *B. anthracis* were reported in at least one organ and often widespread in animals that died (Table 111). The applicant states that in untreated animals, bacteria were found in the vasculature of most or all organs, with hemorrhage, necrosis, and/or inflammation in one or more organs. In a majority of the treated animals, bacteria were mainly restricted to the brain predominantly the meninges leading to meningeal hemorrhage and inflammation; the severity was higher in treated animals compared to the untreated group.

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There were no anthrax-related microscopic findings in NHPs that survived until the end of the 28 day post-challenge period.

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Table 111: Study 1056- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	10 mg/kg Cipro n/N (*)	26 mg/kg Cipro n/N (*)	8 mg/kg ETI-204 + 10 mg/kg Cipro n/N (*)
Adrenal Glands				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	4/ 8 (1.00)	0/13	1/14 (1.00)	1/14 (1.00)
Examined/Unremarkable	4/ 8 (NA)	13/13 (NA)	13/14 (NA)	13/14 (NA)
Aorta				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bone				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bone Marrow				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Brain				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	8/13 (NA)	4/14 (NA)	4/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria, Meninges	6/ 8 (1.33)	9/13 (3.22)	7/14 (2.57)	6/14 (3.00)
Examined/Unremarkable	2/ 8 (NA)	4/13 (NA)	7/14 (NA)	8/14 (NA)
Fibrin, Meninges	0/ 8	5/13 (1.40)	2/14 (1.50)	1/14 (1.00)
Hemorrhage, Meninges	1/ 8 (3.00)	9/13 (3.00)	6/14 (2.67)	4/14 (3.00)
Inflammation, Meninges	1/ 8 (4.00)	8/13 (2.00)	5/14 (2.60)	5/14 (3.20)
Cavity, Abdominal				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	1/ 8 (NA)	0/13 (NA)	0/14 (NA)	1/14 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Cavity, Thoracic				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	1/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2,3}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Cecum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	1/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	1/ 8 (2.00)	0/13	0/14	0/14
Edema	1/ 8 (4.00)	0/13	0/14	0/14
Fibrin	1/ 8 (2.00)	0/13	0/14	0/14
Hemorrhage	1/ 8 (3.00)	0/13	0/14	0/14
Inflammation	1/ 8 (4.00)	0/13	0/14	0/14
Necrosis	1/ 8 (3.00)	0/13	0/14	0/14
Spleen				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	1/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	13/14
Bacteria	5/ 8 (3.60)	0/13	1/14 (3.00)	1/13 (3.00)
Examined/Unremarkable	2/ 8 (NA)	5/13 (NA)	7/14 (NA)	9/13 (NA)
Fibrin	0/ 8	1/13 (3.00)	0/14	0/13
Inflammation	0/ 8	3/13 (2.00)	1/14 (2.00)	1/13 (2.00)
Lymphoid Depletion/Necrosis	6/ 8 (3.67)	8/13 (2.75)	7/14 (2.86)	4/13 (4.00)

NA, not applicable.

*Mean severity of lesion

Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

Control group were not administered with placebo and are all included

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 111 (continued): Study 1056- Incidence of gross, microscopic, and severity of lesions in monkeys bacteremic at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	10 mg/kg Cipro n/N (*)	26 mg/kg Cipro n/N (*)	8 mg/kg ETI-204 + 10 mg/kg Cipro n/N (*)
Cervix				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Colon				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Duodenum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected	5/ 8 (1.00)	0/13	1/14 (1.00)	1/14 (1.00)
Bacteria	3/ 8 (NA)	13/13 (NA)	13/14 (NA)	13/14 (NA)
Examined/Unremarkable				
Epididymis				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Esophagus				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Eyes				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Femur				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Gallbladder				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				
Heart				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	1/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected	5/ 8 (1.00)	0/13	2/14 (1.00)	1/14 (1.00)
Bacteria	3/ 8 (NA)	12/13 (NA)	12/14 (NA)	13/14 (NA)
Examined/Unremarkable				
Ileum				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1, 2, 3}	8/ 8	13/13	14/14	14/14
# Necropsied/Total Infected				

NA, not applicable. *Mean severity of lesion

Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

Control group were not administered with placebo and are all included

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Table 111 (continued): Study 1056-Incidence of gross, microscopic, and severity of lesions in bacteremic monkeys at the time of treatment

Organ/Lesion	Treatment Group			
	Untreated Control n/N (*)	10 mg/kg Cipro n/N (*)	26 mg/kg Cipro n/N (*)	8 mg/kg ETI-204 + 10 mg/kg Cipro n/N (*)
Liver				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	1/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	7/ 8	13/13	14/14	14/14
Bacteria	4/ 7 (1.75)	0/13	1/14 (2.00)	1/14 (2.00)
Examined/Unremarkable	2/ 7 (NA)	4/13 (NA)	8/14 (NA)	8/14 (NA)
Hemorrhage	0/ 7	0/13	0/14	1/14 (2.00)
Necrosis	1/ 7 (2.00)	1/13 (1.00)	1/14 (1.00)	1/14 (2.00)
Necrosis, Multifocal, Hepatocyte	0/ 7	1/13 (2.00)	0/14	0/14
Sinusoidal Leukocytosis	5/ 7 (1.60)	8/13 (1.63)	6/14 (1.00)	6/14 (1.17)
Lung				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	1/ 8 (NA)	1/13 (NA)	1/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	5/ 8 (3.60)	0/13	1/14 (2.00)	1/14 (1.00)
Edema	1/ 8 (3.00)	0/13	2/14 (2.00)	0/14
Examined/Unremarkable	3/ 8 (NA)	12/13 (NA)	11/14 (NA)	13/14 (NA)
Fibrin	1/ 8 (4.00)	0/13	0/14	0/14
Hemorrhage	1/ 8 (4.00)	0/13	1/14 (2.00)	0/14
Inflammation	1/ 8 (3.00)	0/13	2/14 (1.00)	0/14
Necrosis	1/ 8 (2.00)	0/13	0/14	0/14
Lymph Node, Bronchial				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	1/ 8 (NA)	0/13 (NA)	1/14 (NA)	1/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	6/ 8 (2.00)	0/13	2/14 (1.50)	1/14 (1.00)
Edema	2/ 8 (2.00)	0/13	0/14	1/14 (2.00)
Examined/Unremarkable	2/ 8 (NA)	11/13 (NA)	10/14 (NA)	11/14 (NA)
Fibrin	1/ 8 (1.00)	1/13 (1.00)	2/14 (1.50)	1/14 (2.00)
Hemorrhage	4/ 8 (1.25)	1/13 (2.00)	1/14 (2.00)	2/14 (2.00)
Inflammation	2/ 8 (1.00)	1/13 (1.00)	2/14 (1.00)	1/14 (1.00)
Lymphoid Depletion/Necrosis	4/ 8 (2.00)	1/13 (3.00)	3/14 (2.33)	2/14 (2.00)
Vascular Necrosis	0/ 8	0/13	2/14 (2.00)	0/14
Lymph Node, Mandibular				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	5/ 8 (2.40)	0/13	1/14 (1.00)	1/14 (1.00)
Edema	3/ 8 (2.00)	0/13	0/14	0/14
Examined/Unremarkable	3/ 8 (NA)	13/13 (NA)	13/14 (NA)	13/14 (NA)
Hemorrhage	1/ 8 (1.00)	0/13	0/14	0/14
Inflammation	1/ 8 (2.00)	0/13	0/14	0/14
Lymphoid Depletion/Necrosis	3/ 8 (2.67)	0/13	0/14	1/14 (2.00)
Lymph Node, Mediastinal				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	1/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	0/ 8	1/13 (1.00)	0/14	0/14
Edema	0/ 8	1/13 (3.00)	0/14	0/14
Hemorrhage	0/ 8	1/13 (1.00)	0/14	0/14
Lymphoid Depletion/Necrosis	0/ 8	1/13 (3.00)	0/14	0/14
Lymph Node, Mesenteric				
Macroscopic Finding ¹				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Gross Lesions	0/ 8 (NA)	0/13 (NA)	0/14 (NA)	0/14 (NA)
Microscopic Finding ^{1,2}				
# Necropsied/Total Infected	8/ 8	13/13	14/14	14/14
Bacteria	5/ 8 (1.40)	0/13	1/14 (1.00)	1/14 (1.00)
Edema	1/ 8 (2.00)	0/13	0/14	0/14
Examined/Unremarkable	3/ 8 (NA)	13/13 (NA)	13/14 (NA)	13/14 (NA)
Lymphoid Depletion/Necrosis	1/ 8 (1.00)	0/13	0/14	0/14

NA, not applicable. *Mean severity of lesion

Animals bacteremia at any time point prior to treatment (up to and including PTT) and positive by any of the bacteremia assays (qualitative, quantitative).

Control group were not administered with placebo and are all included

¹Gross necropsy and histopathology pathology performed at (b) (4)

²All microscopic findings were graded at (b) (4) according to the following scale, with the associated numerical score:

1 (minimal); 2 (mild); 3 (moderate); 4 (marked)

³Tissues examined microscopically and found unremarkable

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Tissue bacterial assessments:

Histology: The results showed absence of bacteria in the tissues from animals that survived the period of observation. However, bacteria were observed in many of the tissues from the animals that died or were found moribund; there were more tissues from animals from the control group with bacteria compared to the treated animals (Table 112).

Culture: Tissues were not processed for bacterial cultures.

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Table 112: Study 2469-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Untreated Control		Cipro 10mg/kg		Cipro 26mg/kg		ETI-204 8mg/kg+Cipro 10mg/kg		
			Non Survivors (N=2)	Survivors (N=4)	Non Survivors (N=9)	Survivors (N=7)	Non Survivors (N=7)	Survivors (N=8)	Non Survivors (N=6)
Presence of bacteria by microscopy [1, 3, 4]									
Adrenal Glands	0/2	4/6	0/4	0/9	0/7	1/7	0/8	1/6	
Aorta	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Bone	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Bone Marrow	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Brain	0/2	6/6	0/4	9/9	0/7	7/7	0/8	6/6	
Cavity, Abdominal	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Cavity, Thoracic	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Cecum	0/2	1/6	0/4	0/9	0/7	0/7	0/8	0/6	
Cervix	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Colon	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Duodenum	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Epididymis	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Esophagus	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Eyes	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Femur	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Gallbladder	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Heart	0/2	5/6	0/4	0/9	0/7	2/7	0/8	1/6	
Ileum	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Jejunum	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Kidney	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Liver	0/2	4/5	0/4	0/9	0/7	1/7	0/8	1/6	
Lung	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Lymph Node, Bronchial	0/2	6/6	0/4	0/9	0/7	2/7	0/8	1/6	
Lymph Node, Mandibular	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Lymph Node, Mediastinal	0/2	0/6	0/4	1/9	0/7	0/7	0/8	0/6	
Lymph Node, Mesenteric	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Mammary Gland	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Ovaries	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Pancreas	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Parathyroid Gland	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Pituitary Gland	0/2	6/6	0/4	7/9	0/7	6/7	0/8	4/6	
Prostate	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Rectum	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Salivary Gland	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Sciatic Nerve	0/2	4/6	0/4	0/9	0/7	1/7	0/8	1/6	
Seminal Vesicles	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Skeletal Muscle	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Skin	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Spinal Cord	0/2	4/6	0/4	3/9	0/7	4/7	0/8	2/6	
Spleen	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/5	
Sternum	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Stomach	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Testes	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Thymus	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Thyroid Glands	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Tongue	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Trachea	0/2	5/6	0/4	0/9	0/7	1/7	0/8	1/6	
Ureter	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Urinary Bladder	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Uterus	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Vagina	0/2	0/6	0/4	0/9	0/7	0/7	0/8	0/6	
Presence of bacteria by culture [1, 2]									
No culture assessment	ND	ND	ND	ND	ND	ND	ND	ND	
ND=Not Done									
[1] All treated animals irrespective of bacteremia status prior to treatment									
[2] Animal was considered positive if at least 1-5 colonies were present on plate									
[3] Histopathology performed at ^{(b) (4)}									
[4] Not all animals were assessed microscopically; numbers examined are shown									
Animals that were negative for both PA (ECL and ELISA) and bacteremia (qualitative, qualitative enriched, quantitative) at any point prior to treatment: None									
Animals that were negative for bacteremia (qualitative, qualitative enriched, quantitative) only at any point prior to treatment: None									
Animals that were negative for PA (ECL and ELISA) only at any point prior to treatment: None									

Comments:

*Though not statistically significant, the results from this study suggest that antibody treatment in combination with a low dose of ciprofloxacin may provide an added benefit in improving survival compared to ciprofloxacin treatment alone when treatment is delayed following inhalational exposure to *B. anthracis*. Seventy-five percent (6/8) of the untreated animals succumbed to disease. Higher bacteremia levels at the time of trigger were associated with a decreased probability of survival.*

A majority of the animals that survived treatment became culture negative by Day 5 post-challenge. A majority of the animals that died or found moribund were bacteremic.

A majority of the animals treated with ETI-204 + ciprofloxacin approximately 24 hours after a positive PA-ECL result were PA negative by 60 hours post-median challenge. However, all animals that were treated with only ciprofloxacin (10 mg/kg or 26 mg/kg) approximately 24 hours after a positive PA-ECL result remained PA positive by ECL until 72 hours post-median challenge. An increase in PA levels, by ELISA, was observed post-challenge that peaked by 48 hours. The PA levels decreased following treatment in all groups and resolved in survivors following treatment, which is consistent with previous studies.

All animals included in the study were tested for anti-PA IgG antibodies by ELISA and TNA. Of all the animals included in the study, 12 were anti-PA IgG antibody positive by ELISA but none of the animals were TNA positive. There does not appear to be any correlation between presence of anti-PA IgG antibodies and response to challenge or treatment. The reason for anti-PA IgG positive findings in experimentally naïve animals is unclear. The possibility of cross-reactivity or false-positive findings by the anti-PA IgG ELISA used was not examined and cannot be ruled out (for details see microbiology review by Dr Lynette Berkeley).

Bacteria were observed by microscopic examination of many of the tissues from the treated animals, regardless of treatment, that died or found moribund. However, no bacteria were observed in tissues from animals that survived the period of observation. Culture of tissues was not performed.

Clinical and necropsy findings post-challenge and post-treatment were similar to those observed in the natural history and ETI-204 monotherapy studies summarized above.

6.4. Efficacy of ETI-204 - Post-exposure prophylaxis studies

The post-exposure prophylaxis of ETI-204 administered IV or IM was measured in NZW rabbits and cynomolgus monkeys purchased from [REDACTED] (b) (4). All studies were conducted at [REDACTED] (b) (4) unless specified otherwise.

6.4.1. New Zealand White rabbits

The applicant conducted five studies (AR004, AR012, AR0315, AR035, and AR037) in NZW rabbits. All studies, except AR035 and AR037, were conducted at [REDACTED] (b) (4). Studies AR035 and AR037 were conducted at the [REDACTED] (b) (4)

6.4.1.1. Study AR004

This was a randomized, open label, placebo-controlled parallel non-GLP study to evaluate the efficacy of ETI-204 (b) (4) anti-PA MoAb) when administrated IV at increasing times (24, 36, and 48 hours) post-exposure with the Ames strain of *B. anthracis* by inhalation in 36 healthy rabbits.⁶²

Study design:

Animals purchased from (b) (4) were quarantined at (b) (4) as for the animals in the treatment studies summarized above. The animals were 13 to 17 weeks old and weighed between 2 to 2.8 kg at the time of challenge and randomized to four groups (Table 113). Animals were exposed to the spores (spore lot no. not specified) by aerosolization with a targeted 200 LD₅₀ (b) (4) spores) as for the treatment studies summarized above. The average MMAD for the three challenge days ranged from 1.08-1.14 µm.

ETI-204 (10 mg) was administered, intravenously, at 24, 36, or 48 hours post-exposure. Blood was collected at different time points (Study Days 1, 2, 7, 10, 14, 21 and 28) for the detection of bacteremia (qualitative); an approximate 10 µL aliquot sample collected was cultured on TSA plates using an inoculating loop to determine the presence or absence of *B. anthracis*.

Table 113: AR004 - Study design and schedule of blood collection

Study design:

Group #	# per Group	Treatment	Post-Challenge Treatment Time	Study Day / Event ^{1,2}								
				-5	0	1	2	7	10	14	21	28
1	10	10 mg (b) (4) Antibody	24 Hours	B	C	B	B	B	B	B	B	B/E/T
2	10	10 mg Antibody	36 Hours	B	C	B	B	B	B	B	B	B/E/T
3	10	10 mg Antibody	48 Hours	B	C	B	B	B	B	B	B	B/E/T
4	10	PBS	48 Hours	B	C	B	B	B	B	B	B	B/E/T

W = Weigh B = Blood Collection C = Challenge E = Euthanasia T = Tissue Collection

¹ Study Day/Event is based on assigned day of challenge (Study Day 0)

² All animals found moribund/dead will have blood collected for bacteremia culture prior to euthanasia and/or disposal.

ETI-204 dose administered was 10 mg (fixed dose). The body weight of the rabbits in this study ranged from 2 to 2.8 kg at the time of randomization. Therefore, the ETI-204 10 mg dose was approximately equal to 4 mg/kg.

The blood collection and assay schedule:

Sample Type	Study Day							
	-5	1	2	7	10	14	21	28
Serum	X	X	X	X	X	X	X	X
Bacteremia		X	X	X	X	X	X	X
Lung								X
Spleen								X
Intrathoracic Lymph Nodes								X

Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, spleen, and intra-thoracic lymph nodes from each surviving animal were processed for culture as for the studies summarized above.

⁶² (b) (4) Study Number 380-G004907: AR004 - Time response therapeutic efficacy on the (b) (4) monoclonal anti-PA antibody against aerosolized anthrax when administered post-challenge in the rabbit model (January 4, 2005).

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among animals in all the groups (Table 114). The average \pm SD aerosol exposure dose for all animals on study was 183 ± 63 LD₅₀ equivalent; approximately 58% of the animals were challenged with < 200X LD₅₀. At 24 hours post-challenge, all animals were culture negative; bacteremia was not measured at 36 hours (Table 114). At 48 hours post-exposure, however, 4/7 animals in Group 3 were bacteremic (Table 114).

Table 114: Study AR004 - Baseline characteristics and post treatment survival as well as microbiological findings				
Parameters	Placebo 48 hours (Group 4) N=9 ^{†‡}	(b) (4) 10 mg (approximately equal to 4 mg/kg)		
		24 hours (Group 1) N=10	36 hours (Group 2) N=10	48 hours (Group 3) N=7 [†]
Baseline characteristics				
Age (weeks) Range	13-17	13-17	13-17	13-17
Inhaled dose				
cfu $\times 10^7$ Mean \pm SD (Range)	Not included in the data sets			
LD ₅₀ Mean \pm SD (Range)	193.1 \pm 80.4 (86-353)	177.3 \pm 62.4 (103-267)	195.0 \pm 58.7 (91-263)	159.9 \pm 50.2 (62-214)
<200 LD ₅₀ n(%)	5 (55.6)	7 (70.0)	4 (40.0)	5 (71.4)
\geq 200 LD ₅₀ n(%)	4 (44.4)	3 (30.0)	6 (60.0)	2 (28.6)
Bacteremia at the time of treatment				
Bacteremia at the time of treatment n (%)	0	0	ND	4/7
Post-treatment: Survival and bacteremia at the end of study (Day 28)				
Survivors at the end of study	0	8 (80)*	5 (50)*	3 (42.9)*
Post-treatment: number of animals bacteremic (n/N)				
24 hours	0/9	0/10	0/10	0/7
36 hours	ND	ND	ND	ND
48 hours	2/9	0/10	3/10	4/7
Day 7	NA	1/9	0/5	0/3
Day 10, 21, 28	NA	0/8	0/5	0/3
Unscheduled terminal [†]	7/9	1/2	4/5	4/4

[†]There were 40 animals randomized. However, three animals in Group 3 and one animal in Group 4 died prior to the treatment time point and were therefore excluded from the primary survival analysis.

[‡]Group 4: Two of the control animals (K49149 and K49158) in Group 4 were culture negative at all the time points tested.

Group 1: treatment initiated at 24 hours post-exposure, only one animal (K49152) that survived was bacteremic at Day 7 and at terminal time points tested; other animals were culture negative at all the time points.

Group 2: treatment initiated at 36 hours post-exposure, all 5 survivors were culture negative at all the time points tested.

Group 3: treatment initiated at 48 hours post-exposure), 3 animals (K49148, K49151, and K49153) that survived were culture negative at all the time points tested including samples collected at terminal time point.

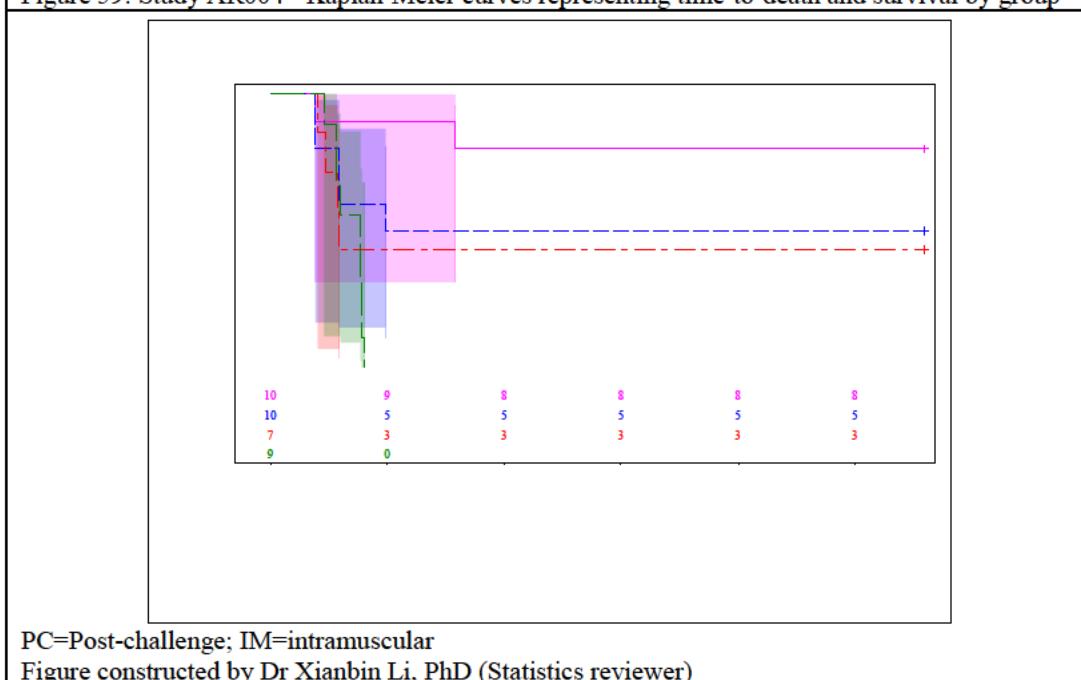
SD=Standard deviation; GM=geometric mean; ND=not done; NA=Not applicable

*Statistically significant between the ETI-204 treated group and the control group by the exact method and Boschloo's test (for details see statistics review by Dr Xianbin Li, PhD).

Effect of treatment on survival: The results show that ETI-204 at a dose of 10 mg (~4 mg/kg), IV, when administered at 24 hours post-exposure was most effective in improving survival (80%); when treatment was administered at 36 or 48 hours post-exposure the survival rate was 50% and 43%, respectively (Table 114 and Figure 59). All the control group animals died within 4 days of challenge. All the non-surviving animals died within 7 days of challenge.

Two of the control animals (K49149 and K49158) in Group 4 were culture negative at all the time points tested. In Group 1 animals (treatment initiated at 24 hours post-exposure), only one of the animal (K49152) that survived was bacteremic at Day 7 and at terminal time points tested; other animals were culture negative at all the time points. Among the Group 2 (treatment initiated at 36 hours post-exposure) animals, all 5 survivors were culture negative at all the time points tested. Of the Group 3 (treatment initiated at 48 hours post-exposure) animals, 3 (K49148, K49151, and K49153) that survived were culture negative at all the time points tested including samples collected at terminal time point. The prevalence of bacteremia is low in this study compared to other studies which could be due to smaller volume of blood processed for culture.

Figure 59: Study AR004 - Kaplan-Meier curves representing time-to-death and survival by group



Clinical Observations: No details included

Necropsy and Histopathology: No details included

Tissue bacterial assessments:

Histology: Not done

Culture: No incidence of positive culture was reported in any of the tissues tested from surviving animals; non-surviving animals were not tested (Table 115).

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Table 115: Study AR004-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 4mg/kg 24hrs PC		ETI-204 4mg/kg 36hrs PC		ETI-204 4mg/kg 48hrs PC	
	Survivors (N=0)	Non Survivors (N=9)	Survivors (N=8)	Non Survivors (N=2)	Survivors (N=5)	Non Survivors (N=5)	Survivors (N=3)	Non Survivors (N=4)
	ND	ND	ND	ND	ND	ND	ND	ND
Presence of bacteria by microscopy [1]								
No microscopic bacterial assessment								
Presence of bacteria by culture [1]								
Lymph Node	0/0	ND	0/8	ND	0/5	ND	0/3	ND
Lung	0/0	ND	0/8	ND	0/5	ND	0/3	ND
Spleen	0/0	ND	0/8	ND	0/5	ND	0/3	ND

ND=Not Done

[1] All treated animals irrespective of bacteremia status prior to treatment

Animals that were negative for bacteremia (qualitative) only at any point prior to treatment: K49131, K49132, K49133, K49134, K49135, K49137, K49138, K49140, K49141, K49143, K49145, K49148, K49149, K49150, K49151, K49152, K49153, K49154, K49156, K49157, K49158, K49159, K49161, K49162, K49163, K49164, K49165, K49168, K49169, K49170

PA (ECL and ELISA) were not performed

Comments:

The study showed that the (b) (4) product ((b) (4) anti-PA MoAb) administered at a dose of ~ 4 mg/kg IV, at 24, 36, or 48 hours post-exposure with the Ames strain of *B. anthracis* was effective in improving survival (delaying or preventing death) in NZW rabbits. The effectiveness was more when administered 24 hours post-exposure compared to the later time points; at 24 hours none of the animals were bacteremic. All the surviving animals were culture negative. All of the animals that became bacteremic at Day 2 ultimately died. In addition, there were no positive cultures reported in any of the tissues from the surviving animals on Day 28.

6.4.1.2. Study AR012

This was a randomized, open label, placebo-controlled, parallel group, dose ranging GLP study conducted at (b) (4) to determine the maximally-effective dose, optimally-effective dose, and lowest effective dose of ETI-204 (Baxter product) when given by the IV and IM routes, 24 hours post-exposure with the *B. anthracis* spores (spore lot no. B30) in 84 specific pathogen free NZW rabbits.⁶³ In addition, the onset of clinical disease was determined retrospectively by significant increases in body temperature, abnormal values in hematology and bacterial cultures. The results were evaluated to determine if abnormalities documented in the clinical and physiological parameters used to determine illness would revert back to normal following treatment with ETI-204.

Study design:

The study design was similar to that summarized above for Study AR004 except that there were 8 groups of animals and different doses (fixed dose and not by body weight) of ETI-204 were administered at 24 hours post-exposure by either IM or IV route (Table 116). Blood was cultured at 18, 24 and 27 hours after challenge and on Study Day 14. An attempt was made to obtain blood samples from moribund animals prior to euthanasia and from rabbits found dead. Blood was cultured to determine the presence or absence of bacteremia.

⁶³ (b) (4) Study Number 704-G005796: AR012 - Rabbit spore challenge ETI-204 post-exposure IV and IM dose-ranging study (April 1, 2008).

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Body temperatures were monitored using an implantable, programmable temperature transponder. Animals were observed for clinical signs at regular interval throughout the study until Day 14. Necropsy and histopathology were performed on rabbits found dead, moribund or euthanized to confirm death or illness due to anthrax infection. At a minimum, brain, liver, spleen, kidney, lung, mediastinal lymph node and gross lesions were collected and processed for histopathology.

Table 116: AR012 - Study design and schedule of blood collection

Study design:

Group	Group ID	Animals per group	Treatment*		Route
			Estimated ETI-204 Dose †	ETI-204 Buffer	
1	ACPC	9	NA	Buffer	IM
2	5-IM	9	5 mg	NA	IM
3	10-IM	9	10.0 mg	NA	IM
4	20-IM	12	20.0 mg	NA	IM
5	40-IM	12	40.0 mg	NA	IM
6	2.5-IV	9	2.5 mg	NA	IV
7	10-IV	12	10.0 mg	NA	IV
8	20-IV	12	20.0 mg	NA	IV

* Treatments were administered in a volume of approximately 1.0 mL 24 hours (\pm 30 min) after anthrax challenge

†The difference between the actual and a target doses of ETI-204 was 2±2% (Appendix M, Table 3).

‡ See Memo "Elusys Investigation on Extinction Coefficient Use"

The blood collection and assay schedule:

Scheduled Time Point	ETI-204 Serum Concentration	Bacteremia*	Hematology	Serum Harvest
Study Day -6	X		X	X
18 hr post challenge		X	X	X
24 hr post challenge		X	X	X
27 hr post challenge		X	X	X
48 hr post challenge	X		X	
Study Day 14		X	X	X

* Blood also taken for bacteremia from animals found dead or moribund euthanized animals if possible.

The body weights ranged from 2.3 to 3.0 kg at the time of randomization. The actual dose of ETI-204 administered was 2.5, 5, 10, 20 or 40 mg per rabbit, which was approximately equal to 1, 2, 4, 8, or 15 mg/kg

The animals were about 3.8 months old and weighed between 2.3 to 3.0 kg at the time of challenge and randomized to eight groups (Table 116). The average MMAD for the three challenge days ranged from 0.95-1.13 μ m.

Results:

Baseline characteristics: Age, gender, and body weights were comparable among the animals in all eight groups (Table 117). The average aerosol exposure (\pm SD) dose for all animals on study was 200(\pm 64) *B. anthracis* LD₅₀ equivalent. Approximately, 39% of the animals were exposed to \geq 200 LD₅₀. However, there was lot of variability in the LD₅₀ among the animals in different groups; the LD₅₀ was lowest in the 20 mg IM treated group of animals (Table 117).

There was an increase in the number of animals bacteremic at 18 hours compared to 27 hours (Table 117). At 24 hours post-challenge, 50% of the animals were bacteremic; the percentage of bacteremic animals varied from 22% and 78% among different groups.

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Table 117: Study AR012 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Group 1 Placebo N=9	ETI-204 [§]						
		Group 6 2.5 mg IV N=9	Group 2 5 mg IM N=9	Group 7 10 mg IV N=12	Group 3 10 mg IM N=9	Group 8 20 mg IV N=12	Group 4 20 mg IM N=12	Group 5 40 mg IM N=12
Baseline characteristics								
Age (months)	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Body weight (kg)	2.63±0.11	2.62±0.13	2.61±0.11	2.56±0.15	2.61±0.11	2.59±0.12	2.59±0.14	2.2±0.12
Inhaled dose								
cfu x 10 ⁷ Mean ± SD (Range)	(b) (4)							
LD ₅₀ Mean ± SD (Range)	205.7±47.4 (111-258)	193.2±34.3 (126-239)	187.2±32.3 (149-248)	189.8±27.3 (151-243)	230.7±87.5 (167-432)	218.5±117.2 (136-567)	180.7±46.4 (111-269)	201.9±62.6 (131-357)
<200 LD ₅₀ n (%)	3 (33.3)	4 (44.4)	5 (55.6)	9 (75.0)	5 (55.6)	8 (66.7)	10 (83.3)	7 (58.3)
≥200 LD ₅₀ n (%)	6 (66.7)	5 (55.6)	4 (44.4)	3 (25.0)	4 (44.4)	4 (33.3)	2 (16.7)	5 (41.7)
Bacteremia prior to treatment								
Bacteremia 18 hours [‡] n (%)	0	0	1	2	2	2	1	1
Bacteremia 24 hours n (%)	4 (44.4)	7 (77.8)	6 (66.7)	6 (50.0)	2 (22.2)	4 (33.3)	5 (41.7)	8 (66.7)
Post-treatment: Survival at the end of study (Day 14)								
Survivors at the end of study n (%)	0	1 (11.1)	1 (11.1)	6 (50)	3 (33.3)	7 (58.3)*	5 (41.7)	4 (33.3)
Post-treatment: Number of animals bacteremic n (%)								
27 hours	6 (66.7)	7 (77.8)	6 (66.7)	7 (58.3)	4 (44.4)	5 (41.7)	8 (66.7)	9 (75.0)
Day 14	NA	0/1	0/1	0/6	0/3	0/7	0/5	0/4
Unscheduled terminal n/N	8/8	8/8	5/6	6/6	5/6	3/4	6/7	6/8

[‡]18 hour data not included in the datasets but included in the study report.

NA=Not applicable

[§]ETI-204 dose was a fixed dose: 2.5 mg= 1.25 mg/kg; 5 mg = 2 mg/kg; 10 mg = 4 mg/kg; 20 mg = 8 mg/kg; 40 mg=15 mg/kg

*Statistically significant between the ETI-204 treated group and the control group by the exact method and Boschloo's test (for details see statistics review by Dr Xianbin Li, PhD).

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The temperature was measured between 23 and 30 hours post-exposure. However, the data were not recorded for some of the animals at a few time points. The first significant mean temperature rise occurred at the 23 hour time point in Group 6. By 24 hours three of eight groups had a significant rise in mean body temperature. Based on an average hourly temperature changes, an increase in temperature was observed in a majority of the animals in all the groups between 28 and 30 hours post-challenge (Table 118).

Table 118: Study AR012 - Summary of increases and decreases in mean body temperature of rabbits from 23 to 30 hours after *B. anthracis* challenge.

Hours after Challenge	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Percentage of Groups with Increased Temperatures
23	-0.5	0.1	0.2	0.2	0.1	0.8 ↑	0.2	0.3	13
24	0.1	0.6	0.6	0.6	0.6	1.3 ↑	0.9 ↑	0.7 ↑	38
25	0.1	1.0 ↑	0.5	0.4	0.6	1.2 ↑	1.3 ↑	0.2	38
26	0.4	1.4 ↑	0.9	0.6	1.1 ↑	2.0 ↑	1.6 ↑	0.2	50
27	0.4	2.0 ↑	1.2	1.1 ↑	1.8 ↑	2.5 ↑	1.8 ↑	1.0 ↑	75
28	1.4 ↑	2.3 ↑	1.0	1.6 ↑	2.3 ↑	3.3 ↑	2.4 ↑	1.3 ↑	88
29	2.0 ↑	2.9 ↑	1.3 ↑	2.0 ↑	3.0 ↑	4.0 ↑	2.7 ↑	1.8 ↑	100
30	2.5 ↑	3.0 ↑	1.6 ↑	2.2 ↑	2.9 ↑	3.8 ↑	2.7 ↑	2.1 ↑	100

Source: BLA submission

Effect of treatment on survival: The results showed ETI-204 at a dose of 20 mg (~8 mg/kg) administered IV, at 24 hours post-exposure was most effective in improving survival (Table 117 and Figure 60). All the animals in the control group died within 5 days of challenge. All of the non-surviving animals died within 7 days of challenge.

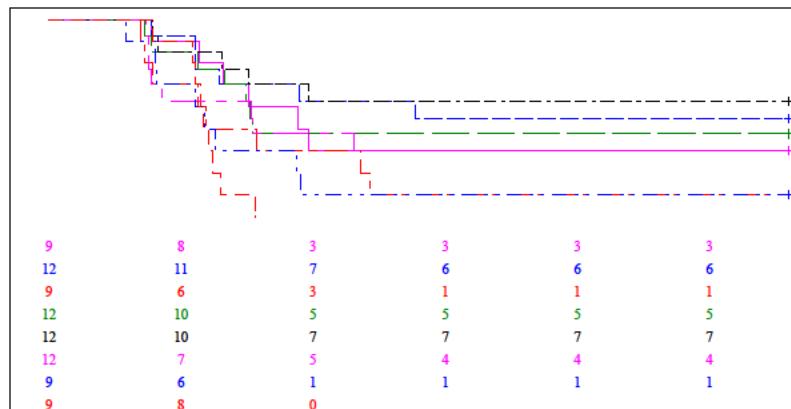
All the animals that died or found moribund were bacteremic. Of the 27 survivors, 8 of the animals were bacteremic (at least one positive culture) post-challenge and treated with ≥ 10 mg dose of ETI-204. At Day 14, all the surviving rabbits were culture negative.

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Figure 60: Study AR012 - Kaplan-Meier curves representing time-to-death and survival by group



PC=Post-challenge; IM=intramuscular; IV=intravenous

Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Hematological parameters: There was a trend towards an increase in neutrophil/lymphocyte ratio after challenge in all the animals as reported in the natural history studies. By Day 14 the cell count returned to normal in a majority of the animals. An increase in the group mean for the neutrophil/lymphocyte (N/L) ratio was reported by 27 hours in all groups.

Clinical Observations: Following challenge, the clinical observations included death, diarrhea, favoring right rear leg, hunched posture, lethargic, minimal stool, moribund, normal, inappetance, no stool, no urine, not using rear legs, ocular discharge, rapid respiration, respiratory distress, seizure, soft stool, swollen face and uncoordinated (Table 119). Lethargy and not-eating were the most common abnormal observations recorded which is consistent with anthrax and summarized above for natural history studies.

No alteration in body weights were reported during the 14 day period of observation.

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Table 119: Study AR012 - Summary of clinical observations

Clinical Observation	Number of animals in each group that demonstrated the clinical observation at least once during the observation period								
	Group 1 (n=9)	Group 2 (n=9)	Group 3 (n=9)	Group 4 (n=12)	Group 5 (n=12)	Group 6 (n=9)	Group 7 (n=12)	Group 8 (n=12)	Total Animals showing observation
Dead/euthanized ^a	9	8	6	7	8	8	6	5	57
diarrhea								1	1
favoring right rear leg							1		1
hunched posture								1	1
lethargic (all animals)	5	5	4	6	2	3	4	6	35
lethargic (died or euthanized)	5	4	3	3	2	3	4	4	28
lethargic (lived)	NA	1	1	3	0	0	0	2	7
minimal stool					1		1		2
moribund		1		1	1		1	2	6
normal	9	9	9	12	12	9	12	11	83
not eating (all animals)	6	6	8	9	5	6	11	9	60
not eating (died or euthanized)	6	5	5	5	3	5	5	4	38
not eating (lived)	NA	1	3	4	2	1	6	5	22
no stool			1	1					2
no urine			1						1
not using rear legs					1				1
ocular discharge				1					1
Rapid respiration								1	1
respiratory distress				1	1	1		2	5
seizure	1						1		2
soft stool								1	1
swollen face	1								1
uncoordinated								1	1

^a Does not include animals that were euthanized on Study Day 14

Source: BLA submission

Necropsy and Histopathology: Complete necropsies were performed on all rabbits that died or were euthanized in moribund condition. Gross lesions were typical of inhalation and included discolorations (hemorrhage) of some of the organs (appendix, brain, kidney, liver, lung, and skin), enlargement of the mediastinal lymph nodes and spleen, edema of the thymus, mediastinum and ventral skin, as well as fluid (transudate) within the pericardial, thoracic and abdominal cavities.

Tissues from three blood culture negative animals treated with ETI-204 5, 20 or 40 mg, IM, were processed for histological examination. Microscopic findings reported are consistent with anthrax in all three rabbits and include presence of large rod-shaped bacteria consistent with *B. anthracis* in at least one organ (Table 120). Necrosis, hemorrhage and lymphoid depletion of some of the organs e.g., brain, lymph nodes, and spleen was observed. There was a trend towards fewer gross lesions in ETI-204 treated animals, aside from foci in the brain and appendix, which were common in rabbits dying from anthrax in this study.

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Table 120: AR012 – Gross necropsy findings by treatment group in animals found dead or euthanized in moribund condition and microscopic finding in selected rabbits with severity

A: Gross necropsy findings

Group	1	2	3	4	5	6	7	8
Number in group	9	9	9	12	12	9	12	12
Mortality (%)	100%	89%	67%	58%	67%	89%	50%	42%
Sex	M	F	M	F	M	F	M	F
ORGAN/lesion	# dead	5	4	3	5	3	3	4
No Gross Lesions					2	3	2	1
APPENDIX								
Discoloration/foci/thick/nodules	4	3	2	1	2	1	3	1
BRAIN								
Discoloration/foci	2	1	1	1	2	2	1*	4
CAVITY, ABDOMINAL								
Fluid	2	1			1			
CAVITY, PERICARDIAL								
Fluid				1	1			
CAVITY, THORACIC								
Fluid	3	2	1		1	2		
KIDNEY								
Discoloration/foci					1	1		
LIVER								
Discoloration/foci					1			
LUNG								
Discoloration/foci	1			1				
LYMPH NODE, MEDIASTINAL								
Enlarged	2	2	3		1		1	
SKIN								
Discoloration/foci						1		
SKIN								
Fluid (ventral edema)	1	1	1					
SPLEEN								
Enlarged								1
THYMUS								
Fluid (edema)	3	2			1			

Cells left blank have an incidence of 0

* Lesion found at trim

B: Microscopic finding in selected blood culture negative rabbits with severity

	Tattoo #	L04941	L04985	L04980
	Histology number	0704296	0704267	0704275
ORGAN/Lesion	Group	2	4	5
BRAIN				
Bacteria		0	3	4
Necrosis		0	2	2
Hemorrhage(s)		0	2	2
Fibrinosuppurative inflammation		0	3	3
Vasculitis		0	2	2
LYMPH NODE, MEDIASTINAL				
Bacteria		3	0	1
Necrosis		2	2	0
Fibrinosuppurative inflammation		0	2	1
Vasculitis		0	1	0
Lymphoid depletion		1	3	3
Hemorrhage(s)		2	0	0
KIDNEY				
Bacteria		2	0	0
Fibrin exudation		0	0	1
Hemorrhage(s)		1	0	0
LIVER				
Bacteria		1	0	1
Sinusoidal leukocytosis		2	0	2
SPLEEN				
Bacteria		4	0	0
Lymphoid depletion		4	3	2
Necrosis		4	0	1
LUNG				
Bacteria		2	0	0
Fibrinosuppurative inflammation		1	2	1

0 = lesion not present

L04941 administered 5 mg ETI-204 IM

L04985 administered 20 mg ETI-204 IM

L044980 administered 40 mg ETI-204 IM

Tissue bacterial assessments:

Histology: The presence of bacteria was examined in only 3 non-surviving animals treated with ETI-24 dose of approximately 2, 8, or 16 mg/kg IM. The results showed the presence of bacteria in some of the tissues (Table 121).

Cultures: Not done.

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Table 121: Study AR012-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 ~1mg/kg IV		ETI-204 ~4mg/kg IV		ETI-204 ~8mg/kg IV		ETI-204 ~2mg/kg IM		ETI-204 ~4mg/kg IM		ETI-204 ~8mg/kg IM		ETI-204 ~15mg/kg IM	
	Non		Non		Non		Non		Non		Non		Non		Non	
	Survivor (N=0)	Survivor (N=9)	Survivor (N=1)	Survivor (N=8)	Survivor (N=6)	Survivor (N=6)	Survivor (N=7)	Survivor (N=5)	Survivor (N=1)	Survivor (N=8)	Survivor (N=3)	Survivor (N=6)	Survivor (N=5)	Survivor (N=7)	Survivor (N=4)	Survivor (N=8)
Presence of bacteria by microscopy [1, 2]																
Brain	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	1/1	0/0	1/1	1/1
Kidney	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/1	0/1
Liver	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/1	1/1
Lung	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/0	0/1
Lymph Node, Mediastinal	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/0	1/1
Spleen	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/0	0/1
Presence of bacteria by culture [1]																
No culture assessment	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[1] All treated animals irrespective of bacteremia status prior to treatment
[2] Histopathology performed at ^{(b) (4)} Not all animal were assessed microscopically; numbers examined are shown

Animals that were negative for bacteremia (qualitative) only at any point prior to treatment: L04901, L04906, L04907, L04914, L04915, L04917, L04919, L04921, L04922, L04925, L04927, L04930, L04931, L04933, L04937, L04942, L04943, L04944, L04945, L04948, L04950, L04953, L04956, L04958, L04959, L04961, L04962, L04963, L04964, L04965, L04972, L04975, L04976, L04981, L04982, L04985, L04989, L04990

PA (ECL and ELISA) were not performed

Comments:

At the time of treatment (24 hours post-challenge), approximately 50% of the rabbits were bacteremic, 63% had a mean increase in the N/L ratio, and 38% had a significant increase in mean temperature over baseline. By 27 hours post-challenge, approximately 63% of the rabbits were bacteremic, 100% of the animals had a mean increase in the N/L ratio, and 75% of the animals had a significant increase in mean temperature over baseline.

ETI-204, administered IV or IM showed a dose dependent increase in survival up to a dose of 20 mg. The highest dose of 40 mg of ETI-204 administered IM was less effective. The percentage of bacteremic animals increased over time through the 27 hour time point. All surviving rabbits treated with ETI-204 irrespective of the dose were culture negative at Day 14. All the animals that died or found moribund were bacteremic.

6.4.1.3. Study AR0315

This was a randomized, open label, placebo-controlled parallel group non-GLP study to evaluate the efficacy of 4 or 16 mg/kg ETI-204 (Baxter product) when administrated IM at 18 or 24 hours post-exposure with the spores (spore lot no. B37) of the Ames strain of *B. anthracis* by inhalation in 58 healthy NZW rabbits.⁶⁴

Study design:

Animals were quarantined at ^{(b) (4)} as for the animals in the treatment studies summarized above. The animals were 6 to 7 month old and weighed between 2.9 to 3.0 kg at the time of challenge and randomized to five groups (Table 122). All animals were culture negative prior to

⁶⁴ ^{(b) (4)} Study Number 142-G924203: AR0315 - An Evaluation of the efficacy of ETI-204 when administered intramuscularly in a rabbit post-exposure spore challenge model (February 10, 2012).

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challenge. Animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The average MMAD for the two challenge days ranged from 1.17 and 1.18 µm.

ETI-204 (4 or 16 mg/kg) was administered IM at 18 or 24 hours post-exposure. Temperatures were measured at regular intervals. Blood was collected at different time points for measuring bacteremia (qualitative and quantitative), and anti-PA IgG by ELISA in order to confirm *B. anthracis* exposure in animals.

Table 122: AR0315 - Study design and schedule of blood collection

Study design:

Group	Number of Animals	ETI-204 Dose (mg/kg)	Dose Volume (mL/kg)	Time of IM dose PC
1	10	0 (Saline)	0.25	24 hrs (±30 min)
2	12	4	0.25	18 hrs (±30 min)
3	12	16	0.25	18 hrs (±30 min)
4	12	4	0.25	24 hrs (±30 min)
5	12	16	0.25	24 hrs (±30 min)

IM: intramuscular, PC: post challenge

The blood collection and assay schedule:

Time Point	Blood Tube Type/~Blood Volume	Bacteremia (Quantitative)	Bacteremia (Culture)	Serum for PA-ELISA (~0.3 mL) ^A	Serum for ETI-204 Assay (~0.5 mL)	Serum for Anti-Drug Antibodies (~0.1 mL)	Anti-PA IgG ELISA (~0.2 mL)
Day -3	EDTA ~0.5 mL SST ~ 4.0 mL		X	X	X	X	X
PTT	EDTA ~0.5mL SST ~ 3.0 mL	X		X	X		
3 hr PTx	SST ~ 2.0 mL				X		
24 hr PTx	EDTA ~0.5 mL SST ~ 3.0 mL		X	X	X		
48 hr PTx	EDTA ~0.5 mL SST ~ 3.0 mL	X		X	X		
4 Days PTx	EDTA ~0.5 mL SST ~ 3.0 mL	X	X	X	X		
7 Days PC	EDTA ~0.5 mL SST ~ 3.0 mL	X		X	X		
14 Days PC	EDTA ~0.5 mL SST ~ 3.0 mL	X		X	X		
Day 28 PC	EDTA ~0.5 mL SST ~ 4.0 mL		X	X		X	X
Terminal ^{1*}	EDTA ~0.5 mL SST ~ 4.0 mL		X			X	X

PITT Prior to Treatment

PTx Post-Treatment

PC Post-Challenge

* If collection was possible

¹ The priority for blood collection was as follows: Bacteremia, PA-ELISA, Serum for ETI-204, Serum for Anti-Drug Antibodies, and Serum for Anti-PA IgG ELISA.

^A Samples were collected for PA-ELISA analysis. However, analysis was not carried out (see Protocol Amendment 7).

All the animals were culture negative and anti-PA IgG negative at Day-3. Although measurement of PA by ELISA was part of the schedule, PA levels were not measured.

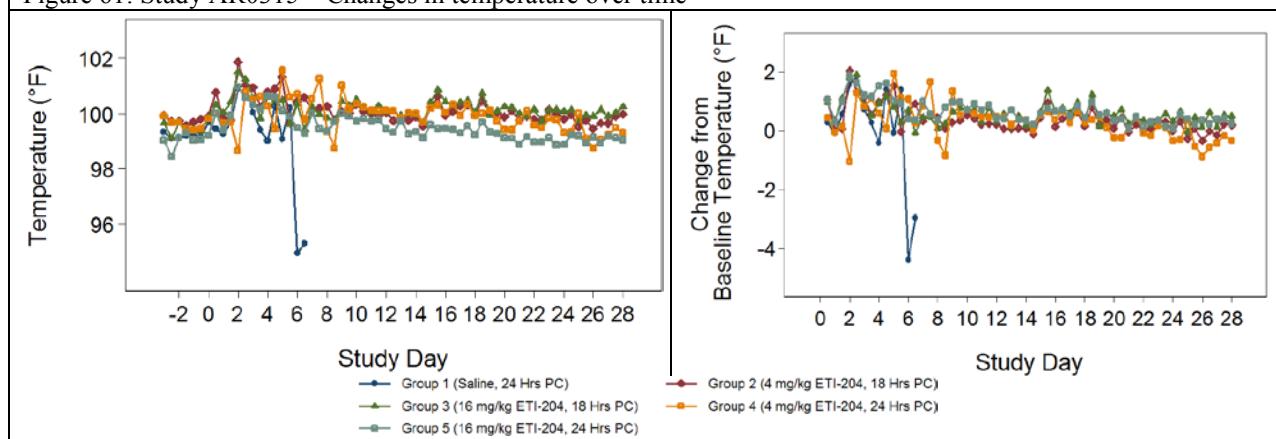
Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, spleen, and intra-thoracic lymph nodes from each surviving animal were processed for culture as for the studies summarized above.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the animals in the five groups (Table 123). The average \pm SD aerosol exposure dose for all animals in the study was 236 ± 34 LD₅₀ equivalent; approximately, 91% of the animals were exposed to ≥ 200 LD₅₀. The baseline characteristics (disease stage) of animals were similar in animals in the four groups.

The animals in all groups experienced increasing temperature post challenge; most of the significant increases in temperature compared to baseline occurred prior to Study Day 2 were similar in all the groups (Figure 61). About 64% of the animals were bacteremic at 24 hours post-challenge (Table 123). At 24 hours, the proportion of bacteremic animals was higher in groups treated at 24 hours compared to those treated at 18 hours animals.

Figure 61: Study AR0315 – Changes in temperature over time



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Table 123: Study AR0315-Baseline characteristics, inhaled dose of *B. anthracis*, and survival at the end of the study

Parameters	Groups and time of initiation of treatment	Placebo, 24 hours (Group 1) N=10		ETI-204 4 mg/kg		ETI-204 16 mg/kg	
		18 hours (Group 2) N=12	24 hours (Group 4) N=12	18 hours (Group 3) N=12	24 hours (Group 5) N=12		
Baseline characteristics							
Age (months)							
Mean± SD (Range)	6-7	6-7	6-7	6-7	6-7	6-7	6-7
Body weight (kg)	2.9±0.2	3.0±0.1	3.0±0.2	2.9±0.3	2.9±0.3		
Mean ±SD							
Inhaled dose							
Inhaled Dose cfu x 10 ⁷ Mean ± SD (Range)	(b) (4)						
LD ₅₀ Mean ± SD (Range)	245.5±16.2 (218-270)	235.3±27.6 (197-278)	221.6±15.7 (197-253)	223.5±31.0 (141-261)	255.7±53.0 (150-337)		
<200 LD ₅₀ n(%)	0	1 (8.3)	1 (8.3)	1 (8.3)	2 (16.7)		
≥200 LD ₅₀ n(%)	10 (100)	11 (91.7)	11 (91.7)	11 (91.7)	10 (83.3)		
Bacteremia prior to treatment (24 hours)							
Qualitative bacteremia n (%)	8 (80.0)	4 (33.3)	10/10 (100)	3 (25.0)	8 (66.7)		
Quantitative n (%)	5 (50.0)	5 (41.7)	11 (91.7)	5 (41.7)	11 (91.7)		
Log ₁₀ (cfu/mL) Mean±SD (0.30-2.98)	1.39±1/17 (0.30-2.98)	0.88±0.72 (0.30-1.70)	2.87±1.17 (0.30-5.01)	0.97±0.87 (0.30-2.74)	2.75±1.25 (0.30-5.01)		
Geometric mean (cfu/mL)	24.4	7.6	735.5	9.3	556.3		
Time to bacteremia (hours)							
Time to bacteremia Mean±SD (Range)	10 47.7±25.0 (23.8-72.4)	9 49.7±47.7 (17.6-160.7)	12 28.0±13.9 (23.6-72.3)	5 18.0±0.5 (17.2-18.4)	12 28.1±13.8 (23.7-72.0)		
End of Study (Day 28 or Day 56)							
Survivors at the end of study	0	11 (91.7)*	5 (41.7)	11 (91.7)*	8 (66.7)*		
Post-treatment: number of animals bacteremic qualitative [quantitative]							
48 hours	ND [10/10]	ND [5/5]	ND [10/10]	ND [1/12]	ND [7/11]		
Day 4	3/3 [3/3]	3/11 [3/11]	7/7 [7/7]	2/12 [2/12]	6/10 [6/10]		
Day 7	NA	ND [4/11]	ND [6/6]	ND [1/11]	ND [5/9]		
Day 14	NA	ND [0/11]	ND [0/5]	ND [0/11]	ND [0/9]		
Day 28	NA	0/11 [ND]	0/5 [ND]	0/11 [ND]	0/8 [ND]		
Unscheduled terminal	9/10 [ND]	1/1 [ND]	7/7 [ND]	1/1 [ND]	2/4 [ND]		
Anti-PA IgG antibodies n/N (Mean±SD)							
Day 28	NA	11/11 (338.3±470.9)	5/5 (336.7±174.5)	11/11 (120.3±90.2)	8/12 (242.8±215.5)		
Unscheduled terminal	0/4	ND	1/6 (124.8)	0/1 (<LOD)	1/2 (146.9)		

SD Standard deviation

Quantitative bacteremia values less than the limit of detection (LOD, 3 cfu/mL) were reported as "0," and values less than the limit of quantification (LOQ, 100 cfu/mL) were reported as "+"PA

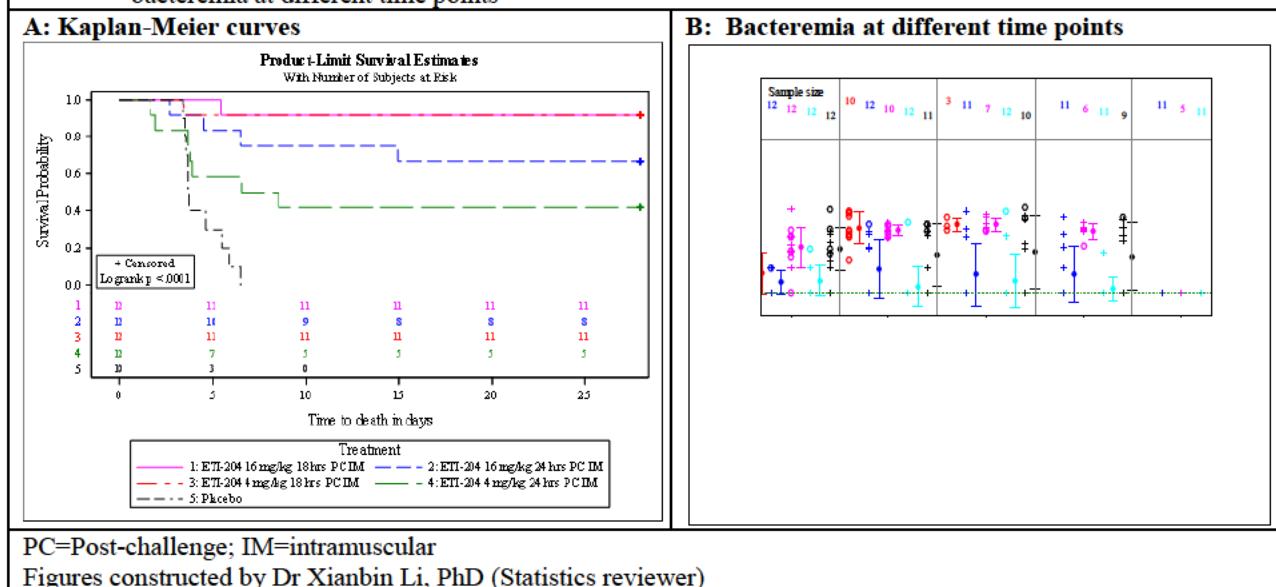
*Statistically significant between the ET-204 treated group and the control group by the exact method and Boschloo's test (for details see statistics review by Dr Xianbin Li, PhD).

Effect of treatment on survival and microbial burden: The results show that ETI-204 at a dose of either 4 or 16 mg/kg, IM, was effective in improving survival when administered at 18 hours post-exposure; the survival rate was similar (92%) in animals treated with 4 or 16 mg/kg of ETI-204 (Table 123 and Figure 62A). However, when administered at 24 hours post-exposure, the 16

mg/kg dose of ETI-204 was more effective than the 4 mg/kg dose. All the control group animals died within 7 days of challenge. All of the non-surviving animals treated with ETI-204 died within 2 weeks of challenge.

All the surviving rabbits were culture negative at Day 28 (Figure 62B); all the rabbits (except two treated with 16 mg/kg at 24 hours) found dead or moribund were bacteremic at the unscheduled terminal time point. The animals administered ETI-204 at 24 hours post-challenge had higher levels of bacteremia compared to those treated at 18 hours.

Figure 62: Study AR0315 – (A) Kaplan-Meier curves representing time-to-death and survival by group (B) bacteremia at different time points



Anti-PA IgG antibodies: All the animals were anti-PA IgG antibody negative (<LOD of 1.0 µg/mL), by ELISA, at Day-3. On Day 28, the anti-PA IgG levels were detected in all the animals that survived (Table 123). Anti-PA IgG antibodies were measured in the terminal sample from 13 of the animals that died; of the 13 animals, antibodies were detected in 2 animals treated at 24 hours post-challenge with either 4 mg/kg or 16 mg/kg of ETI-204 (Table 123).

Clinical Observations: The most common clinical observations made post-challenge include decrease in food consumption, lethargy, no stool, soft stool and respiratory abnormalities. Generally, animals surviving to the end of the study returned to normal between 5 - 8 days post-exposure.

Necropsy and Histopathology: Gross lesions reported in rabbits that died post-exposure included discolorations and/or foci in the appendix, brain, lung and large intestine; enlargement of multiple lymph nodes; and fluid in multiple body cavities (effusion) and the ventral skin (edema). Lesions typical of anthrax in this study included acute fibrinous to heterophilic inflammation, necrosis, hemorrhage, edema, and the presence of large rod-shaped bacteria in the brain (meninges), heart, kidney, liver, lung, spleen, cecum/appendix and multiple lymph nodes. Slightly more bacteria were reported in organs from control (Group 1) animals as compared to the treated animals in Groups 2, 3, 4, and 5. No permanent gross or neurological adverse effect in

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animals surviving to the end of the study as no brain lesions were found in any surviving animals, regardless of their bacteremia status during the study. The applicant states that there were no other qualitative differences in lesions among the control and treated group animals. No significant microscopic lesions were present in the rabbits that survived until Study Day 28.

Tissue bacterial assessments:

Histology: No bacteria were observed in the animals that survived. However, bacteria were observed in most of the organs from non-surviving animals (Table 124).

Culture: Spleen and bronchial lymph nodes from surviving and non-surviving animals were processed for culture. Tissues from all the surviving animals were culture negative except for lymph node from one animal, treated with 16 mg/kg dose of ETI-204, was culture positive. Both lymph nodes and spleen from all of the control group animals as well as most of non-surviving animals that were treated were culture positive (Table 124).

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Table 124: Study AR0315-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 4mg/kg 18hrs PC		ETI-204 16mg/kg 18hrs PC		ETI-204 4mg/kg 24hrs PC		ETI-204 16mg/kg 24hrs PC	
	Non Survivors (N=0)		Non Survivors (N=10)		Non Survivors (N=11)		Non Survivors (N=11)		Non Survivors (N=5)	
	Non Survivors (N=0)	Non Survivors (N=10)	Non Survivors (N=11)	Non Survivors (N=1)	Non Survivors (N=11)	Non Survivors (N=1)	Non Survivors (N=5)	Non Survivors (N=7)	Non Survivors (N=8)	Non Survivors (N=4)
Presence of bacteria by microscopy [1, 3]										
Brain [4]										
Brain: Total [5] (Tox Path)	0/0	10/10	0/11	1/1	0/11	1/1	0/5	3/7	0/8	3/4
Brain, Cerebellum: extravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	0/4
Brain, Cerebellum: intravascular (Tox Path)	0/0	3/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	1/4
Brain, Cerebral Cortex: extravascular (Tox Path)	0/0	4/10	0/11	1/1	0/11	0/1	0/5	1/7	0/8	2/4
Brain, Cerebral Cortex: intravascular (Tox Path)	0/0	8/10	0/11	0/1	0/11	0/1	0/5	2/7	0/8	3/4
Brain, Hippocampus: extravascular (Tox Path)	0/0	2/10	0/11	0/1	0/11	1/1	0/5	0/7	0/8	2/4
Brain, Hippocampus: intravascular (Tox Path)	0/0	5/10	0/11	0/1	0/11	1/1	0/5	1/7	0/8	2/4
Brain, Medulla Oblongata: extravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	1/7	0/8	0/4
Brain, Medulla Oblongata: intravascular (Tox Path)	0/0	2/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	0/4
Brain, Meninges: extravascular (Tox Path)	0/0	4/10	0/11	1/1	0/11	0/1	0/5	1/7	0/8	2/4
Brain, Meninges: intravascular (Tox Path)	0/0	9/10	0/11	1/1	0/11	0/1	0/5	2/7	0/8	3/4
Brain, Midbrain: extravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	0/4
Brain, Midbrain: intravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	0/4
Brain, Pons Region/Pontine Nuclei: extravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	2/4
Brain, Pons Region/Pontine Nuclei: intravascular (Tox Path)	0/0	3/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	1/4
Brain, Thalamus/Hypothalamus: extravascular (Tox Path)	0/0	1/10	0/11	0/1	0/11	0/1	0/5	1/7	0/8	0/4
Brain, Thalamus/Hypothalamus: intravascular (Tox Path)	0/0	6/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	1/4
Brain, Ventricular System: extravascular (Tox Path)	0/0	3/10	0/11	0/1	0/11	0/1	0/5	1/7	0/8	0/4
Brain, Ventricular System: intravascular (Tox Path)	0/0	7/10	0/11	0/1	0/11	0/1	0/5	0/7	0/8	1/4
Brain: Bacteria (Batelle)	ND	9/10	ND	1/1	ND	1/1	ND	2/7	ND	3/4
Cecum (Appendix)	ND	4/10	ND	0/1	ND	0/1	ND	2/7	ND	1/4
Heart	ND	4/10	ND	0/1	ND	0/1	ND	2/7	ND	1/4
Intestine, Small	ND	1/10	ND	0/1	ND	0/1	ND	0/7	ND	0/4
Kidney	ND	7/10	ND	0/1	ND	0/1	ND	4/7	ND	3/4
Liver	ND	6/10	ND	0/1	ND	0/1	ND	0/7	ND	1/4
Lung	ND	9/10	ND	0/1	ND	0/1	ND	3/7	ND	1/4
Lymph Node, Bronchial	ND	9/9	ND	0/1	ND	1/1	ND	6/6	ND	1/4
Lymph Node, Mediastinal	ND	10/10	ND	0/1	ND	1/1	ND	5/7	ND	1/4
Spleen	ND	9/10	ND	0/1	ND	0/1	ND	0/7	ND	2/4
Presence of bacteria by culture [1, 2]										
Bronchial Lymph Node	0/0	10/10	0/11	1/1	0/11	0/1	0/5	7/7	1/8	2/4
Spleen	0/0	10/10	0/11	1/1	0/11	0/1	0/5	7/7	0/8	2/4

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate

[3] Histopathology performed at (b) (4) Not all animal were assessed microscopically; numbers examined are shown

[4] Neuropathology performed at (b) (4)

[5] Animals that were positive in at least one area for either extra- or intra-vascular bacteria

Animals that were negative for bacteremia (qualitative and quantitative) only at any point prior to treatment: 33600, L33601, L33603, L33604, L33608, L33611, L33622, L33623, L33627, L33633, L33638, L33642, L33644, L33645, L33650, L33651, L33652, L33654, L33657, L33658, L33661

PA (ECL and ELISA) were not performed

Comments:

The study showed that ETI-204 at a dose of either 4 or 16 mg/kg was effective in improving survival when administered at 18 hours post-exposure; the survival rate was similar (92%) in animals treated with 4 or 16 mg/kg of ETI-204. However, when administered at 24 hours post-exposure, the 16 mg/kg dose of ETI-204 was more effective than the 4 mg/kg dose. All the control group animals died within 7 days of challenge. All of the non-surviving animals treated with ETI-204 died within 2 weeks of challenge. All the rabbits that survived were anti-PA IgG antibody positive at Day 28. Of the 13 animals that died, 2 animals treated at 24 hours post-challenge with either 4 mg/kg or 16 mg/kg of ETI-204 were anti-PA IgG antibody positive.

All the surviving rabbits were culture negative at Day 28; all the rabbits (except two treated with 16 mg/kg at 24 hours) found dead or moribund rabbits were bacteremic at the unscheduled terminal time point.

The animals administered ETI-204 at 24 hours post-challenge had higher levels of bacteremia compared to those treated at 18 hours.

Animals in all groups experienced an increase in body temperature and clinical observations following challenge.

ETI-204 resulted in no permanent gross or neurological adverse effect in animals surviving to the end of the study as no brain lesions were found in any surviving animals, regardless of their bacteremia status during the study.

Overall, the study suggests that ETI-204 is effective in preventing death due to anthrax when administered post-exposure in NZW rabbits; the earlier the post exposure treatment is administered the higher survival rate.

6.4.1.4. Study AR035

This was a randomized, open label, placebo-controlled GLP study to evaluate the efficacy of 16 mg/kg ETI-204 (Lonza product) when administered IM at different time points post-exposure with the Ames strain of *B. anthracis* (obtained from the University of New Mexico) by inhalation in 38 healthy NZW rabbits at the [REDACTED] (b) (4)

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Study design:

Animals purchased from [REDACTED] (b) (4) were quarantined at the [REDACTED] (b) (4) as for the studies conducted at [REDACTED] (b) (4) summarized above. The animals were 6 to 7 month old and weighed between 3.0 and 3.7 kg at the time of challenge and randomized to four groups (Table 125). All animals were culture negative and anti-PA IgG antibody negative prior to challenge (Day-7). Animals were tested for presence of *Bordetella bronchiseptica* by real-time polymerase chain reaction (RTPCR) analysis [REDACTED] (b) (4). Nineteen out of 43 rabbits were PCR-positive. Results were not used for excluding animals from the study.

Animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The spore lot no. used was not specified. The average MMAD for the two challenge days ranged from 0.88 and 3.46 µm; this is higher than those reported for the studies conducted at [REDACTED] (b) (4)

ETI-204 (16 mg/kg) was administered, IM, at 18, 24 or 30 hours post-exposure. Temperatures were measured at regular intervals. The pre-study baseline body temperature for each animal

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[REDACTED] (b) (4) Study Number FY12-033: AR035 – Pharmacokinetics of intramuscularly administered ETI-204 in inhalational anthrax challenged rabbits at various post-exposure time points (November 19, 2013).

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was the mean body temperature of the animal from Day -7 through Day -1. SIBT was defined for each animal as the body temperature higher than the pre-study baseline body temperature plus two standard deviations. Blood was collected at different time points for measuring bacteremia and anti-PA IgG antibodies.

Bacteremia (qualitative and quantitative) was measured by culture of 100 µL of different dilutions of blood collected in EDTA tubes on to TSA plates. The LLOQ was defined as 1 cfu across the three replicate plates inoculated with 100 µL, which resulted in a calculated LLOQ of 3.33 cfu/mL i.e., 0.52 cfu/mL after Log₁₀ transformation.

The anti-PA IgG antibodies were measured by ELISA; the method used captured both ETI-204 and endogenous anti-PA IgG antibodies. Therefore, the reported anti-PA IgG value for a given sample represents residual ETI-204, if any, and endogenous anti-PA IgG antibodies. The LLOQ was 50 ng/mL.

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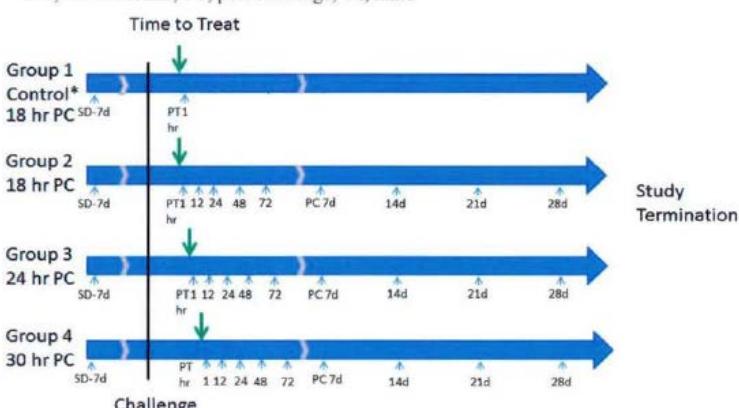
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Table 125: Study AR035 - Study design and schedule of blood collection

Study design:

Group	Animal Numbers Randomized	ETI-204 Dose (mg/kg, i.m.)	Time of Dosing Relative to Challenge*
1	10M	0 (vehicle)	18 hrs ± 15 min PC
2	10M	16	18 hrs ± 15 min PC
3	10M	16	24 hrs ± 15 min PC
4	10M	16	30 hrs ± 15 min PC

*Time of dosing was determined based on the challenge start time for each animal
i.m., intramuscular; PC, post challenge; M, male



The blood collection and assay schedule:

	SD-7	SD 0	18 hr PC	19 hr PC	24 hr PC	25 hr PC	30 hr PC	31 hr PC	36 hr PC	42 hr PC	48 hr PC	54 hr PC	66 hr PC	72 hr PC	78 hr PC	90 hr PC	96 hr PC	102 hr PC	SD 7	SD 14	SD 21	SD 28	
Temperature	X	X	Hourly (± 15 min) observations and temperature collections occurred from 18 to 72 hr post-challenge, clinical observations and body temperatures every 6 hr (± 1 hr) occurred from 78 hr to SD 7, twice daily obs and temperatures were performed from SD-7 to SD 0, and from SD 8 to SD 27, and once daily on SD 28 prior to the study termination.																				
BW	X	Every other day from SD 0 until Study Termination.																					
Challenge	X																						
ETI-204 Dose		X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}		
QT-BAC ^{*,1}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}	X ^{a,b,c}		
ETI-204 ^{2,3}	X ^{a,b,c}		X ^a	X ^b	X ^{c,d}	X ^e	X ^b	X ^{c,d}	X ^b	X ^c	X ^d	X ^b	X ^c	X ^d	X ^b	X ^c	X ^d	X ^b	X ^c	X ^{b,c,d}	X ^{b,c,d}		
ATA ²	X																					X	
Anti-PA IgG ²	X																					X	
Tissue Fixation		Tissues were collected from animals found dead, euthanized due to moribund status or at completion of study.																					
Tissue Burden		Bacterial load determination was performed on tissues collected from animals found dead, euthanized due to moribund status, or at completion of study.																					

* Quantitative bacteremia, prior to treatment as applicable. Collection occurred as described in Section 6.0, Experimental Design.

SD, Study day; PC, post challenge; ATA, anti-therapeutic antibody; PA, protective antigen

¹ Blood was collected in EDTA tube.

² Blood was collected in SST tube.

³ Blood collection for ETI-204 for Group 1 at SD -7 and 19 hr ± 15 min only (collections for bacteriology continued as specified in Section 6.0, Experimental Design)

^a Groups 1 and 2

^b Group 3

^c Group 4

^d Group 2 only

Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, brain, spleen, mediastinal lymph nodes and other tissues from animals were processed for culture as for the studies summarized above. No histopathology examination was performed.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the animals in four groups (Table 126). All animals were culture and anti-PA IgG antibody negative prior to challenge. The average ± SD aerosol exposure dose for all animals on study was 286±82 *B. anthracis* LD₅₀ equivalent; the LD₅₀ was ≥200 in approximately 90% of the animals.

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About 29% of the animals were bacteremic at the time of treatment (Table 126). Although baseline characteristics (disease stage) of animals were similar in animals in the four groups at the time of treatment, all animals treated at 18 hour post-exposure were culture negative; at 24 and 30 hours post-exposure, the proportion of bacteremic animals was 40% and 88%, respectively. An increase in temperature was observed in 2, 7, and 6 of the animals in Group 2, 3, and 4, respectively, but not in any of control animals at the time of treatment.

Table 126: Study AR035 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Placebo 18 hours (Group 1) N=10	ETI-204 16 mg/kg		
		18 hours (Group 2) N=10	24 hours (Group 3) N=10	30 hours (Group 4) N=8
Baseline characteristics				
Age (months) Range	6-7	6-7	6-7	6-7
Body weight (kg) Mean ± SD	3.3±0.2	3.3±0.2	3.3±0.2	3.3±0.2
Anti-PA IgG (Day-7)	0/10	0/10	0/10	0/8
Inhaled dose				
Inhaled Dose cfu x 10 ⁷ Mean ± SD (Range)	(b) (4)			
LD ₅₀ dose Mean ± SD (Range)	283.1±84.9 (151-427)	281.7±84.2 (151-424)	281.7±84.4 (151-423)	297.9±89.4 (150-424)
<200 LD ₅₀ dose n(%)	1 (10.0)	1 (10.0)	1 (10.0)	1 (12.5)
≥200 LD ₅₀ dose n(%)	9 (90.0)	9 (90.0)	9 (90.0)	7 (87.5)
Bacteremia prior to treatment and time to bacteremia				
Bacteremia at the time of treatment n (%)	0	0	4 (40.0)	7 (87.5)
Log ₁₀ bacteremia cfu/mL Mean±SD (Range)	<LOD	<LOD	0.93±1.19 (<LOD-4.02)	4.51±2.65 (<LOD-7.70**)
Geometric mean (cfu/mL)	<LOD	<LOD	8.5	32574.7**
Time to quantitative bacteremia (hours) n Mean±SD (Range)	10 30.0±13.3 (24-66)	7 25.7±4.5 (24-36)	7 26.6±3.2 (24-30)	8 27.0±4.5 (24-36)
SIBT at the time of treatment				
n/N (%)	0/10 (0)	2/10 (20)	7/10 (70)	6/7 (85.7)
Post-treatment: Survival and bacteremia at the end of study (Day 28)				
Survivors at the end of study	0	6 (60.0)*	6 (60.0)*	0
Number of animals bacteremic post-treatment				
6 hours PT	7/10	6/9	7/10	5/5
12 hours PT	7/9	4/10	3/8	ND
24 hours PC	5/6	1/5	0/7	2/2
48 hours PC	2/2	0/7	1/7	ND
72 hours PT	NA	1/6	1/7	ND
Day 7	NA	0/7	1/7	
Day 14, 21, 28 PC	NA	0/6	0/6	ND
Unscheduled terminal	NA	1/2	4/5	ND
Anti-PA IgG at Day 28 n/N (Range - ng/mL)				
Day 28	NA	6/6 (820-32800)	6/6 (1520-9830)	NA
Unscheduled terminal	ND	ND [‡]	ND	

**One animal's bacteremia was truncated at 3E7 because the value was >3E7.

[‡]Animal 2008 the terminal serum sample collected on SD 20 was analyzed and was below LOQ.

NA= Not applicable; SD Standard deviation;

*Statistically significant between the ETI-204 treated group and the control group.

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Effect of treatment on survival and microbial burden: ETI-204 at a dose of 16 mg/kg, when administered at 18 or 24 hours post-exposure was effective in improving survival in 60% of the animals; when administered at 30 hours post-exposure none of the animals survived (Table 126 and Figure 63). All of the control group animals and those treated at 30 hours post-exposure died within 4 days of challenge.

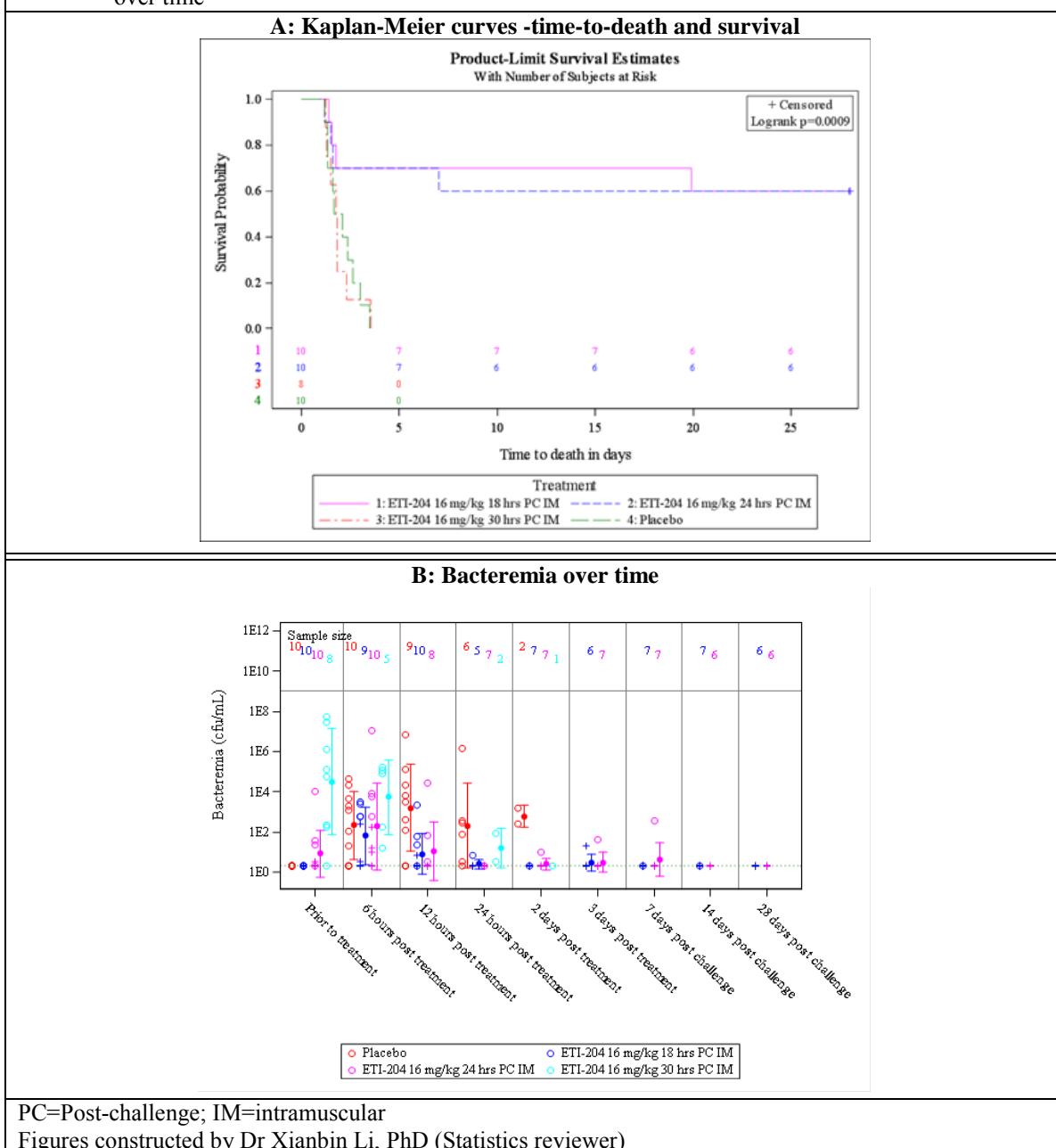
Bacteremia was reported as early as 24 hours post-exposure in some of the animals in all the 4 groups (Table 126 and Figure 63). The number of animals bacteremic and bacteremia levels at 6 hour post-treatment were similar among the four groups; at later time points (between Days 1 and 3) all animals treated with ETI-204 had lower bacteremia than the control group. By Day 7, blood from all the surviving animals was culture negative.

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Figure 63: Study AR035 – A: Kaplan-Meier curves representing time-to-death and survival. B: Bacteremia over time



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Clinical Observations: The most common clinical observations post-challenge include decrease in food consumption, lethargy, no stool, soft stool and respiratory abnormalities; animals surviving to the end of the study returned to normal between 5 - 8 days post-challenge.

Necropsy and Histopathology: Gross lesions in rabbits dying post-challenge include discolorations and/or foci in the lungs, brain and some of the other organs; these observations are consistent with other studies summarized above. Slightly more bacteria were reported in organs from control group animals compared to the treated animals. There were no other qualitative differences in lesions of anthrax among the control and experimental groups. No significant microscopic lesions were reported in the 36 rabbits terminated on Day 28.

Tissue bacterial assessments:

Histology: Not done.

Cultures: All the tissues from the animals that survived in groups 2 and 3 were culture negative. Tissues from all animals that succumbed to disease were culture positive (Table 127). The bacterial burden appears to be higher in tissues from control animals compared to those in the treated groups (Table 128).

Table 127: Study AR035-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 16mg/kg 18hrs PC		ETI-204 16mg/kg 24hrs PC		ETI-204 16mg/kg 30hrs PC			
			Survivors (N=0)	Non Survivors (N=10)	Survivors (N=6)	Non Survivors (N=4)	Survivors (N=6)	Non Survivors (N=4)	Survivors (N=0)	Non Survivors (N=8)
	Presence of bacteria by microscopy [1]	ND	ND	ND	ND	ND	ND	ND	ND	
No microscopic bacterial assessment										
Presence of bacteria by culture [1, 2, 3]										
Brain	0/0	10/10	0/6	0/3	0/6	4/4	0/0	8/8		
Heart	0/0	10/10	0/6	2/4	0/6	3/4	0/0	8/8		
Kidney	0/0	10/10	0/6	1/4	0/6	4/4	0/0	8/8		
Lung	0/0	10/10	0/6	3/4	0/6	4/4	0/0	8/8		
Mediastinal Lymph Node	0/0	10/10	0/6	1/4	0/6	4/4	0/0	7/8		
Spleen	0/0	10/10	0/6	2/4	0/6	4/4	0/0	8/8		

ND=Not Done

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Tissue bacteremia was assessed quantitatively. Animal was considered positive if bacteremia was above limit of detection (LOD= 17.8 cfu/g)

Animals that were negative for bacteremia (quantitative) only at any point prior to treatment: 1001, 1002, 1003, 1004, 1005, 1006, 1007, 1008, 1009, 1010, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 3002, 3004, 3006, 3007, 3008, 3009, 4010

PA (ECL and ELISA) were not performed

[3] Tissue burden was assessed in mesenteric lymph node for animal 2008 only at Study Director request and was below limit of detection.

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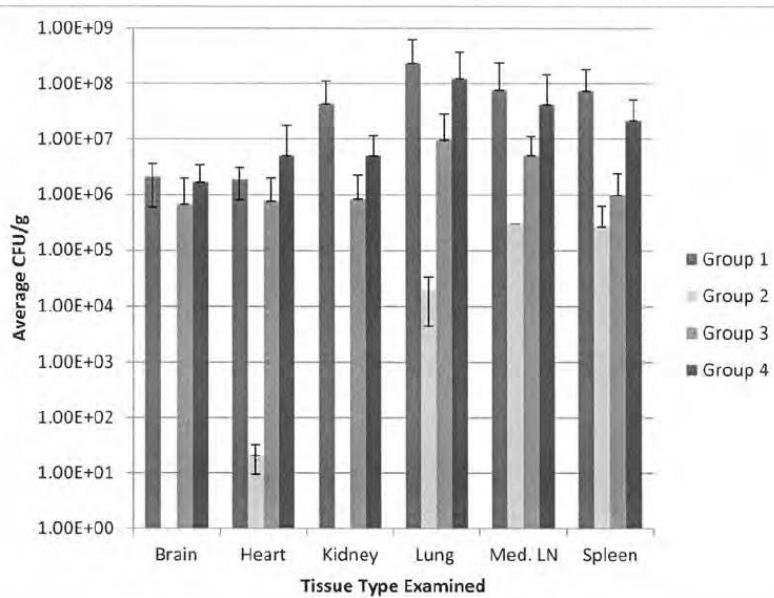
Table 128: AR035 - Bacterial burden in tissues of anthrax-infected rabbits at necropsy after ETI-204 administration at different time points

Group geometric mean of tissue bacterial burden (CFU/g Tissue)								
	N	Brain	Heart	Kidney	Lung	Med. LN	Spleen	
Succumbed	Group 1	10	1.10E+06	1.26E+06	9.56E+06	9.05E+07	9.66E+06	2.55E+07
	Group 2	3	BLD/NA*	7.21E+00	2.33E+00	1.29E+04	6.69E+01	2.71E+02
	Group 3	4	1.03E+04	3.51E+04	1.25E+05	2.19E+05	1.69E+05	4.63E+05
	Group 4	10	5.85E+05	6.57E+04	6.00E+05	4.49E+06	8.22E+05	4.81E+05
Survived	Group 2[#]	7	BLD	BLD	BLD	BLD	BLD	BLD
	Group 3	6	BLD	BLD	BLD	BLD	BLD	BLD

BLD, below limit of detection (< 17.8 CFU/g tissues), i.e., all animals in the subgroup lacked detectable bacterial burden. The LLOQ for tissue bacterial burden analysis was 17.8 CFU/g tissues.

*Two brain samples had BLD and the third brain had positive (+) result for *B. anthracis*. However, the result calculation to CFU/g analysis for the third brain was not applicable (NA) because no tissue weight was recorded for the brain portion used for bacterial analysis.

[#]The mesenteric lymph node was also collected from Animal 2008 at Study Director's request, and no bacteria were detected in the lymph node.



Data for the study animals were averaged and the SD was calculated and graphed. In order to calculate averages for data representation, 6.65 cfu/g was assigned for all BLD values.

Source: BLA submission

Comments:

ETI-204 (16 mg/kg) administered IM at 18 or 24 hours post-challenge was effective in improving survival of 60% of the animals until Day 28. None of the animals survived when ETI-204 was administered at 30 hours post-exposure. All rabbits in the control group died within 4 days of post-challenge.

The bacteremia levels were reduced by 12 hours post-treatment. All the surviving rabbits were culture negative at Day 28; all the animals that died were bacteremic. All tissues collected at Day 28, from surviving animals, were culture negative. All tissues from animals that died were culture positive.

Anti-PA IgG antibodies were reported in all animals that survived. Anti-PA IgG antibodies were not measured in the animals that died. The anti-PA IgG antibodies detected in this assay represent residual ETI-204 and endogenous anti-PA antibodies produced. However, the method for analysis of serum ETI-204 concentration used human specific agents and only detected (human-origin) ETI-204 and did not detect endogenous anti-PA IgG antibodies of rabbit origin. Since ETI-204 was not measurable, in most of the animals, at Day 28, the results are a reflection of anti-PA IgG antibody response.

6.4.1.5. Study AR037

This was a randomized, open label, placebo-controlled GLP study to evaluate the efficacy of 8, 16, and 32 mg/kg ETI-204 (Lonza product) when administrated IM at 24 hours post-exposure with the Ames strain of *B. anthracis* (obtained from the University of New Mexico) by inhalation in 38 healthy NZW rabbits at the [REDACTED]^{(b) (4)}.⁶⁶ The spore lot no. used was not specified. The primary objective of the study was to assess the effect on survival of a single IM dose of ETI-204 administered in NZW rabbits at 24 hours PC with a lethal dose of *B. anthracis* spores administered by inhalation. The secondary objective was to assess time to death of rabbits after challenge. The dose response of ETI-204 on overall mortality rate, time to death, bacteremia, tissue bacterial burden, and free circulating PA levels were evaluated.

Study design:

The study design was similar to that of Study AR035 summarized above. The animals were 6 to 7 month old and weighed between 2.9 and 4.0 kg at the time of challenge and randomized to four groups (Table 129). All animals were culture negative prior to challenge; 3 animals [#1003 (117 ng/mL) in the control group; # 2009 (65.8 ng/mL) and #2014 (223 ng/mL) treated with 8 mg/kg ETI-204] were anti-PA IgG antibody positive prior to challenge (Day-7).

Like for Study AR035, animals were tested for presence of *B. bronchiseptica* by RTPCR analysis. However, animals were also tested for *B. bronchiseptica* by microbiological culture at [REDACTED]^{(b) (4)}. Fourteen out of 64 rabbits tested were PCR-positive. Of the 64 rabbits, 45 were positive for *B. bronchiseptica* by the microbiological test; 25 had rare/few colonies, 11 had several conies, 2 had moderate growth, and 7 had heavy growth. Among the 14 PCR-positive animals, 11 were positive and the remaining three negative by culture. The results were not used for excluding animals from the study.

Animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The average MMAD for the two challenge days ranged from 0.60 to 0.93 µm.

Different doses of ETI-204 (8, 16, and 32 mg/kg) were administered IM, at 18, 24 or 30 hours post-exposure. Temperatures were measured at regular intervals. The pre-study and post-study measurements were similar to those summarized above for the Study AR035 except that serum PA levels were measured by ELISA at [REDACTED]^{(b) (4)}

⁶⁶ [REDACTED]

[REDACTED]^{(b) (4)}) Study Number FY12-097: AR037 –

Evaluating the post-exposure effect of intramuscularly administered ETI-204 in inhalational anthrax challenged rabbits (April 11, 2014).

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Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, brain, spleen, mediastinal lymph nodes and other tissues from animals were processed for culture as for the studies summarized above.

Table 129: AR037 - Study design and schedule of blood collection

Study design:

Group	Numbers of Animals Randomized	Animal ID ¹	ETI-204 Dose (mg/kg, IM)	Time of Dosing (PC ²)
1	5M/5F	M (1001-1005) F (1006-1010)	0 (vehicle)	24 hrs ± 15 min
2	8M/8F	M (2001-2008) F (2009-2016)	8	24 hrs ± 15 min
3	8M/8F	M (3001-3006, 4001, 4002) F (3009-3014, 4009, 4010)	16	24 hrs ± 15 min
4	8M/8F	M (4003-4008, 3007, 3008) F (4011-4016, 3015, 3016)	32	24 hrs ± 15 min

¹Animals 4001, 4002, 4009 and 4010 were initially randomized to Group 4 and switched to Group 3 as a consequence of mis-dosing, and to maintain number of animals in each dose group, Animals 3007, 3008, 3015 and 3016 initially randomized to Group 3 were switched to Group 4.

²Time of dosing was determined based on the challenge start time for each animal

IM: intramuscular; PC: post challenge

The blood collection and assay schedule:

	SD -7	SD 0	24 hrs PC	12 hrs PT (36 hrs PC)	24 hrs PT (48 hrs PC)	48 hrs PT (72 hrs PC)	72 hrs PT (96 hrs PC)	SD 7	SD 14	SD 21	SD 28	Moribund/ Found Dead
Temp	X	X										
BW	X											
Challenge		X										
ETI-204 Dose			X									
QT-BAC¹	X		X	X	X	X	X	X	X	X	X	
ETI-204^{a,2}	X			X	X	X	X	X	X	X	X	
PA^{b,2}	X		X	X	X	X	X ^c	X ^c	X ^c	X ^c	X	
ATA²	X										X	
Anti-PA IgG²	X										X	
Tissue Fixation/ Histology												
Tissue Bacterial Burden												

Note that bold line separates model establishing disease phase and treatment phase, and at 24 hrs PC endpoints were assessed prior to treatment. BW, Body weight; QT-BAC, quantitative bacteremia; PA, protective antigen; ATA, anti-therapeutic antibody; PC, post challenge; PT, post treatment

¹ Blood collected in EDTA tube.

² Blood collected in SST tube.

^a For control group, only samples from SD -7, 12 hrs ± 15 min PT, 24 hrs ± 15 min PT and 48 hrs ± 15 min PT were analyzed for ETI-204

^b For control group, only samples from SD -7, 24 hrs ± 15 min PC, 12 hrs ± 15 min PT, 24 hrs ± 15 min PT, and 48 hrs ± 15 min PT were analyzed for PA

^c Samples were not analyzed for animals that survived to study termination at 28d PC.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the animals in the four groups (Table 130). The average aerosol exposure dose of the spores for all animals on study was 143 ± 45 *B. anthracis* LD₅₀ equivalent; the LD₅₀ was ≥200 in approximately 10% of the animals. The baseline characteristics (disease stage) of animals were similar among the animals in the four groups at the time of treatment.

About 31% of the animals were bacteremic and 12% PA positive at the time of treatment (24 hours post-exposure). An increase in temperature was observed in 1 of the animal in the control group, 4 animals treated with ETI-204 16 mg/kg, 2 animals treated with ETI-204 32 mg/kg and none of the animals treated with 8 mg/kg of ETI-204 (Table 130).

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Table 130: Study AR037 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Placebo (Group 1) N=10	ETI-204		
		8 mg/kg (Group 2) N=16	16 mg/kg (Group 3) N=16	32 mg/kg (Group 4) N=16
Baseline characteristics				
Age (weeks) Range	28.2±1.2	28.1±1.2	27.4±0.9	28.9±0.8
Body weight (kg) Mean ± SD	3.5±0.3	3.5±0.3	3.5±0.3	3.5±0.3
Anti-PA IgG (Day-7)	1/10	2/16	0/16	0/16
Inhaled dose				
Inhaled Dose cfu x 10 ⁷ Mean ± SD (Range)				(b) (4)
LD ₅₀ dose Mean ± SD (Range)	153.1±50.5 (101.0-271.0)	142.1±44.7 (76.0-268.0)	156.2±54.0 (76.0-269.0)	124.7±22.6 (64.0-151.0)
<200 LD ₅₀ dose n(%)	8 (80.0)	15 (93.8)	13 (81.3)	16 (100.0)
≥200 LD ₅₀ dose n(%)	2 (20.0)	1 (6.3)	3 (18.8)	0 (0.0)
Bacteremia, PA and body temperature at 24 hours (time of treatment) and time to bacteremia				
Bacteremia at the time of treatment n (%)	2 (20.0)	5 (31.3)	5 (31.3)	6 (37.5)
Log ₁₀ bacteremia cfu/mL Mean±SD (Range)	0.70±0.85 [<LOD (0.30)-2.44]	1.10±1.42 [<LOD (0.30)-4.33]	1.33±1.65 [<LOD (0.30)-5.00]	1.16±1.31 [<LOD (0.30)-3.87]
GM bacteremia cfu/mL Mean±SD (Range)	5.1	12.7	21.3	14.4
Time to quantitative bacteremia (hours) n Mean±SD (Range)	9 40.0±14.7 (24-72)	14 38.6±20.6 (24-96)	11 30.5±6.3 (24-36)	11 34.9±21.1 (24-96)
PA-ELISA Positivity 24 hours post challenge	0	1 (6.3)	3 (18.8)	3 (18.8)
Log ₁₀ PA-ELISA 24 hours post challenge Mean±SD (Range)	0.70±0.00 (0.70 - 0.70)	0.72±0.09 (0.70 - 1.07)	0.92±0.53 (0.70-2.36)	0.85±0.32 (0.70-1.59)
PA-ELISA 24 hours post challenge (ng/mL) Geometric mean	5.0 (<LOD)	5.3	8.3	7.0
Body temperature (°F; SIBT) prior to treatment				
N (%)	1 (10.0)	0 (0)	4 (25.0)	2 (12.5)
Geometric mean	100.4	100.6	101.0	100.8
Post-treatment: Survival and bacteremia at the end of study (Day 28)				
Survivors at the end of study	0	5 (31.3)*	5 (31.3)*	5 (31.3)*
Number of animals bacteremic or (PA positive) post-exposure				
36 hours	5/9 (5/9)	12/16 (5/16)	10/14 (2/14)	8/12 (1/36)
48 hours	5/9 (5/7)	3/10 (0/10)	4/9 (0/9)	3/9 (1/9)
72 hours	1/1 (1/1)	5/9 (0/9)	4/9 (0/9)	2/7 (1/6)
96 hours	NA	4/6 (1/1)	4/9 (2/3)	2/6 (0/1)
Day 7	NA	2/5 (ND)	0/5 (ND)	0/5 (ND)
Day 14, 21	NA	0/5 (ND)	0/5 (ND)	0/5 (ND)
Unscheduled terminal	ND	5/10 (3/7)	4/9 (2/8)	0/5 (0/5)
Anti-PA IgG antibodies n/N (Range)				
Day 28	NA	5/5 (475-106000)	5/5 (2910-14900)	5/5 (1130-26400)
Unscheduled terminal	ND	1/1 (5410)**	2/2 (50200-57600)**	ND

SD=Standard deviation; GM=geometric mean; NA=not applicable; ND=not done

*Statistically significant between the ETI-204 treated group and the control group.

**Animals died by 4 days post-challenge; may represent ETI-204 and not anti-PA antibodies

Effect of treatment on survival and microbial burden: ETI-204 was effective in improving survival time in about one-third of the animals at either of the doses; a dose-dependent effect was

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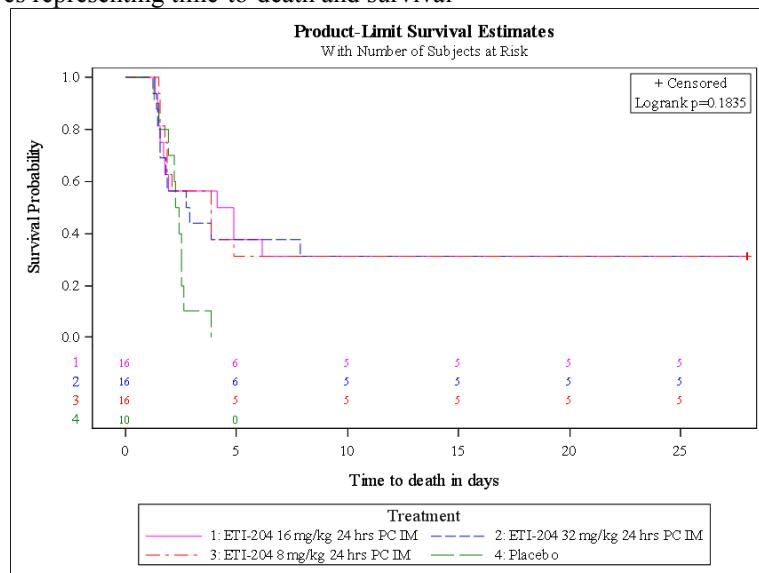
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not observed (Table 130 and Figure 64). All the control group animals died within 4 days of challenge.

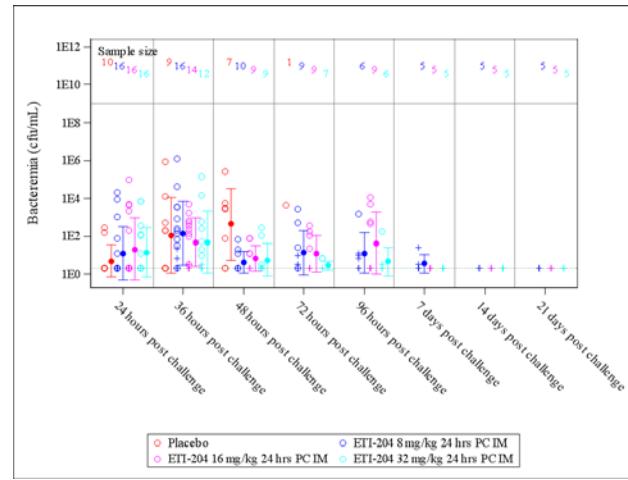
The bacterial burden and PA levels showed a trend towards decrease in animals treated with ETI-204 at 48 hours post-treatment compared to the untreated group of animals; however, there does not appear to be any dose-dependent effect (Figure 64). All the surviving rabbits were culture negative and PA negative at the time of treatment. Most of the animals that died in all 4 groups were bacteremic prior to or at the time of death.

Figure 64: Study AR037 - Kaplan-Meier curves representing time-to-death and survival, bacteremia and PA levels over time

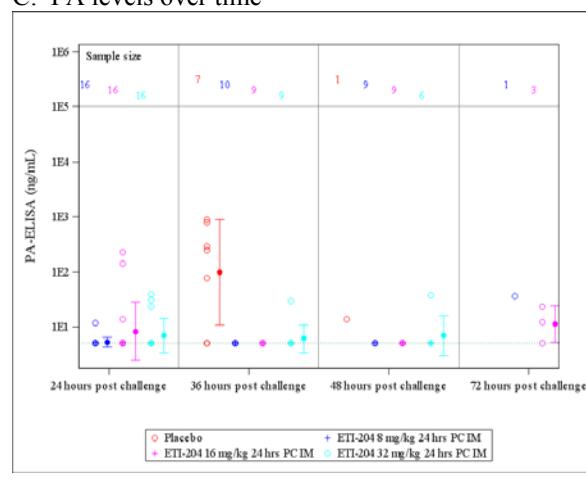
A: Kaplan-Meier curves representing time-to-death and survival



B: Bacteremia over time



C: PA levels over time



PC=Post-challenge; IM=intramuscular

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

Anti-PA IgG antibodies: As stated above, 3 animals (one in the control group and 3 treated with ETI-204 8 mg/kg) were anti-PA IgG positive prior to challenge; all 3 animals died on Days 2, 4, and 2, respectively, post-challenge.

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Anti-PA IgG antibodies were reported in all the surviving rabbits (Table 130); the antibody titers in the two animals (#3003 and #4010) treated with 16 mg/kg ETI-204 were really high (~50,000 ng/mL).

Three rabbits treated with either 8 or 16 mg/kg of ETI-204 were reported to be anti-PA IgG positive at the time of death; these three animals died by Day 4. The positive findings may be a reflection of ETI-204 as the method used for detection of anti-PA IgG antibodies detected ETI-204 as well as endogenous anti-PA IgG antibodies; also the antibody response after primary challenge at Day 4 is likely to be IgM and not IgG.

Clinical Observations: The most common clinical observations were decrease in food consumption, lethargy, no stool, soft stool and respiratory abnormalities post-challenge. Body weight was not altered by exposure to *B. anthracis* or treatment with ETI-204.

Necropsy and Histopathology: Gross lesions in rabbits that died post-challenge included discolorations and/or foci in the lungs, brain and some of the other organs; these observations were consistent with those reported in other studies summarized above. No gross or microscopic lesions were reported in the surviving animals.

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the organs from surviving animals; bacteria were observed in most of the organs from the animals that died (Table 131).

Culture: All the tissues from the animals that survived in the three ETI-204 treated groups were culture negative. However, tissues from all of the non-surviving animals were culture positive (Table 131).

Bacterial burden in tissues from treated animals was lower than the controls; bacterial burden was lowest in animals treated with the 8 mg/kg dose of ETI-204 compared to the other groups (Table 132).

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Table 131: Study AR037-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 8mg/kg		ETI-204 16mg/kg		ETI-204 32mg/kg	
	Survivors (N=0)	Non Survivors (N=10)	Survivors (N=5)	Non Survivors (N=11)	Survivors (N=5)	Non Survivors (N=11)	Survivors (N=5)	Non Survivors (N=11)
Presence of bacteria by microscopy [1, 3]								
Brain	0/0	9/10	0/5	7/11	0/5	7/11	0/5	8/11
Kidney	0/0	9/10	0/5	7/11	0/5	6/11	0/5	7/11
Large Intestine, Cecum	0/0	0/10	0/5	0/11	0/5	0/11	0/5	0/11
Liver	0/0	9/10	0/5	3/11	0/5	5/11	0/5	5/11
Lung	0/0	10/10	0/5	6/11	0/5	7/11	0/5	8/11
Lymph Node, Mediastinal	0/0	4/5	0/5	2/8	0/4	8/9	0/4	8/10
Mediastinum	0/0	1/10	0/5	1/11	0/5	0/11	0/5	1/11
Site, Injection	0/0	3/10	0/5	0/11	0/5	0/11	0/5	0/11
Spleen	0/0	9/10	0/5	5/11	0/5	6/11	0/5	7/11
Presence of bacteria by culture [1, 2]								
Brain	0/0	10/10	0/5	11/11	0/5	11/11	0/5	11/11
Kidney	0/0	10/10	0/5	11/11	0/5	11/11	0/5	11/11
Liver	0/0	10/10	0/5	11/11	0/5	11/11	0/5	10/11
Lung	0/0	10/10	0/5	9/9	0/5	11/11	0/5	10/10
Lymph Node, Mediastinal	0/0	10/10	0/5	11/11	0/5	11/11	0/5	10/11
Spleen	0/0	10/10	0/5	11/11	0/5	11/11	0/5	11/11

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Tissue bacteremia was assessed quantitatively. Animal was considered positive if bacteremia was above limit of detection (LOD = 14.8 cfu/g)

[3] Histopathology performed at ^{(b) (4)} Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ELISA) and bacteremia (quantitative) at any point prior to treatment:

1002, 1003, 1004, 1005, 1006, 1007, 1008, 1010, 2001, 2002, 2004, 2005, 2006,
2007, 2009, 2010, 2011, 2012, 2014, 3002, 3003, 3006, 3007, 3008, 3009, 3010, 3011, 3012, 3013, 3014, 3015,
3016, 4002, 4003, 4006, 4007, 4010, 4014, 4015, 4016

Animals that were negative for bacteremia (quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ELISA) only at any point prior to treatment: 1001, 1009, 2003, 2013, 2015,
2016, 3004, 3005, 4004, 4005, 4008

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Table 132: AR037 - Bacterial burden in tissues at necropsy after ETI-204 administration at different time points

Group geometric mean (95% confidence interval) of tissue bacterial burden (CFU/g Tissue)							
	N	Brain	Liver	Kidney	Lung	Med. LN	Spleen
Succumbed	Group 1	1.11E+06 (1.01E05-1.23E+07)	5.72E+06 (2.84E+05-1.15E+08)	7.59E+06 (4.94E+05-1.17E+08)	3.03E+07 (2.36E+06-3.89E+08)	4.21E+06 (9.66E+05-1.83E+07)	1.81E+07 (1.56E+06-2.09E+08)
		1.37E+04 (7.74E+02-2.42E+05)	5.77E+03 (4.59E+02-7.24E+04)	6.31E+04 (7.99E+03-4.98E+05)	6.27E+04 ^a (1.22E+04-3.23E+05)	2.24E+04 (1.91E+03-2.62E+05)	8.45E+04 (5.29E+03-1.35E+06)
	Group 2	11					
	Group 3	11	1.71E+04 (9.01E+02-3.24E+05)	9.81E+04 (7.68E+03-1.25E+06)	2.85E+05 (1.96E+04-4.14E+06)	2.70E+05 (1.72E+04-4.23E+06)	7.78E+04 (5.94E+03-1.02E+06)
Survived	Group 4	11	9.94E+04 ^b (1.38E+04-7.14E+05)	8.95E+04 ^b (3.69E+03-2.18E+06)	1.19E+06 (2.12E+05-6.72E+06)	5.67E+05 (5.34E+04-6.01E+06)	7.28E+04 (3.89E+03-1.36E+06)
	Group 2	5	BLD	BLD	BLD	BLD	BLD
	Group 3	5	BLD	BLD	BLD	BLD	BLD
	Group 4	5	BLD	BLD	BLD	BLD	BLD

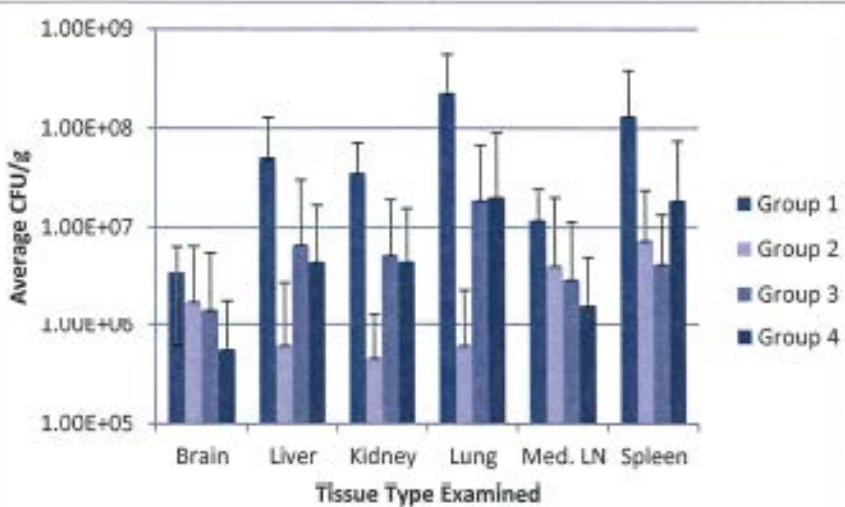
Succumbed, animals succumbing to disease; Survived, animals surviving to the end of the study

BLD, below limit of detection (<14.8 CFU/g tissue), i.e., all animals in the subgroup lacked detectable bacterial burden.

In order to do the geometric mean calculation, 5.65 CFU/g tissue was assigned to a "BLD" value for individual animal data (by following the tradition in the Microbiology report).

^a Lung culture weights of two animals (Animals 2008 and 2016) were not documented at sample collection, therefore no CFU/g was calculable. Both samples were positive for *B. anthracis* growth.

^b Animal 4005 brain, Animal 4016 liver, and Animal 4009 spleen had a value of above limit of detection (ALD), i.e. the highest sample dilution had a lawn of bacterial growth. Therefore no results were obtained for those samples.



Comments:

ETI-204 was effective in improving survival time in about one-third of the animals at either of the doses; a dose-dependent effect was not observed. All the control group animals died within 4 days of challenge. All the surviving rabbits were culture negative and PA negative at the time of treatment. Most of the animals that died in all 4 groups were bacteremic prior to or at the time of death.

The bacterial burden and PA levels showed a trend towards decrease in animals treated with ETI-204 at 48 hours post-treatment compared to the untreated group of animals; however, there does not appear to be any dose-dependent effect. All the animals that survived were culture negative at Day 28; no bacteria were reported in any of the organs. Anti-PA IgG antibodies were detected.

The animals that died were bacteremic and bacteria were present in different tissues. Three rabbits treated with either 8 or 16 mg/kg of ETI-204 were reported to be anti-PA IgG positive at the time of death; these two animals died by Day 4. These positive findings between Days 1 and 4 may be a reflection of ETI-204 as serum ETI-204 concentration peaked between 48-72 hours post dosing. Additionally, the primary antibody response is likely to be IgM and not IgG.

6.4.2. Cynomolgus monkeys

The post-exposure efficacy of ETI-204 was measured in three studies (AP301, 307, and AP107) in cynomolgus monkeys.

6.4.2.1. Study AP301

This was a randomized, blinded, placebo-controlled GLP study to evaluate the pharmacokinetics of ETI-204 (Lonza product) when administered IM at increasing times post-exposure to *B. anthracis* spores (spore lot no. B39) by inhalation in 42 naïve cynomolgus monkeys at [REDACTED] (b) (4)⁶⁷. The secondary objective of this study was to evaluate the impact of the time of treatment on the PK of ETI-204 administered IM.

Study design:

Animals purchased from [REDACTED] (b) (4) were quarantined at [REDACTED] (b) (4) as for the animals in the treatment studies summarized above; additionally, the health surveillance information collected was similar. The mean age of the animals was 2.9 (range 2.6-4.2) years and mean body weight 2.8 kg (range 2.5-3.3kg) at the time of challenge. Animals were randomized to seven groups (Table 133). *Klebsiella* was reported in 12 animals at the time of screening; however, the method used for screening was not specified. All the *Klebsiella* positive animals were in either Groups 3, 4, 5, 6, or 7.

Animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The average MMAD for the three challenge days ranged from 1.14-1.30 µm.

ETI-204 was administered, IM, at 18, 24, or 36 hours post-exposure. Blood was collected at different time points for measuring bacteremia (Table 133). Although the applicant stated that serum samples for measurement of PA levels by ELISA were shipped to [REDACTED] (b) (4) and for anti-PA IgG levels shipped to [REDACTED] (b) (4) for storage) it appears that PA or anti-PA IgG levels were not measured.

⁶⁷ [REDACTED] (b) (4) Study Number 2720 -100014200: AP301: Study to evaluate the pharmacokinetics of ETI -204 administered via intramuscular (IM) route in a time of treatment post -exposure prophylaxis model of cynomolgus monkey anthrax infection (July 31, 2014).

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Table 133: AP301 - Study design and schedule of blood collection

Study design:

Group	Number of Animals	ETI-204 Dose (mg/kg, IM)	Target Concentration of the final formulation (mg/mL)	Time of Dose (hrs PC)	Last Day of Observations (PC)
1	6	0 (vehicle)	0	18	28
2	6	8	61.5	18	28
3	6	16	120	18	56
4	6	8	61.5	24	28
5	6	16	120	24	56
6	6	8	61.5	36	28
7	6	16	120	36	56

The blood collection and assay schedule:

Time-point either post challenge (PC) or post treatment (PT)	Collection Window	Blood Tube/Approx. Volume	Bacteremia (Quantitative)	Sera for PA ELISA	Sera for ETI-204 Levels	Sera for anti-drug antibodies (ADA) and Potential Future Use ^d
Day -7	-	EDTA ~1.0mL SST ~3.0mL	X ^c	X	X	X
18hr PC ^b	± 60 min	EDTA ~1.0mL SST ~1.5mL	X	X		
24hr PC ^b	± 60 min	EDTA ~1.0mL SST ~1.5mL	X	X		
36hr PC ^b	± 60 min	EDTA ~1.0mL SST ~1.5mL	X	X		
1hr PT	± 15 min	SST ~2.0mL		X	X	
6hr PT	± 60 min	SST ~2.0mL		X	X	
12hr PT	± 60 min	SST ~2.0mL		X	X	
24hr PT	± 60 min	EDTA ~1.0mL SST ~2.0mL	X	X	X	
48hr PT	± 60 min	SST ~1.5mL		X		
72hr PT	± 60 min	EDTA ~1.0mL SST ~2.5mL	X	X	X	
7 days PC	-	EDTA ~1.0mL SST ~2.5mL	X	X	X	
14 days PC	-	EDTA ~1.0mL SST ~2.5mL	X	X	X	
21 days PC	-	EDTA ~1.0mL SST ~2.5mL	X	X	X	
28 days PC	-	EDTA ~1.0mL SST ~4.0mL	X	X	X	X
56 days PC ^c	-	EDTA ~1.0mL SST ~4.0mL	X	X	X	X
Terminal	-	EDTA ~1.0mL SST ~2.5mL	X	X		

PC = post-challenge; PT = post-treatment (based on first treatment) | Bold line separates model establishing phase and treatment phase | ^a Qualitative bacteremia only | ^b Immediately prior to treatment for the animals being treated | ^c Only animals from 16 mg/kg group | ^d Samples were shipped to a Sponsor-designated facility for potential future analysis not part of this study.

Animals were followed for clinical observations for up to 56 days post-challenge. At the time of necropsy, tissue sections of spleen, brain, liver, and bronchial lymph node were processed for histology and culture as for the studies summarized above.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among animals in different groups (Table 134). The average ± SD aerosol exposure dose for all animals on study was 402 ± 153 LD₅₀ equivalent; the LD₅₀ was ≥ 200 in approximately 98% of the animals. The baseline characteristics (disease stage) of animals were similar in animals in the seven groups.

All animals were bacteremic between 18 and 90 hours post-challenge (Table 134). At the time of initiation of therapy, all animals in Groups 2 and 3 i.e., those treated at 18 hours post-exposure) were culture negative. However, one third of the animals treated at 24 hours (Groups 4 and 5) and all the animals treated at 36 hours (Groups 6 and 7) post-exposure were bacteremic (Table 134).

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Table 134: Study AP301 - Baseline characteristics, inhaled dose of <i>B. anthracis</i> , time to treatment and survival							
Parameters	Placebo (Group 1) N=6	ETI-204: 8 mg/kg			ETI-204: 16 mg/kg		
		18 hours (Group 2) N=6	24 hours (Group 4) N=6	36 hours (Group 6) N=6	18 hours (Group 3) N=6	24 hours (Group 5) N=6	36 hours (Group 7) N=6
Baseline characteristics							
Age (years) Mean± SD (Range)	2.9±0.5 (2.6-4.0)	2.8±0.1 (2.6-2.9)	2.8±0.2 (2.7-3.1)	2.8±0.1 (2.7-3.0)	3.0±0.6 (2.6-4.2)	3.1±0.7 (2.6-4.6)	2.8±0.1 (2.7-2.9)
Body weight (kg) Mean ±SD	2.77±0.2	2.68±0.18	2.78±0.15	2.75±0.22	2.78±0.26	2.88±0.19	2.78±0.16
Inhaled dose							
Inhaled Dose cfu $\times 10^7$ Mean ± SD (Range)	(b) (4)						
LD ₅₀ dose Mean ± SD (Range)	395.7±166.9 (257-725)	461.7±151.6 (278-673)	385.5±133.4 (250-602)	409.5±131.1 (266-584)	422.8±157.8 (290-700)	305.2±130.2 (152-501)	431.8±215.4 (216-810)
<200 LD ₅₀ dose n(%)	0	0	0	0	0	1 (16.7)	0
≥200 LD ₅₀ dose n(%)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	5 (83.3)	6 (100)
Bacteremia prior to treatment							
Bacteremia at the time of treatment n (%)	0	0	2 (33.3)	6 (100)	0	2 (33.3)	6 (100)
Log ₁₀ (cfu/mL) Mean±SD	0.30±0.00 (0.30-0.30)	0.30±0.00 (0.30-0.30)	1.11±1.40 (0.30-3.73)	4.78±0.46 (4.21-5.40)	0.30±0.00 (0.30-0.30)	1.13±1.45 (0.30-3.85)	4.51±2.00 (1.70-7.79)
cfu/mL) GM (range)	2.0 (<LOD)	2.0 (<LOD)	12.8 (0.4-378.6)	60287.8 (19996- 181766)	2.0 (<LOD)	13.4 (0.4-442.5)	32327.4 (255.4- 4091563)
Time to bacteremia							
Time to bacteremia (hours) Mean±SD (Range)	6 41.7±1.0 (40.8-43.2)	5 51.0±21.6 (40.5-89.6)	6 40.5±12.9 (23.1-49.7)	6 28.9±8.2 (17.7-36.8)	3 42.3±1.3 (41.3-43.7)	3 31.8±13.8 (23-47.7)	6 30.2±7.1 (23.4-36.8)
End of Study (Day 28^s or Day 56^t)							
Survivors at the end of study	0	6 (100) ^{§*}	5 (83.3) ^{§*}	0	6 (100) ^{**}	5 (83.3) ^{**}	3 (50) ^{**}

Effect of treatment on survival: The results show that ETI-204 at a dose of 8 mg/kg or 16 mg/kg when administered at 18 or 24 hours post-exposure was effective in improving survival, compared to the control group; however, when treatment was administered at 36 hours post-exposure the 8 mg/kg dose of ETI-204 mg/kg was not effective in improving survival whereas 16 mg/kg dose was effective (Figure 65 and Table 134). All of the non-surviving animals died within 7 days of challenge.

Effect of treatment on microbial burden: The bacterial load steadily increased as the time from challenge to treatment increased; bacteremia levels decreased in all groups following ETI-204 treatment. At 72 hours post-treatment, the bacteremia level in the control group (Group 1) was significantly greater compared to those in other groups treated with ETI-204 at either 18 and 24 hours post-challenge (Groups 2 through 5) (Figures 65 and 66). For all animals surviving to the end of the study, the quantitative bacteremia levels at Days 14, 21, 28 days, and 56 post-challenge were below than the LOD (Figure 66).

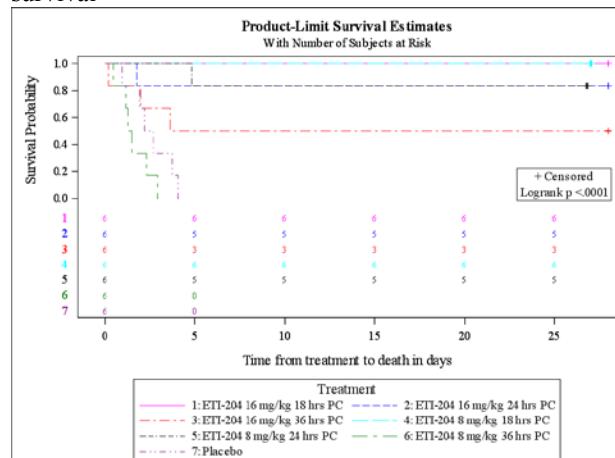
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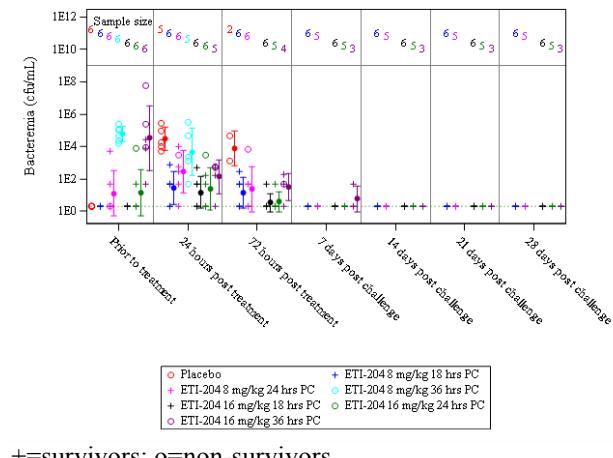
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Figure 65: Study AP301 - Survival and relationship between microbial burden and time to death

A: Kaplan-Meier curves representing time-to-death and survival



B: Bacteremia by survival status



+=survivors; o=non-survivors

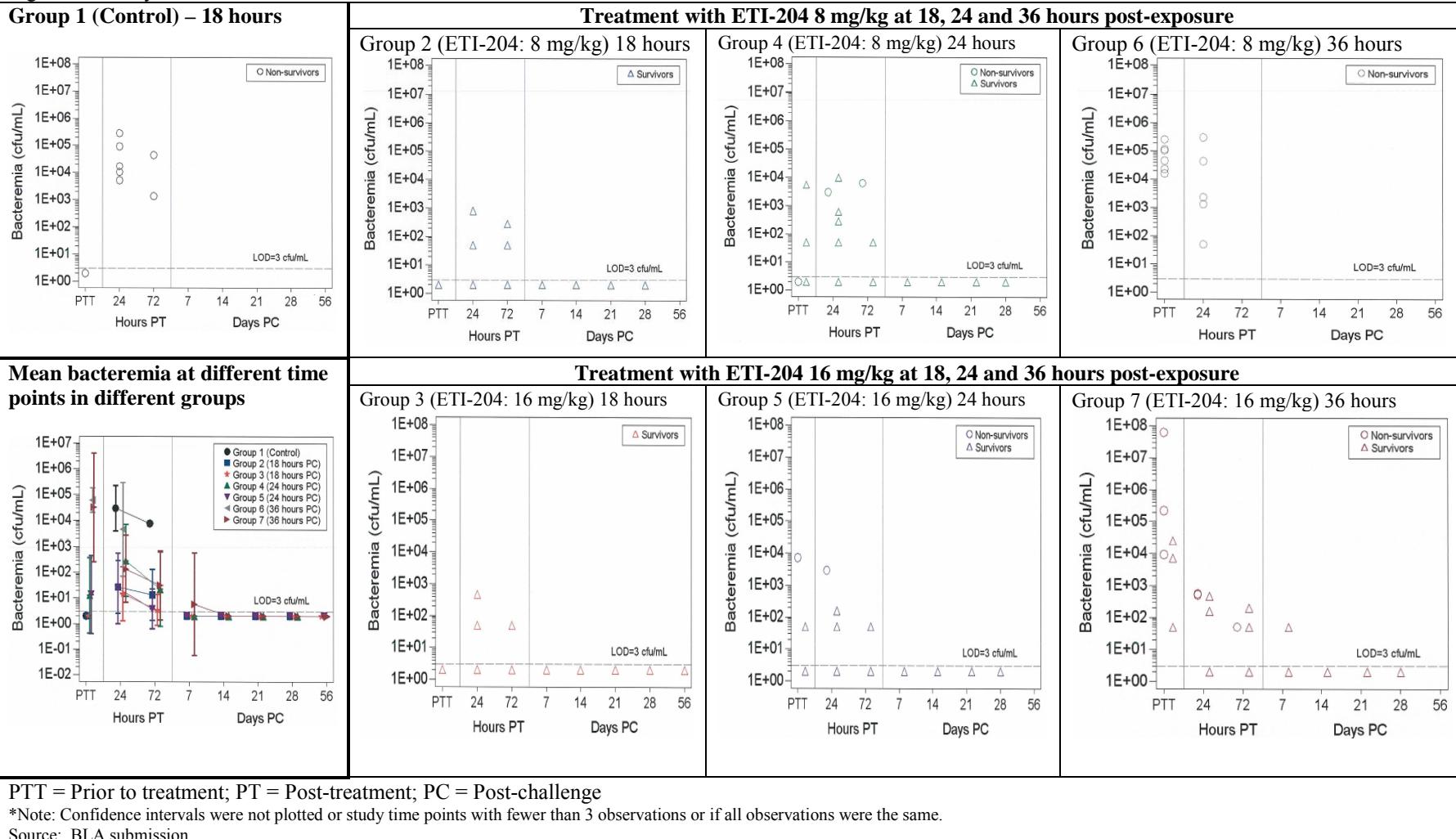
Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

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Figure 66: Study AP301 - Observed bacteremia over time in survivors and non-survivors



Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these signs were consistent with those reported in other studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is common with laboratory housed non-human primates) after Day 22 post-challenge (Figure 67).

Figure 67: Study AP301 – Clinical observations in animals treated with ETI-204 16 mg/kg

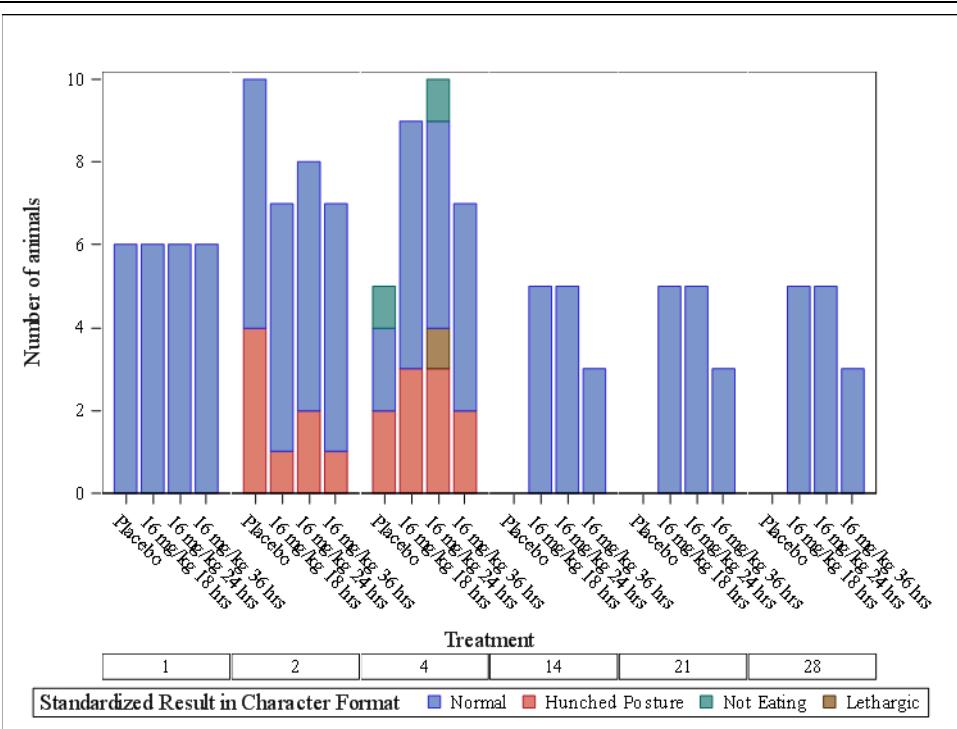


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

Necropsy and Histopathology: No gross lesions were reported in animals that survived the period of observation. Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included discoloration of the brain and/or skin crust. Microscopic findings considered consistent with anthrax were present in all gross lesions examined; these were acute lesions typical of fulminant anthrax infection and similar to those summarized above for other studies.

Tissue bacterial assessments:

Histology: No bacteria were observed in organs from any of the surviving animals and majority of the non-surviving animal examined (Table 135).

Culture: Tissues tested from all of the animals that died on study were culture positive. However, tissues from animals that survived to their scheduled sacrifice on Day 56 post-challenge were culture negative (Table 135).

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Table 135: Study AP301-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 8mg/kg 18hrs PC		ETI-204 16mg/kg 18hrs PC		ETI-204 8mg/kg 24hrs PC		ETI-204 16mg/kg 24hrs PC		ETI-204 8mg/kg 36hrs PC		ETI-204 16mg/kg 36hrs PC	
			Non Survivor (N=0)		Non Survivor (N=6)		Non Survivor (N=6)		Non Survivor (N=5)		Non Survivor (N=1)		Non Survivor (N=0)	
		Survivor (N=0)	Survivor (N=6)	Survivor (N=0)	Survivor (N=6)	Survivor (N=6)	Survivor (N=5)	Survivor (N=1)	Survivor (N=5)	Survivor (N=1)	Survivor (N=0)	Survivor (N=3)	Survivor (N=3)	Survivor (N=3)
Presence of bacteria by microscopy [1, 3, 4]														
Brain	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	2/ 2	0/ 0	1/ 2
Site, Injection	0/ 0	0/ 6	0/ 6	0/ 0	0/ 6	0/ 0	0/ 5	0/ 1	0/ 5	0/ 1	0/ 0	2/ 6	0/ 3	1/ 3
Skin	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 2	0/ 0	1/ 2
Presence of bacteria by culture [1, 2]														
Brain	0/ 0	6/ 6	0/ 6	0/ 0	0/ 6	0/ 0	0/ 5	1/ 1	0/ 5	1/ 1	0/ 0	6/ 6	0/ 3	3/ 3
Liver	0/ 0	6/ 6	0/ 6	0/ 0	0/ 6	0/ 0	0/ 5	1/ 1	0/ 5	1/ 1	0/ 0	6/ 6	0/ 3	2/ 3
Lymph Node	0/ 0	5/ 6	2/ 6	0/ 0	0/ 6	0/ 0	3/ 5	1/ 1	0/ 5	1/ 1	0/ 0	6/ 6	0/ 3	3/ 3
Spleen	0/ 0	6/ 6	0/ 6	0/ 0	0/ 6	0/ 0	0/ 5	1/ 1	0/ 5	1/ 1	0/ 0	6/ 6	0/ 3	3/ 3

[1] All treated animals irrespective of bacteremia status prior to treatment
[2] Animal was considered positive if at least 1-5 colonies were present on plate
[3] Histopathology performed at (b) (4)
[4] Injection sites were examined for all animals; other tissues were examined only when gross lesions were identified; numbers examined/with gross lesions are shown
Animals that were negative for bacteremia (quantitative) only at any point prior to treatment: C52573, C53565, C53869, C53908, C53912, C54041, C54106, C54107, C54115, C54133, C54158, C54159, C54160, C54226, C54236, C54237, C54238, C54244, C54260, C54262, C54292, C54293, C54294, C54305, C54308, C54340
PA (ECL and ELISA) were not performed

Comments:

The results of this study suggest a dose-dependent effect of ETI-204 on survival; when intervention was delayed to 36 hours post-challenge, the proportion of animals that survived decreased. When the treatment was delayed, dose also played a role in survival. For example, at 36 hours post-exposure, administration of 8 mg/kg ETI-204 resulted in no survivors (0/6) while 16 mg/kg ETI-204 protected 3 of the 6 animals. All of the control animals died by Day 5 post-exposure. The animals that died were bacteremic and bacteria were present in the tissues examined. All the animals that survived were culture negative until terminal sacrifice (Day 28 or 56) and no bacteria were observed in the tissues examined.

No gross lesions were reported in the animals that survived. However, in the animals that died, all the gross lesions examined were consistent with acute *B. anthracis* infection.

6.4.2.2. Study AP307

This was a randomized, open label, placebo-controlled parallel non-GLP study conducted at (b) (4) to evaluate the pharmacokinetics of ETI-204 (Lonza product) when administrated IM at increasing times post-exposure with the spores (spore lot no. B39) of *B. anthracis* by inhalation in 52 experimentally naïve cynomolgus monkeys at (b) (4)⁶⁸. The secondary objectives were to determine pharmacokinetics of ETI-204 when administered via the IM route to evaluate the impact of the time of ETI-204 administration on PA levels and to evaluate the numbers of *B. anthracis* in the blood.

⁶⁸ (b) (4) Study Number 597-100011517: AP307: Study to evaluate the post-exposure efficacy of ETI-204 via intramuscular (IM) administration in the cynomolgus macaque inhalation anthrax model (January 20, 2014).

Study design:

Animals purchased from [REDACTED] were quarantined at [REDACTED] (b) (4) as for the animals in the treatment studies summarized above; additionally, the health surveillance information collected was similar. The mean age of the animals was 3.8 (range 3.0-4.0) years and mean body weight 3.21 kg (range 2.6-5.6 kg) at the time of challenge. Animals were randomized to four groups (Table 136) and exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The average MMAD for the three challenge days ranged from 1.08-1.14 µm.

ETI-204 (16 mg/kg) was administered, IM, at 24, 36, or 48 hours post-exposure. Blood was collected at different time points for measuring bacteremia (qualitative and quantitative), serum PA by ELISA, and serum anti-PA IgG (shipped to [REDACTED] (b) (4) for storage) levels by ELISA (Table 136).

Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of spleen, brain, liver, and bronchial lymph node were processed for histology and culture as for the studies summarized above.

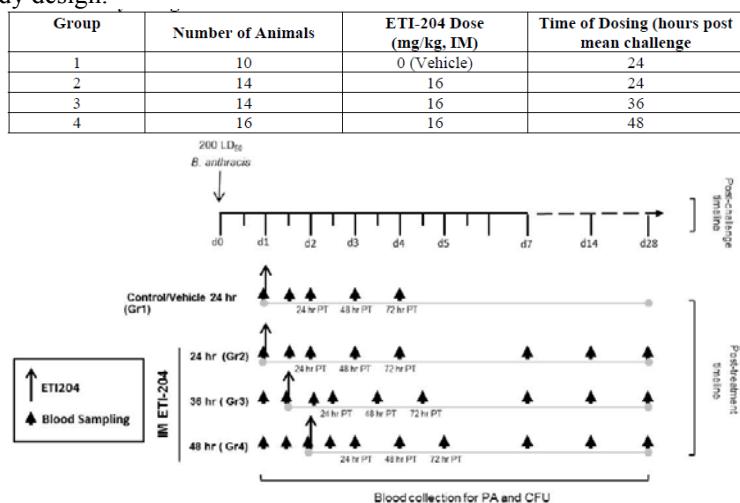
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Table 136: AP307 - Study design and schedule of blood collection

Study design:



The blood collection and assay schedule:

Time-point	Collection Window	Blood Tube/Approx. Volume	Bacteremia (Quantitative)	Sera for PA ELISA	Serum for ETI-204 Levels	Serum for Retention and anti-therapeutic IgG
Day -7	-	EDTA ~1.0 mL SST ~3.0 mL	X*	X	X	X
24hr PC ^{a,b}	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X		
36hr PC ^{a,b}	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X		
48hr PC ^{a,b}	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X		
12hr PT	± 30 min	SST ~1.5 mL			X	
24hr PT	± 60 min	EDTA ~1.0 mL SST ~2.5 mL	X	X	X	
48hr PT	± 60 min	SST ~1.5 mL		X		
72hr PT	± 60 min	EDTA ~1.0 mL SST ~2.5 mL	X	X	X	
7 days PC	-	EDTA ~1.0 mL SST ~2.5 mL	X	X	X	
14 days PC	-	EDTA ~1.0 mL SST ~2.5 mL	X	X	X	
21 days PC	-	EDTA ~1.0 mL SST ~1.5 mL	X	X		
28 days PC	-	EDTA ~1.0 mL SST ~4.0 mL	X	X	X	X
Terminal	-	EDTA ~1.0 mL SST ~2.5 mL	X	X		

PC = post-challenge; PT = post-treatment; SST = Serum Separator Tubes; EDTA = Ethylenediaminetetraacetic Acid; PA = Protective Antigen;

ELISA = Enzyme-Linked Immunosorbent Assay

Bold line separates model establishing phase and treatment phase.

* Qualitative bacteremia only.

^a Based on mean challenge time.

^b: Only for animals that had not been treated. These time points served as the immediately prior to treatment samples for animals scheduled for treatment at that time point.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among three groups (Table 137). The average ± SD aerosol exposure dose for all animals on study was 205±68 *B. anthracis* LD₅₀ equivalent; the LD₅₀ was ≥200 in approximately 50% of the animals. The baseline characteristics (disease stage) of animals were similar in animals in the four groups.

At the time of treatment (24 hours) of Group 2 animals, 13 animals were culture negative whereas all animals were PA negative; at 36 hours, 2 animals were culture negative and 7 animals were PA negative. At 48 hours, however, all animals were PA positive as well as bacteremic (Table 137). The baseline characteristics (disease stage) of animals were similar in animals in the four groups.

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Table 137: Study AP307 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Placebo 24 hours (Group 1) N=10	ETI-204 16 mg/kg		
		24 hours (Group 2) N=14	36 hours (Group 3) N=14	48 hours (Group 4) N=14 ¹
Baseline characteristics				
Age (years) Mean± SD (Range)	3.8±0.4 (3.0-4.0)	3.7±0.5 (3.0-4.0)	3.8±0.4 (3.0-4.0)	4.0±0.0 (3.0-4.0)
Body weight (kg) Mean ±SD	3.21±0.31	3.16±0.35	3.12±0.24	3.35±0.78
Inhaled dose				
cfu x 10 ⁷ Mean ± SD (Range)				(b) (4)
LD ₅₀ dose Mean ± SD (Range)	200.7±46.0 (131-265)	209.0±56.8 (112-310)	197.6±92.4 (84-346)	211.6±70.1 (131-329)
<200 LD ₅₀ dose n (%)	4 (40.0)	6 (42.9)	8 (57.1)	8 (57.1)
≥200 LD ₅₀ dose n (%)	6 (60.0)	8 (57.1)	6 (42.9)	6 (42.9)
Bacteremia prior to treatment				
Bacteremia at the time of treatment n (%)	5 (50)	1 (7.1)	12 (85.7)	14 (100)
Log ₁₀ (cfu/mL) Mean±SD	1.14±0.93 (0.30-2.57)	0.48±0.66 (0.30-2.78)	3.73±2.21 (0.30-6.86)	4.79±1.75 (2.26-7.94)
cfu/mL Geometric mean	13.8	3.0	5380.0	61537.9
PA prior to treatment				
n (%)	0	0	7 (50)	14 (100)
Log ₁₀ PA-n/g/mL Mean±SD (Range)	0.70±0.00 (0.70, 0.70) <LOQ	0.70±0.00 (0.70, 0.70) <LOQ	1.30±0.77 (0.70-3.15)	2.36±0.86 (1.20-4.20)
Geometric Mean (Range)	5.0 <LLOQ	5.0 <LLOQ	19.8 (7.1-55.5)	228.5 (72.7-720.3)
Post treatment: Time to bacteremia and PA				
Time to bacteremia (hours) Mean±SD (Range)	10 [‡] 39.8±22.7 (22.2-95.9)	7 50.1±20.8 (25.2, 93.5)	12 31.2±5.3 (21.9-36.3)	14 36.6±7.5 (21.9-49.8)
Post-treatment: Survival and bacteremia at the end of study (Day 28)				
Survivors at the end of study	1 (10.0)	13 (92.9)*	6 (42.9)	4 (28.6)
Bacteremia at different time points n/N (Range cfu/mL x 10³)				
24 hours PT	8/10 (0.5-5400)	5/13 (0.001-0.3)	9/13 (0.001-43.7)	11/11 (0.001-3000)
72 hours PT	6/6 (0.001-76)	2/13 (0.001-78.7)	3/7 (1-0.6)	2/6 (0.001-0.2)
7, 14, 21, and 28 Days PC	0/1 (<LOD)	0/13 (<LOD)	0/6 (<LOD)	0/4 (<LOD)
PA levels at different time points n/N (Range ng/mL)				
24 hours PT	8/9 (74-5080)	0/13<LOQ	2/13 (12.8-104)	2/10 (13.5-15.5)
48 hours PT	7/7 (49-493)	1/13 (14.8)	1/10 (82.7)	0/7 (<LOQ)
72 hours PT	6/6 (63-849)	0/13 (<LOQ)	1/8 (66.2)	0/6 (<LOQ)
7 Days PC	0/1 (<LOQ)	0/13 (<LOQ)	1/6 (19.9)	0/4 (<LOQ)
14, 21, and 28 Days PC	0/1 (<LOQ)	0/13 (<LOQ)	0/6 (<LOQ)	0/4 (<LOQ)

¹There were 16 animals randomized to Group 4. However, two animals (C49209 and C51315) in the group treated at 48 hours post-challenge died prior to treatment and were therefore excluded from the primary survival analysis.

[‡]The control animal C50738 that survived was PA negative and culture negative at all the time points tested except PA positive (PA titer 48.7 ng/mL) at 48 hours and PA (PA titer 62.8 ng/mL) and bacteremic (1 cfu/mL) at 72 hours post-exposure. Tissues were culture positive.

There was one animal (C51350) in the 24 treatment group treated group which had a reportable PA value above the lower limit of quantitation at the Day-7 collection, but this sample was below the lower limit of quantitation when repeated

Quantitative bacteremia LOD Limit of detection=3 cfu/mL.

PA ELISA LLOQ Lower limit of quantification=9.68 ng/mL.

*Statistically significant between the ET-204 treated group and the control group.

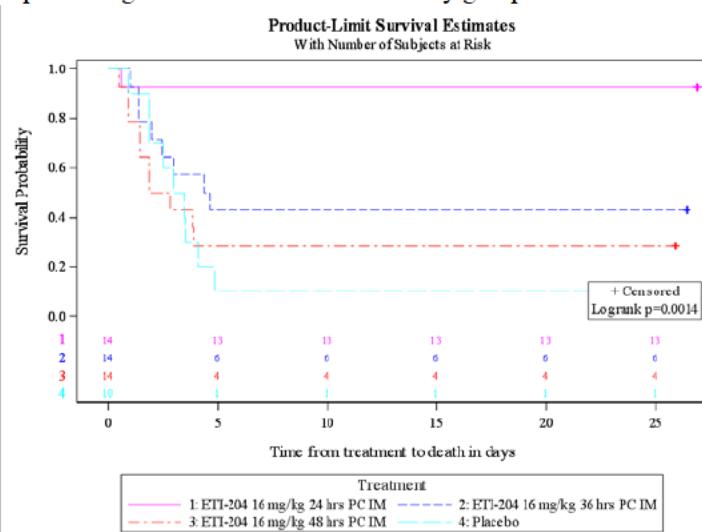
SD=Standard deviation; PT=post-treatment; PC=post challenge

Effect of treatment on survival and microbial burden: The results show that ETI-204 at a dose of 16 mg/kg, administered 24 hours post-exposure, was most effective in improving survival (93%); when treatment was delayed to 36 or 48 hours post-exposure the survival rate was 43% and 29%, respectively (Table 137 and Figure 68). One (10%) of the control group animal survived. All of the non-surviving animals died within 7 days of challenge. A majority of the animals surviving to the end of the study were culture negative (i.e., less than the LOD) by 7 days post-exposure (Table 137 and Figure 68).

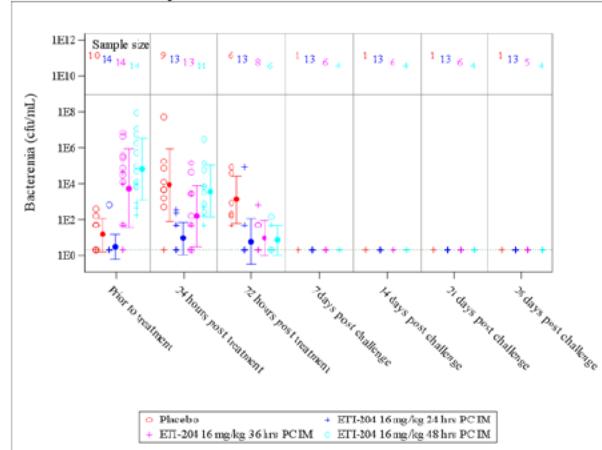
The bacterial load and PA levels, prior to treatment, steadily increased as the time from challenge to treatment increased; bacteremia and PA levels decreased in all groups following ETI-204 treatment (Figure 68). At 72 hours post-treatment, the mean bacteremia level in the control group (Group 1) was significantly greater than those in Groups 2, 3 and 4 treated with ETI-204 at 24, 36 or 48 hours post-exposure (Figures 68 and 69).

Figure 68: Study AP307 - Survival and relationship between microbial burden and time to death

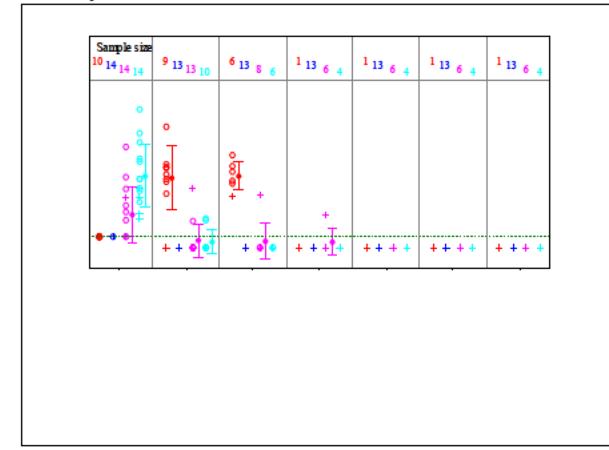
A: Kaplan-Meier curves representing time-to-death and survival by group



B: Bacteremia by survival status



C: PA by survival status



PC=Post-challenge; IM=intramuscular; +=survivors; o=non-survivors at Day 28

Figures constructed by Dr Xianbin Li, PhD (Statistics reviewer)

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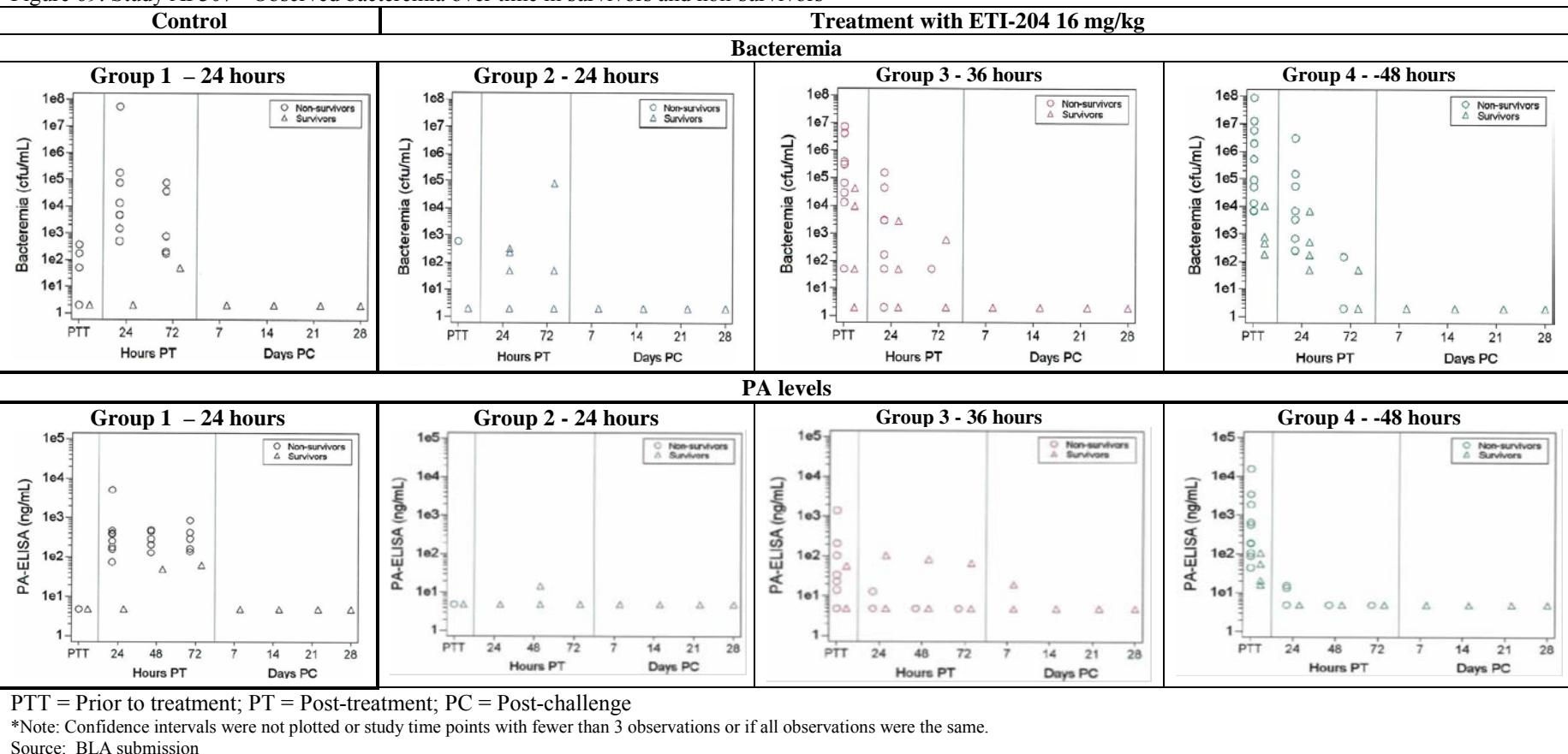
Effect of microbial burden on survival: The effect of treatment time on survival was probably due to the prior to treatment bacteremia and PA levels (Figures 68 and 69). All deaths occurred in animals that were confirmed bacteremic prior to treatment. All animals that were culture negative prior to treatment including 2 animals in the 36 hour treatment group survived.

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Figure 69: Study AP307 - Observed bacteremia over time in survivors and non-survivors



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Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these signs were consistent with those reported in other studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is common with laboratory housed non-human primates) after Day 22 post-challenge (Figure 70).

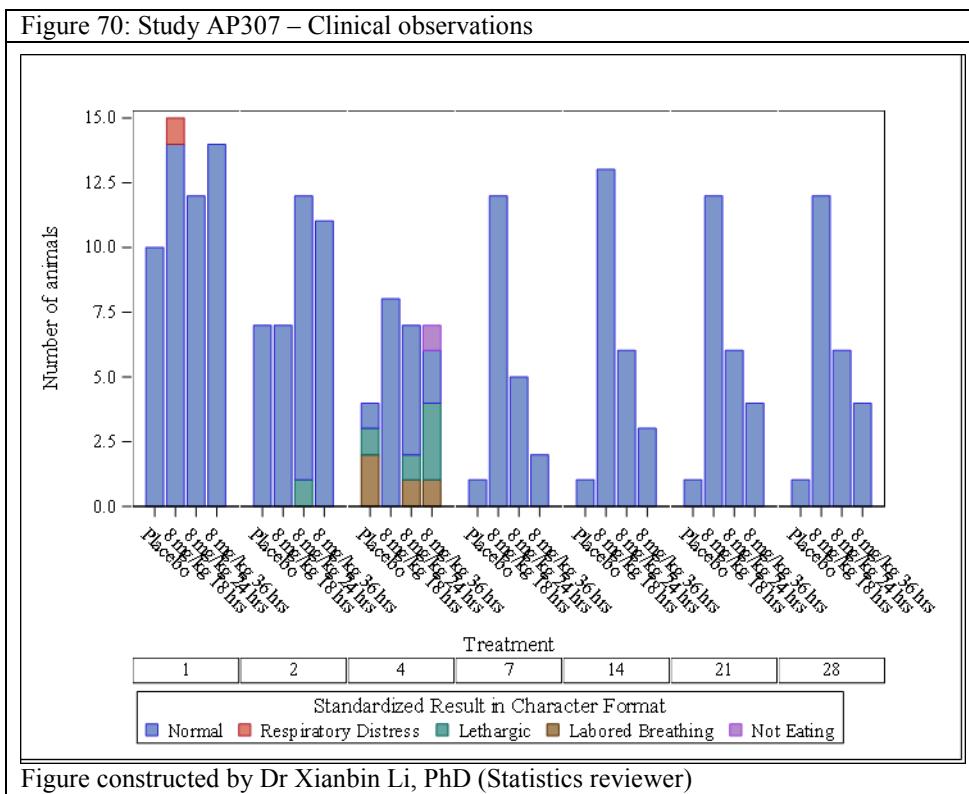


Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge were consistent with those reported in other studies summarized above.

Microscopic findings considered consistent with anthrax were present in all monkeys found dead and included acute lesions typical of fulminant anthrax infection to include inflammation, hemorrhage, fibrin exudation, necrosis, hepatic sinusoidal leukocytosis, and/or the presence of large rod-shaped bacteria consistent with *B. anthracis* in multiple organs. However, the animals that survived to scheduled termination typically had lesions consistent with previous inflammation including: hyperplasia (bronchial and/or mediastinal lymph nodes and/or lymphoid follicles in the spleen); hemosiderin pigment accumulation and histiocytic cellular infiltration (bronchial and/or mediastinal lymph nodes); minimal inflammation in the lung, bronchial and/or mediastinal lymph nodes, spleen, and/or kidneys; and sinusoidal leukocytosis in the liver.

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Tissue bacterial assessments:

Histology: No bacteria were observed in any of the tissues from animals that survived whereas bacteria were observed in many of the tissues from non-surviving animals (Table 138).

Culture: All of the animals that died on study were culture positive in at least three of the tissues tested; exception was one animal (#C51400 - Group 3, treated at 36 hours post-challenge) that only had a positive brain culture. Of the animals that survived until Day 28, 17% (4/24) had positive culture findings in brain specimens, which included three animals (C48999, C50097, C50256) that were treated at 24 hours post-challenge and one animal (C50405) treated at 36 hours post-challenge; liver from the animal C50405 was also culture positive. In addition, lymph nodes were culture positive in 33% (8/24) of survivors; brain and spleen tissues were culture positive from one of the survivors (#C50256) treated at 24 hours post-challenge. The applicant states that a majority of the survivors had low bacterial burden in the tissues (Table 138).

Tissue	Placebo		ETI-204 16mg/kg 24hrs PC		ETI-204 16mg/kg 36hrs PC		ETI-204 16mg/kg 48hrs PC	
	Survivors (N=1)	Non Survivors (N=9)	Survivors (N=13)	Non Survivors (N=1)	Survivors (N=6)	Non Survivors (N=8)	Survivors (N=4)	Non Survivors (N=10)
Presence of bacteria by microscopy [1, 3]								
Bronchial	0/1	0/9	0/13	0/1	0/6	0/8	0/4	0/10
Kidney	0/1	6/9	0/13	1/1	0/6	1/8	0/4	7/10
Liver	0/1	5/9	0/13	0/1	0/6	2/8	0/4	5/10
Lung	0/1	6/9	0/13	0/1	0/6	1/8	0/4	5/10
Lymph Node	0/1	0/9	0/13	0/1	0/6	0/8	0/4	0/10
Lymph Node, Bronchial	0/1	5/9	0/13	0/1	0/6	3/8	0/4	6/10
Lymph Node, Mediastinal	0/1	6/9	0/13	0/1	0/6	3/8	0/4	6/10
Mediastinal	0/1	0/9	0/13	0/1	0/6	0/8	0/4	0/10
Spleen	0/1	6/9	0/13	0/1	0/6	2/8	0/4	5/10
Presence of bacteria by culture [1, 2]								
Brain	0/1	9/9	3/13	1/1	1/6	8/8	0/4	10/10
Bronchial Lymph Node	0/1	9/9	5/13	0/1	2/6	7/8	1/4	9/10
Liver	0/1	9/9	0/13	1/1	1/6	7/8	0/4	9/10
Spleen	0/1	9/9	1/13	1/1	0/6	7/8	0/4	10/10

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Animal was considered positive if at least 1-5 colonies were present on plate

[3] Histopathology performed at ^{(b) (4)} Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for both PA (ELISA) and bacteremia (quantitative) at any point prior to treatment:

C48036, C48378, C48999, C49314, C50097, C50235, C50256, C50738, C50740, C50743, C50814, C51332, C51350, C51352, C51354, C51371, C51396, C51397, C52438, C52442

Animals that were negative for bacteremia (quantitative) only at any point prior to treatment: None

Animals that were negative for PA (ELISA) only at any point prior to treatment: C47505, C48802, C50099, C50823, C51317, C51335, C51353, C51382, C51386, C51472, C51799

Comments:

The study showed that as the time of treatment with ETI-204 (16 mg/kg, IM) is delayed, the survival probability decreases; the survival rates were 93%, 43%, and 29% in animals treated with ETI-204 at 24 hours, 36 hours, or 48 hours post-challenge, respectively. The survival rate decreased in animals with higher bacteremia or PA levels prior to treatment. One control animal (10%) survived until Day 28.

All the animals that died or were moribund, had microscopic acute lesions consistent with *B. anthracis* infection and their deaths were attributed to anthrax infection. All animals that survived to scheduled termination had lesions consistent with a previous bacterial infection (anthrax) as evidenced by chronic inflammatory changes; it should be noted that, unlike other studies summarized above, some of the animals that survived to their scheduled sacrifice had positive bacterial culture results in the brain and other tissues.

6.4.2.3. Study AP107

This was a randomized, open label, placebo-controlled GLP study to evaluate the efficacy of ETI-204 (Lonza product) when administrated IV or IM at 24 hours post-exposure with *B. anthracis* spores (spore lot no. B39) by inhalation in 40 naïve cynomolgus monkeys at [REDACTED] (b) (4)⁶⁹ Additionally, the results were evaluated to determine if abnormalities documented in the clinical and physiological parameters used to determine illness returned to normal following treatment with the monoclonal antibody.

Study design:

The study design was similar to that summarized above for Study AP301 except that ETI-204 IM was administered at a dose of 4 or 8 mg/kg and ETI-204 IV was administered at a dose of 2 and 8 mg/kg (Table 139). All treatments were administered at 24 hours post-exposure. Additionally, only qualitative cultures were performed to measure bacteremia at different time intervals by plating 40 µL of whole blood.

⁶⁹ [REDACTED] (b) (4) Study Number 766-G924201: AP107-Post-exposure prophylaxis dose ranging study in cynomolgus macaques exposed to *Bacillus anthracis* spores followed by treatment intravenously or intramuscularly with ETI-204 (January 19, 2009).

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Table 139: AP107 - Study design and schedule of blood collection

Study design:

Group	Number of Animals	ETI-204 Dose	Route
1	6	Control (0.5 mL/kg)	IV
2	9	8.0 mg/kg	IV
3	9	2.0 mg/kg	IV
4	9	8.0 mg/kg	IM
5	8	4.0 mg/kg	IM

The blood collection and assay schedule:

Approximate Collection Time Point	Blood Tube type/ Approximate Blood volume	Bacteremia (Culture)	Serum ETI-204 Concentration (serum shipped to Elusys)	CBC
Study day -5	EDTA ~1.5 mL + SST ~2.0 mL	X	X	X
24 hours PC (prior to treatment)	EDTA ~1.5 mL	X		X
32 hours PC	EDTA ~1.5 mL	X		X
40 hours PC	EDTA ~1.5 mL	X		X
48 hours PC	EDTA ~1.5 mL + SST ~2.0 mL	X	X	X
14 days PC	EDTA ~1.5 mL	X		X
Terminal *	EDTA ~0.5 mL + SST ~2.0 mL	X	X	

One animal (A04766) was administered ETI-204 2 mg/kg subcutaneously rather than intravenously; decision was made to administer an additional 6.0 mg/kg IV and move this animal to Group 2. This animal was excluded from analysis.

All animals were culture negative prior to challenge. The age of the animals ranged from 2 to 5 years and mean body weight 2.5 kg (range 2.4-3.0 kg) at the time of challenge. Animals were randomized to five groups and were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The average MMAD for the three challenge days ranged from 1.21-1.22 μ m.

Animals were followed for clinical signs for up to 30 days post-challenge. At the time of necropsy, tissue sections of spleen, brain, liver, bronchial lymph node and other organs with gross lesions from few animals were processed for histology as for the studies summarized above.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the animals in the different groups (Table 140). The average \pm SD aerosol exposure dose for all animals on study was 315 \pm 78 *B. anthracis* LD₅₀ equivalent; the LD₅₀ was \geq 200 in approximately 98% of the animals. At 24 hours post-exposure, 7 (17%; 1 to 2 animal per group) animals were bacteremic (Table 140). The baseline characteristics (disease stage) of animals were similar among animals in all groups.

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Table 140: Study AP107 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Placebo IV (Group 1) N=6	ETI-204			
		2 mg/kg IV (Group 3) N=9	8 mg/kg IV (Group 2) N=8	8 mg/kg IM (Group 4) N=9	4 mg/kg IM (Group 5) N=8
Baseline characteristics					
Age (years) Range	2-5	2-5	2-5	2-5	2-5
Body weight (kg) Mean ±SD	2.4±0.2	2.4±0.2	2.6±0.4	2.5±0.3	2.4±0.2
Inhaled dose					
cfu x 10 ⁷	(b) (4)				
Mean ± SD (Range)					
LD ₅₀ Mean ± SD (Range)	324.2±70.6 (254-458)	315.6±83.4 (213-451)	293.8±49.9 (225-370)	289.0±51.8 (222-351)	366.0±113.6 (198-551)
<200 LD ₅₀ n(%)	0	0	0	0	1 (12.5)
≥200 LD ₅₀ n(%)	6 (100)	9 (100)	8 (100)	9 (100)	7 (87.5)
Bacteremia prior to treatment (24 hours)					
n (%)	1 (16.7)**	2 (22.2)	1 (12.5)	2 (22.2)	1 (12.5)
Post treatment: Time (hours) to bacteremia					
n	6	9	7	7	7
Mean±SD (Range)	33.3±7.9 (23.9-48.1)	30.2±3.5 (24.0-32.1)	32.0±4.6 (24.1-40.1)	29.7±3.9 (24-32)	33.2±5.6 (24.1-40.2)
Post-treatment: Survival and bacteremia at the end of study (Day 30)					
Survivors	1 (16.7)	4 (44.4)	6 (75.0)*	5 (55.6)	6 (75.0)*
Bacteremia (number of animals positive) by number of animals tested					
32 hours	5/6	8/9	6/8	7/9	5/8
40 hours	5/6	7/9	6/8	7/9	7/8
48 hours	6/6	4/9	5/8	6/9	5/8
Day 14	0/1	0/4	0/6	0/5	0/6
Terminal	4/5	5/5	2/2	4/4	2/2

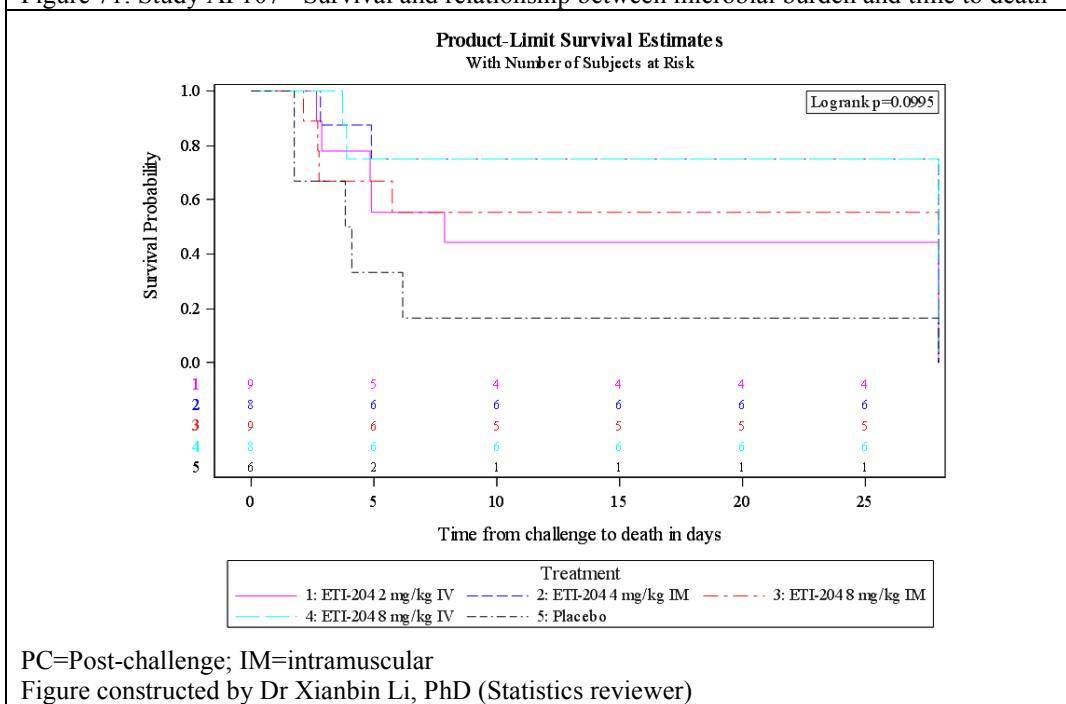
** One of the control group animals (#A02398) that survived the inhaled inoculum dose (LD₅₀) was [REDACTED] fu.
One animal randomized to 8 mg/kg IV group but receiving 6 mg/kg IV and 2 mg/kg subcutaneously. It was included in the 8 mg/kg IV group for analysis.

SD Standard deviation;

*Statistically significant between the ET-204 treated group and the control group.

Effect of treatment on survival: The results show that ETI-204 at a dose of 8 mg/kg when administered IV or 4 mg/kg administered IM at 24 hours post-exposure were most effective in improving survival (Table 140 and Figure 71). All of the non-surviving animals died within 10 days of exposure and were bacteremic. All animals surviving to the end of the study were culture negative (i.e., < LOD) by 30 days post-exposure (Table 140).

Figure 71: Study AP107 - Survival and relationship between microbial burden and time to death



Clinical Observations: The majority of placebo treated animals exhibited abnormal clinical signs consistent with anthrax following challenge; these signs were consistent with those observed in the other studies summarized above. In ETI-204 treated animals that survived to the end of the study, most of these abnormal observations were not observed (other than sporadic stool abnormalities which is common with laboratory housed NHPs) after Day 22 post-challenge.

In all animals that survived to the end of the study, body weights on Day 28 were greater than or equal to Day 0 weights.

Hematological parameters: WBC count increased in some of the animals at 32, 40 and 48 hours and Day 14 post-challenge compared to baseline (prior to challenge) suggesting that animals were exhibiting signs of infection following exposure to *B. anthracis*. Neutrophil and lymphocyte counts also increased until 48 hours followed by a decrease to baseline. Monocyte counts were not altered.

Necropsy and Histopathology: Gross lesions in animals found dead or euthanized due to moribund condition post-challenge included pericardial and thoracic effusions; enlargement and dark discoloration of bronchial, inguinal, mediastinal and pancreatic lymph nodes (hemorrhage, edema and fibrin exudation); dark discoloration, foci, or accumulation in the brain (hemorrhage and necrosis); and enlargement and dark discoloration of the adrenal glands (hemorrhage and necrosis) (Table 141). These findings are consistent with those observed in other studies summarized above.

Microscopic findings considered consistent with anthrax were present in two anthrax spore-exposed animals (Table 141). Lesions typical of anthrax in this study included hemorrhage, fibrin exudation, edema, neutrophilic inflammation, lymphoid depletion, and the presence of

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large rod-shaped bacteria consistent with *B. anthracis* in many of the organs evaluated histologically.

Table 141: Study AP107 - Incidence summary of gross necropsy findings and microscopic observations (in select animals) by treatment group in animals found dead or euthanized in moribund condition.

Gross observations										Microscopic observations in select animals					
Group	1		2		3		4		5		(b) (4)	A05193	A04051	A03226	
Number in group	6		9		9		9		8*		Histology #	0803463	0803482	0804464	
Mortality (%)	83%		33%		56%		44%		25%		Group	1	1	N/A	
Sex	M	F	M	F	M	F	M	F	M	F					
ORGAN/lesion	# dead	2	3	2	1	2	3	3	1	2	0				
ADRENAL GLAND		1													
Enlarged															
BRAIN															
Accumulation/discoloration/foci		1		1	1	1	2	3	1	1					
CAVITY, ABDOMINAL								1							
Fluid															
CAVITY, PERICARDIAL								1	1	1					
Fluid															
CAVITY, THORACIC					1										
Fluid															
LIVER								1							
Foci															
LYMPH NODE, BRONCHIAL		1	2	2		1	1			2					
Enlarged															
LYMPH NODE, INGUINAL				1											
Enlarged															
LYMPH NODE, MEDIASTINAL		2	2	1	1					1					
Enlarged/discoloration															
LYMPH NODE, PANCREATIC										1					
Enlarged															
THYROID			1												
Enlarged															
Cells left blank have an incidence of 0.															
*Group size was decreased from 9 to 8 animals after one animal died during quarantine, prior to placement on study.															

Microscopic observations in select animals

(b) (4)	A05193	A04051	A03226	
ORGAN/Lesion	Group	1	1	N/A
BRAIN				
Bacteria		2	1	0
Hemorrhage		2	0	0
Meningitis		3	0	0
HEART				
Neutrophilic inflammation, coronary fat		-	-	1
Myocardial fiber degeneration/mineralization		-	-	1
KIDNEY				
Bacteria		0	1	0
Tubular degeneration/necrosis		0	1	0
LIVER				
Bacteria		0	2	0
Hepatocellular atrophy		0	0	2
Sinusoidal leukocytosis		1	1	0
LUNG				
Bacteria		1‡	4	0
Edema		0	3	0
Fibrin exudation		1	3	0
Hemorrhage		0	1	0
Neutrophilic inflammation		0	1	0
Pleural fibrosis		0	0	1
LYMPH NODE, BRONCHIAL				
Hemorrhage		1	-	-
Fibrin exudation		1	-	-
Lymphocyte necrosis/depletion		2	-	-
LYMPH NODE, MEDIASTINAL				
Bacteria		0	2	0
Edema		0	0	0
Fibrin exudation		1	1	0
Hemorrhage		1	4	0
Lymphocyte necrosis/depletion		0	4	3
LYMPH NODE, MESENTERIC				
Lymphocyte necrosis/depletion		-	-	3
SPLEEN				
Bacteria		0	3	0
Fibrin exudation		1	4	0
Neutrophilic inflammation		0	1	0
Red pulp depletion		0	0	2
Lymphocyte necrosis/depletion		1	3	2
STERNUM (BONE MARROW)				
Hyperplasia, myeloid (granulocytic)		-	-	4†

0 = lesion not present

Dashed (-) finding = tissue not examined in this animal.

*This animal died during quarantine, prior to placement on study. Other organs examined (jejunum, ileum, cecum, colon, muscle and sciatic nerve) had no significant microscopic findings.

‡Multiple types of bacteria present in alveoli (agonal aspiration).

†Marrow is 70-80% cellular with all cell lines represented; M:E ratio estimated 15:1 - 20:1.

Tissue bacterial assessments:

Histology: Bacteria were observed in tissues from 2 non-surviving animals from the control group; tissues from animals treated with ETI-204 were not examined (Table 142).

Culture: Not done

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Table 142: Study AP107-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 2mg/kg IV		ETI-204 8mg/kg IV		ETI-204 4mg/kg IM		ETI-204 8mg/kg IM	
	Non		Non		Non		Non		Non	
	Survivor (N=1)	Non (N=5)	Survivor (N=4)	Non (N=5)	Survivor (N=6)	Non (N=2)	Survivor (N=6)	Non (N=2)	Survivor (N=5)	Non (N=4)
Presence of bacteria by microscopy [1, 2]										
Brain	0/0	2/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Kidney	0/0	1/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Liver	0/0	1/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Lung	0/0	2/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Lymph Node, Bronchial	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Lymph Node, Mediastinal	0/0	1/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Spleen	0/0	1/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Presence of bacteria by culture [1]										
No culture assessment	ND	ND								

ND = Not Done

[1] All treated animals irrespective of bacteremia status prior to treatment

[2] Histopathology performed at ^{(b) (4)} Not all animals were assessed microscopically; numbers examined are shown

Animals that were negative for bacteremia (qualitative) only at any point prior to treatment: A02398, A03207, A03463, A03511, A03855, A03899, A03918, A04021, A04049, A04060, A04067, A04149, A04150, A04174, A04319, A04341, A04741, A04803, A04929, A05191, A05193, A05206, A05299, A05315, A05337, A05365, A05367, C22731, C22817, C22819, C22820, C22826, C22852

PA (ECL and ELISA) were not performed

Comments:

The study showed increased survival rates in all ETI-204 treated animals compared to control animals. Administration of ETI-204 at a dose of 8 mg/kg IV or 4 mg/kg IM at 24 hours after aerosol exposure to *B. anthracis* improved survival in 75% of the animals at Day 30. About 17% of the animals were bacteremic at the time of treatment and 80% of the animals were bacteremic at some time post-challenge. All the animals that survived were culture negative by Day 30 post-challenge; all the animals that died were bacteremic.

Significant increases in white blood cell counts and neutrophils were observed post-challenge. Lymphocyte count decreased by Day 14 post-challenge. Most of the hematologic abnormalities in surviving monkeys returned to normal levels.

Overall, the results suggest that ETI-204 is effective in protecting against death due to anthrax when administered IV or IM 24 hours post-challenge.

6.5. Efficacy of ETI-204 - Pre-exposure prophylaxis studies

The pre-exposure prophylaxis studies were conducted in NZW rabbits and cynomolgus monkeys to evaluate the effectiveness of ^{(b) (4)} (^{(b) (4)} anti-PA MoAb) or the Lonza product of ETI-204. All studies were conducted at ^{(b) (4)}

6.5.1. New Zealand White rabbits

The efficacy of ^{(b) (4)} MoAb or Lonza product of ETI-204 was measured in two studies (AR001 and AR003) in NZW rabbits.

6.5.1.1. Study AR001

This was a randomized, open label, placebo-controlled non-GLP study to evaluate the efficacy of 10 mg of ^{(b) (4)} product (^{(b) (4)} anti-PA MoAb) administered IV, 30-45 minutes prior to

exposure to the spores (spore lot no. not specified) *B. anthracis* by inhalation in 14 healthy NZW rabbits at [REDACTED]^{(b)(4)}70

Study design:

Animals purchased from [REDACTED] were quarantined for 7 days at [REDACTED]. The study design was similar to that for NZW rabbit studies summarized above except that the animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 100X LD₅₀ ([REDACTED] spores) which is lower than that for the studies summarized above. The average MMAD for the three challenge days ranged from 1-2 µm. [REDACTED] (b)(4) anti-PA MoAb (10 mg) was administered, IV, 30 to 45 minutes prior to exposure.

The animals were 13 to 17 weeks old and weighed between 2.2 to 2.6 kg at the time of challenge and randomized to four groups (Table 143). Blood was collected at different time points for measuring bacteremia (qualitative) by culture; an aliquot of the blood sample collected on study Days 1, 2, 7, 10, 14, 21 and 28 was cultured on TSA plates using an inoculating loop to determine the presence or absence of *B. anthracis*.

Table 143: AR001 - Schedule of blood collection and tissues for bacterial cultures

Sample Type	Study Day							
	-1	1	2	7	10	14	21	28
Serum	X	X	X	X	X	X	X	X
Bacteremia		X	X	X	X	X	X	X
Lung								X
Spleen								X
Intrathoracic Lymph Nodes								X

Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, spleen, and intra-thoracic lymph nodes from each surviving animal were processed for culture.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the animals in the two groups (Table 144). The average ± SD aerosol exposure dose for all animals on study was 162±47 LD₅₀ equivalent (Table 144). The LD₅₀ was <200 in a majority (86%) of the animals. This is expected as the target challenge dose was 100X LD₅₀. The baseline characteristics (disease stage) of animals were similar among the animals in the two groups (Table 144).

⁷⁰ [REDACTED] (b)(4) Study Number 357-G004819: AR001 – Assessment of the effectiveness of a monoclonal anti-PA antibody candidate as therapeutic protection against a bacillus anthracis aerosol challenge in the rabbit model (January 19, 2004).

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Table 144: Study AR001 - Baseline characteristics and survival as well as microbiological findings

Parameters	Placebo (Group 2) N=5	ETI-204 (Group 1) 10.16 mg [‡] ~35 min Pre-exposure N=9
Baseline characteristics		
Age (weeks) Range	13-17	13-17
Body weight (kg) Mean ± SD	2.4±0.2	2.3±0.1
Inhaled Dose		
cfu x 10 ⁷ Mean ± SD (Range)	Not in the data sets	
LD ₅₀ Mean ± SD (Range)	171.9±55.2 (96.4-244.0)	156.0±43.9 (106.1-217.5)
<200 LD ₅₀ dose n(%)	4 (80.0)	8 (88.9)
≥200 LD ₅₀ dose n(%)	1 (20.0)	1 (11.1)
Bacteremia at the time of treatment n (%)	0	0
Time to bacteremia (hours)		
N	5	NA
Mean±SD (Range)	72±33.9 (48-120)	
Survivors at the end of study (Day 28)		
n (%)	0	9 (100)*
Bacteremia post-exposure		
24 hours	0/5	0/10
48 hours	2/4	0/10
Day 7	NA	0/10
Day 10, 21, 28	NA	0/10
Unscheduled terminal	4/5	NA

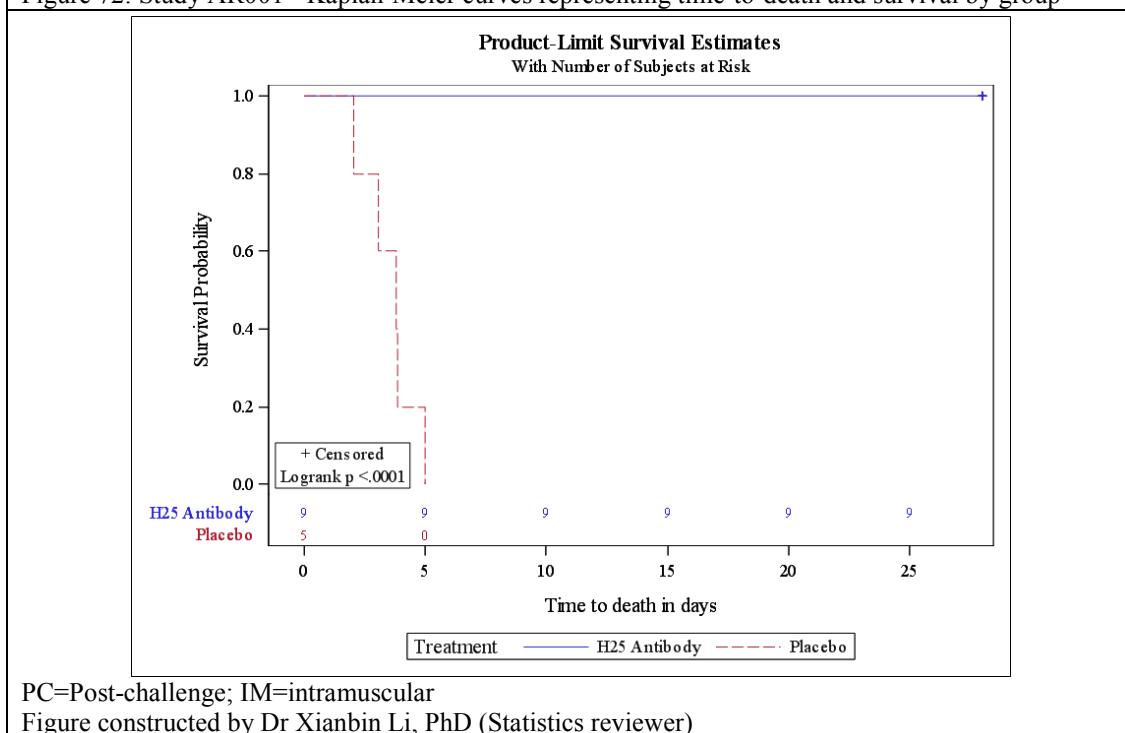
[‡]One animal (K29312) in the treatment group received 3.2 mL of antibody (concentration 8.13 mg). 10 mg is approximately 4 mg/kg

SD Standard deviation; GM geometric mean; NA=not applicable.

*Statistically significant between the ETI-204 treated group and the control group.

Effect of treatment on survival and bacteremia: The results show that ^{(b) (4)} MoAb at a dose of 10 mg (one animal was administered 8 mg) administered 30 to 45 minutes prior to exposure was effective in improving survival in all the animals (Table 144 and Figure 72); all animals were culture negative at all the time points tested. All of the control group animals died within 5 days of challenge and all the rabbits were bacteremic at least at one of the time point tested (Table 144).

Figure 72: Study AR001 - Kaplan-Meier curves representing time-to-death and survival by group



Clinical Observations: Although the protocol specified monitoring of animals as for the studies summarized above, no details were included.

Necropsy and Histopathology: Not done

Tissue bacterial assessments:

Histology: Not done.

Culture: Presence of bacteria was reported by culture of some of the tissues from 3 of the surviving animals (lymph nodes - one animal; lung - two animals) treated with ^{(b) (4)} anti-PA MoAB (Table 145). Tissues from non-surviving animals were not processed culture.

Table 145: Study AR001-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 ~4mg/kg	
	Survivors (N=0)	Non Survivors (N=5)	Survivors (N=9)	Non Survivors (N=0)
Presence of bacteria by microscopy [1]				
No microscopic bacterial assessment	ND	ND	ND	ND
Presence of bacteria by culture [1]				
Lymph Node	0/0	ND	1/9	ND
Lung	0/0	ND	2/9	ND
Spleen	0/0	ND	0/9	ND

ND=Not Done
[1] All treated animals

Comments:

^{(b) (4)} anti-PA MoAb at a dose of 10 mg (approximately 4 mg/kg) administered IV to NZW rabbits 30 to 45 minutes prior to anthrax spore challenge was effective in improving survival in all the 9

animals until Day 28 post-challenge. All of the control group animals died by Day 5 post-challenge. No bacteremia was observed in any of the treated animals. At necropsy, lymph node or lung from 3 animals showed evidence of bacteria by culture. Tissues from non-surviving animals were not processed for culture.

6.5.1.2. Study AR003

This was a randomized, open label, placebo-controlled non-GLP study to evaluate the efficacy of different doses of ETI-204 ((b) (4) anti-PA MoAb), administrated IV or IM, approximately 30 minutes prior to exposure to *B. anthracis* by inhalation in 48 healthy NZW rabbits at (b) (4)
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Study design:

The animals were 13 to 17 weeks old, weighed between 2.2 to 2.6 kg at the time of challenge, and randomized to six groups. The study design was similar to that for the Study AR001 except that the animals were exposed to the spores (spore lot no. not specified) of the Ames strain of *B. anthracis* by aerosolization with a targeted 200 LD₅₀ ((b) (4) spores). The average MMAD for the three challenge days ranged from 1-2 µm.

Different doses of (b) (4) anti-PA MoAb were administered, intravenously, 30 to 45 minutes prior to exposure. Blood was collected at different time points for measuring qualitative bacteremia; an aliquot of the blood sample collected on study Days 1, 2, 7, 10, 14, 21 and 28 was cultured on TSA plates using an inoculating loop to determine the presence or absence of *B. anthracis* (Table 146).

Table 146: AR003 - Schedule of blood collection and tissues collected for histology

Sample/Test Type	Study Day							
	-5	1	2	7	10	14	21	28
Serum	X	X	X	X	X	X	X	X
Bacteremia		X	X	X	X	X	X	X
Lung								X
Spleen								X
Intrathoracic Lymph Nodes								X

Animals were followed for clinical observations for up to 28 days post-challenge. At the time of necropsy, tissue sections of lung, spleen, and intra-thoracic lymph nodes from each surviving animal were processed for culture as for the studies summarized above.

Results:

Baseline characteristics: Age, gender, body weight, and spore challenge dose were comparable among the six groups (Table 147). The average ± SD aerosol exposure dose for all animals on study was 287±81 LD₅₀ equivalent; the LD₅₀ was ≥200 in 81% of the animals (Table 147). The

71 (b) (4) Study Number 397-G004957: AR003 – Minimum effective dose of the (b) (4) monoclonal anti-PA antibody when administered immediately prior to challenge against aerosolized anthrax in the NZW rabbit model (January 25, 2005).

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baseline characteristics (disease stage) of animals were similar among the animals in all groups (Table 147).

Table 147: Study AR003 - Baseline characteristics and survival as well as microbiological findings

Parameters	Placebo IV (Group 5) N=8	ETI-204 ^{(b) (4)}				
		1.25 mg IV (Group 4) N=8	2.5 mg IV (Group 3) N=8	5.0 mg [*] IV (Group 2) N=8	10.0 mg IV (Group 1) N=8	20.0 mg IM (Group 6) N=8
Baseline characteristics						
Age (weeks) Range	13-17	13-17	13-17	13-17	13-17	13-17
Body weight (kg) Mean ± SD	2.5±0.0	2.4±0.2	2.4±0.1	2.4±0.1	2.5±0.1	2.5±0.1
Inhaled dose						
cfu x 10 ⁷	Information not included in the data sets					
LD ₅₀ Mean ± SD (Range)	301.0±117.8 (163.2-434.6)	282.1±84.8 (91.8-358.8)	296.6±53.1 (228.1-401.5)	303.8±78.0 (180.3- 413.7)	269.8±99.6 (106.2-404.6)	268.1±56.6 (187.1-352.5)
<200 LD ₅₀ dose n(%)	3 (37.5)	1 (12.5)	0	1 (12.5)	2 (25.0)	2 (25.0)
≥200 LD ₅₀ dose n (%)	5 (62.5)	7 (87.5)	8 (100)	7 (87.5)	6 (75.0)	6 (75.0)
Time (hours) of bacteremia						
n Mean±SD (Range)	7 65.1±18.1 (48-96)	6 128±29.1 (96-168)	1 (192)	1 (168)	NA [§]	NA [§]
Survivors at the end of study (Day 28)						
n (%)	0	1 (12.5)	5 (62.5)*	5 (62.5)*	7 (87.5)*	8 (100)*
Proportion of animals bacteremic (%) post-exposure						
24 hours	2 (25.0)	0	1 (12.5)	1 (12.5)	0	0
48 hours	8/8	0/8	1/8	1/8	0/8	1/8
Day 7	NA	1/2	1/6	1/7	0/7	0/8
Day 10, 21, 28	NA	0/1	0/5	**	0/7	0/8
Unscheduled terminal	7/8	6/7	1/3	1/3	0/1	NA

[†]One animal (K49292) in the 5 mg treatment group was dosed with the antibody lot previously provided by the Applicant for study number 380-0004907.

[§]Not applicable as none of the animals became bacteremic

**0/6 at Day 10; 1/5 at Day 21 and 0/5 at Day 28

SD Standard deviation; GM geometric mean; NA=not applicable

*Statistically significant between the ETI-204 treated group and the control group.

Note: 1.25 mg= ~0.5 mg/kg; 2.5 mg= ~1 mg/kg; 5.0 mg= ~2 mg/kg; 10.0 mg= ~4 mg/kg; 20.0 mg= ~8 mg/kg

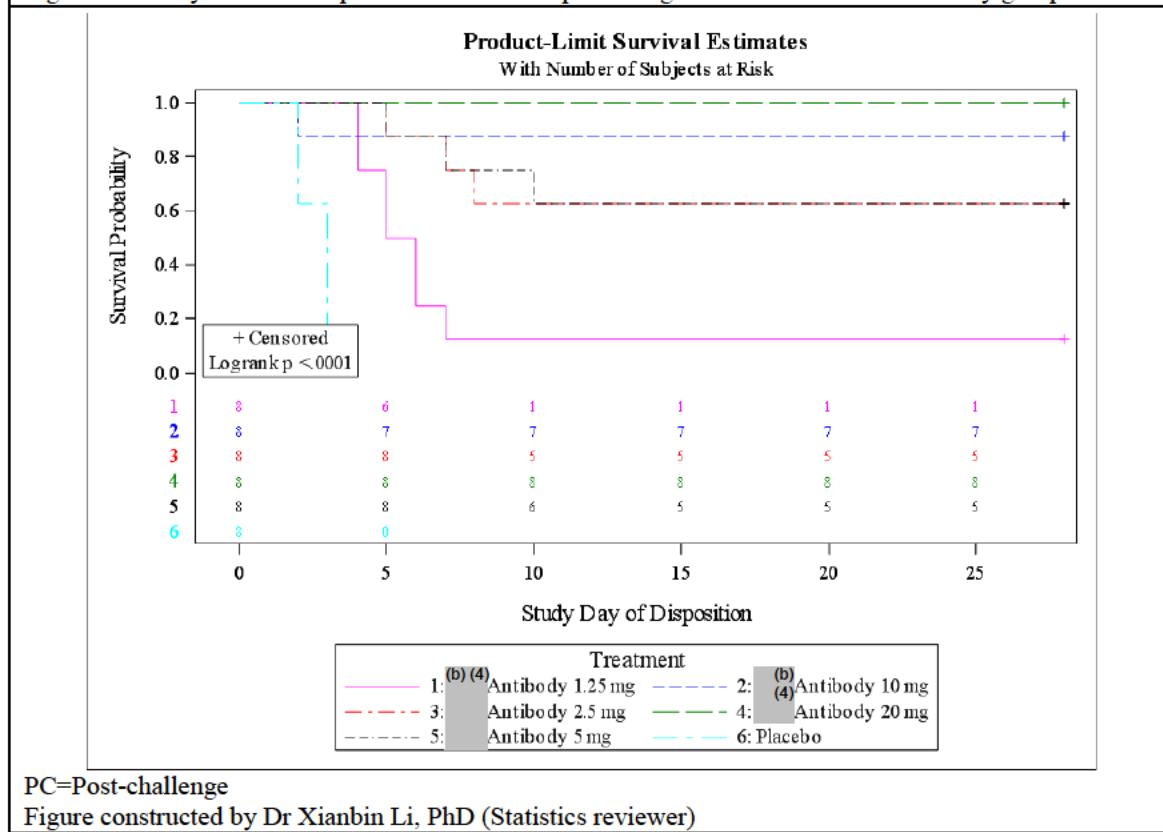
Effect of treatment on survival: The results show that ^{(b) (4)} Anti-PA MoAb at a dose of 10 mg, administered IV, approximately 30 to 45 minutes pre-exposure, improved survival in 88% of the animals; all the animals survived when 20 mg of ^{(b) (4)} MoAb was administered IM. Survival rate at the 2.5 and 5 mg dose was 63% and at 1.25 mg dose 13% (Table 147 and Figure 73). All the control group animals died within 4 days of challenge.

Effect on bacteremia: A majority of the surviving ^{(b) (4)} anti-PA MoAb-treated animals were blood culture negative at some of the time point analyzed during the course of the study (Table

147). Only 2 rabbits that survived were culture positive within 48 hours. Of the 14 treated rabbits that died, 8 were culture positive at some of the time points.

All the control group animals were bacteremic by 48 hours post-challenge (Table 147).

Figure 73: Study AR003 - Kaplan-Meier curves representing time-to-death and survival by group



Clinical Observations: No details included

Necropsy and Histopathology: Not done

Tissue bacterial assessments:

Histology: Not done

Cultures: Of all of the tissues processed for bacterial cultures from surviving animals (n=26), presence of bacteria was reported in lung from nine animals; spleen and lymph nodes were culture negative (Table 148). Tissues from non-surviving animals were not processed.

Table 148: Study AR003-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 ~0.5mg/kg		ETI-204 ~1.0mg/kg		ETI-204 ~2.0mg/kg		ETI-204 ~4.0mg/kg		ETI-204 ~8.0mg/kg	
	Non		Non		Non		Non		Non		Non	
	Survivors (N=0)	Survivors (N=8)	Survivors (N=1)	Survivors (N=7)	Survivors (N=5)	Survivors (N=3)	Survivors (N=5)	Survivors (N=3)	Survivors (N=7)	Survivors (N=1)	Survivors (N=8)	Survivors (N=0)
Presence of bacteria by microscopy [1]												
No microscopic bacterial assessment	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Presence of bacteria by culture [1]												
Lymph Node	0/0	ND	0/1	ND	0/5	ND	0/5	ND	0/7	ND	0/8	ND
Lung	0/0	ND	0/1	ND	1/5	ND	0/5	ND	4/7	ND	4/8	ND
Spleen	0/0	ND	0/1	ND	0/5	ND	0/5	ND	0/7	ND	0/8	ND

ND=Not Done

[1] All treated animals

Comments:

The study showed a dose dependent improvement in survival of NZW rabbits when (b) (4) anti-PA MoAb was administered IV between doses of 2.5 mg to 10 mg approximately 35 minutes prior to challenge; IM dose of 20 mg was effective in improving survival of all the animals. All animals in the control group died by Day 4 post-challenge.

All the treated animals that survived were culture negative. However, lung from some (35%) of the treated animals were culture positive; no bacteria were observed in the cultures of spleen and lymph nodes. Tissues from the animals that died were not processed for culture.

6.5.2. Cynomolgus monkeys

6.5.2.1. Study AP305

This was a randomized, blinded, placebo-controlled GLP study conducted at (b) (4) to evaluate the pre-exposure prophylaxis of ETI-204 (Lonza product) when administrated IM at Day -3, Day -2, and Day -1 prior to exposure by inhalation in 53 cynomolgus monkeys at (b) (4)⁷². The secondary objectives included evaluation of the impact of time of IM ETI-204 administration on bacteremia levels.

Study design:

Animals purchased from (b) (4) were quarantined at (b) (4) as for the animals in the treatment studies summarized above; additionally, the health surveillance information collected was similar. Four of the animals (#C56534, C52493, C52507, C52528) were stated to be *Klebsiella* positive at the time of screening. The age of the animals ranged between 2.4 and 4.0 years and mean body weight was 2.6 kg (range 2.2-3.0 kg) at the time of challenge. Animals were randomized to 4 groups (Table 149). Animals were exposed to the spores of the Ames strain of *B. anthracis* by aerosolization with a targeted 200X LD₅₀ as for the studies summarized above. The spore lot no. used for the study was B39. The average MMAD for the three challenge days ranged from 1.21-1.22 µm.

⁷² (b) (4) Study Number 2778 -100018326: AP305 - Study to evaluate the prophylactic effect of a single intramuscular ETI-204 dose administered at various times prior to anthrax challenge in a cynomolgus macaque aerosol challenge model of *B. anthracis* (May 29, 2014).

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ETI-204 was administered, IM, at Day -3, Day -2, or Day -1 pre-exposure. Blood was collected at different time points for quantitating bacteremia using 100 µL of blood (Table 149). Although measurement of anti-PA IgG was planned it appears testing was not done as the results were not included.

Table 149: Study AP305 - Schedule of blood collection

Blood Collection Event	Day	Time-point	Collection Window	Blood Tube/Approx. Volume	Bacteremia (Quantitative)	Sera for Retention ^c	Serum for ETI-204 Levels	Serum for Anti-PA IgG ^b and anti-therapeutic IgG
1	-7	None	-	EDTA ~1.0 mL SST ~3.0 mL	X*	X	X	X
2	-3	6 hr post 1 st dose**	± 15 min	SST ~1.0 mL			X	
3	-2	Prior to 2 nd dose**	- 10 min	SST ~1.0 mL			X	
4	-2	6 hr post 2 nd dose**	± 15 min	SST ~1.0 mL			X	
5	-1	Prior to 3 rd dose**	- 60 min	SST ~1.0 mL			X	
6	-1	6 hr post 3 rd dose**	± 15 min	SST ~1.0 mL			X	
7	0	Prior to challenge	- 60 min	SST ~1.0 mL			X	
8	1	24 hr PC ^a	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X	X	
9	2	54 hr PC ^a	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X	X	
10	4	96 hr PC ^a	± 60 min	EDTA ~1.0 mL SST ~1.5 mL	X	X	X	
11	7	7 days PC	-	EDTA ~1.0 mL SST ~1.5 mL	X	X	X	
12	14	14 days PC	-	EDTA ~1.0 mL SST ~1.5 mL	X	X	X	
13	28	28 days PC	-	EDTA ~1.0 mL SST ~4.0 mL	X	X	X	X
14	56	56 days PC	-	EDTA ~1.0 mL SST ~4.0 mL	X	X	X	X
-	-	Unscheduled Death (Terminal)	-	EDTA ~1.0 mL SST ~1.0 mL	X	X		

PC = post-challenge
Bold line separates model establishing phase and dosing phase.
* Qualitative bacteremia only.
** 1st dose = Day -3 dose, 2nd dose = Day -2 dose, 3rd dose = Day -1 dose
^a: Based on mean challenge time.
^b: Samples that were collected for anti-PA IgG ELISA were shipped to a Sponsor-designated facility and not analyzed as a part of this study.
^c: Sera saved as retention for potential future use; The sera is stored at a Sponsor-designated facility and not analyzed as part of this study.

Animals were followed for clinical observations for up to 56 days post-challenge. At the time of necropsy, tissue sections of spleen, brain, liver, and bronchial lymph node were processed for histology and culture as for the studies summarized above.

Results:

Baseline characteristics: Age, gender, body weight, and challenge dose were comparable among the four groups (Table 150). The average ± SD aerosol exposure dose for all animals on study was 221±78 LD₅₀ equivalent; the LD₅₀ was ≥200 for 53% of the animals. The baseline characteristics (disease stage) of animals were similar among the animals in the four groups (Table 150).

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Table 150: Study AP305 - Baseline characteristics and post treatment survival as well as microbiological findings

Parameters	Placebo (Group 1) N=10	ETI-204 16 mg/kg		
		Day -3 (Group 4) N=15	Day -2 (Group 3) N=14	Day -1 (Group 2) N=14
Baseline characteristics				
Age (years) Range	2.36-3.96	2.36-3.96	2.36-3.96	2.36-3.96
Body weight (kg) Mean ± SD	2.7±0.2	2.5±0.2	2.5±0.2	2.5±0.2
Inhaled dose				
cfu x 10 ⁷ Mean ± SD (Range)	(b) (4)			
LD ₅₀ Mean ± SD (Range)	217.8±65.2 (144-330)	220.2±86.7 (126-490)	209.3±61.6 (103-315)	237.3±96.1 (138-440)
<200 LD ₅₀ n(%)	5 (50.0)	7 (46.7)	7 (50.0)	6 (42.9)
≥200 LD ₅₀ n(%)	5 (50.0)	8 (53.3)	7 (50.0)	8 (57.1)
Bacterial burden (cfu/mL) and time (hours) to bacteremia				
cfu/mL	10	15	14	14
Log ₁₀ Mean±SD (Range)	1.1±1.4 (0.3-3.5)	0.4±0.4 (0.3-1.7)	0.3±0.0 (0.3-0.3)	0.3±0.0 (0.3-3.5)
Geometric mean	13.7	2.5	2.0	2.0
Time to bacteremia n Mean±SD (Range)	10 44.3±14.5 (22.3-55.3)	2 39.7±22.3 (23.9-55.4)	3 54.3±0.8 (53.4-54.9)	1 95.6 (95.6)
Survivors at the end of study (Day 56)				
n (%)	1 (10.0) [‡]	15 (100.0)*	14 (100.0)*	14 (100.0)*
Bacteremia (quantitative) post-exposure				
24 hours	3 (30.0)	1 (6.7)	0/15	0/15
54 hours	8/8	1/15	3/15	0/15
96 hours	4/5	1/15	0/15	1/15
Day 7	1/2	0/15	0/15	0/15
Day 14, 28, 56	0/1	0/15	0/15	0/15
Unscheduled terminal	6/7	ND	ND	ND

[‡]One animal (C53558) that survived in the control group was culture negative at all the time points tested except 54 and 96 hours; also, tissues were culture negative.

Animal C52448 (control group). This animal was bacteremic at the 54 hour and Day 7 post-challenge time points and found dead on Day 14 post-challenge. While the terminal blood sample and all tissues were negative for *B. anthracis*, a contaminant was observed on the plates. A colony was isolated that was representative of the contamination from each of the brain, spleen, liver, and bronchial lymph node plates and these isolates were plated on TSA and blood agar. All isolates were described as white, shiny, round, and P-hemolysis on blood agar and white, shiny, and round on TSA agar. The isolates were then Gram stained and all were Gram positive cocci. While cultures were inconclusive regarding the reason for death, the pathology results attributed the death of this animal to anthrax

* Statistically significant between the ETI-204 treated group and the control group.

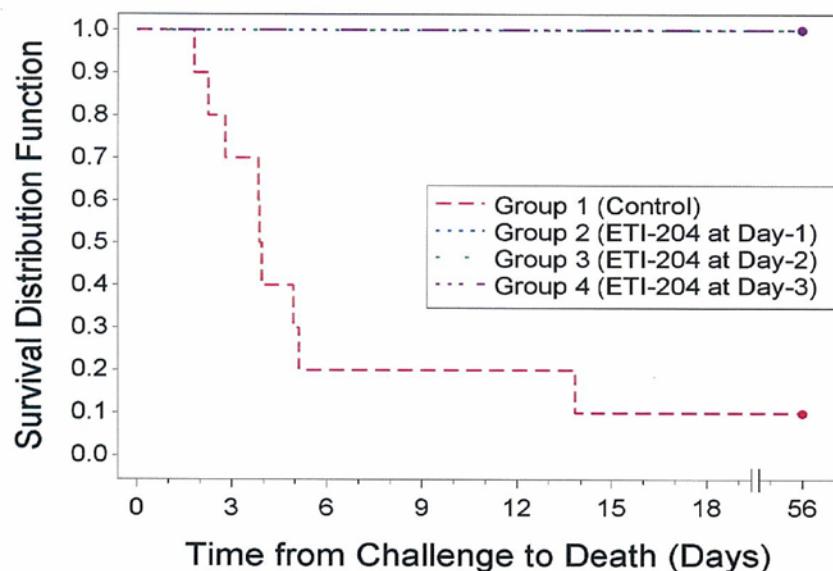
Effect of treatment on survival: The results show that ETI-204 at a dose of 16 mg/kg when administered either on Day 3, 2 or 1 prior to exposure was effective in improving survival in all the animals; of the 10 control group animals, one survived the period of observation (Table 150 and Figure 74A).

Effect of treatment on microbial burden: The bacterial load between 1 and 7 days post-treatment was lower in treated animals compared to the animals in the control group (Figures 74B and 75). In addition, the bacteremia in the ETI-204-treated animals was transient and only present in low

numbers (≤ 267 cfu/mL); complete resolution of bacteremia occurred by 7 days post-challenge in all monkeys that survived to scheduled termination.

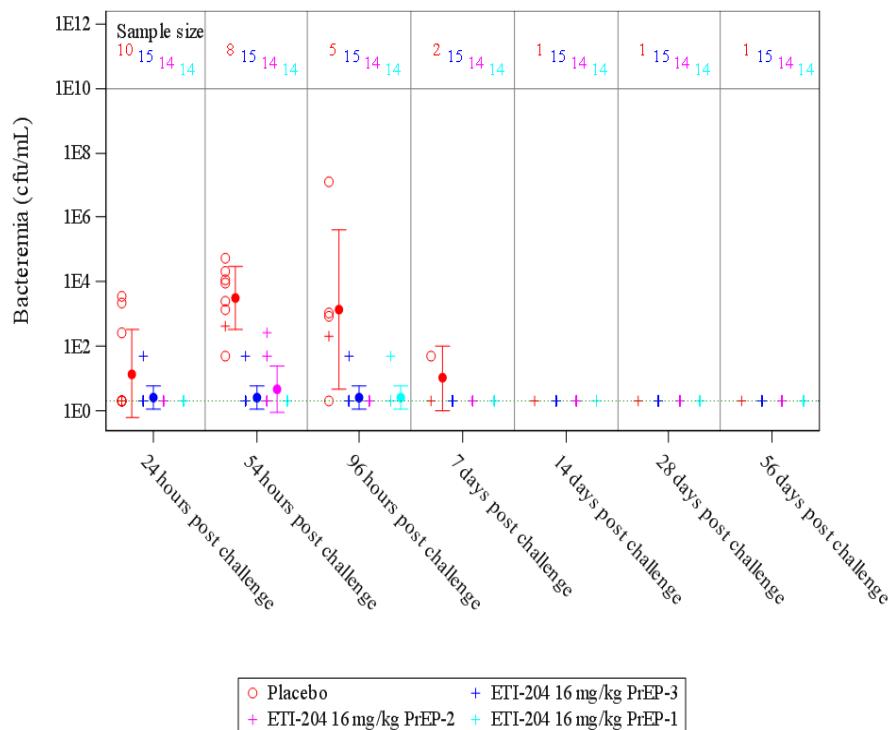
Figure 74: Study AP305 - Survival and relationship between microbial burden and time to death

A: Kaplan-Meier curves representing time-to-death and survival



Source: BLA submission

B: Bacteremia by survival status



+ = survivors; o = non-survivors

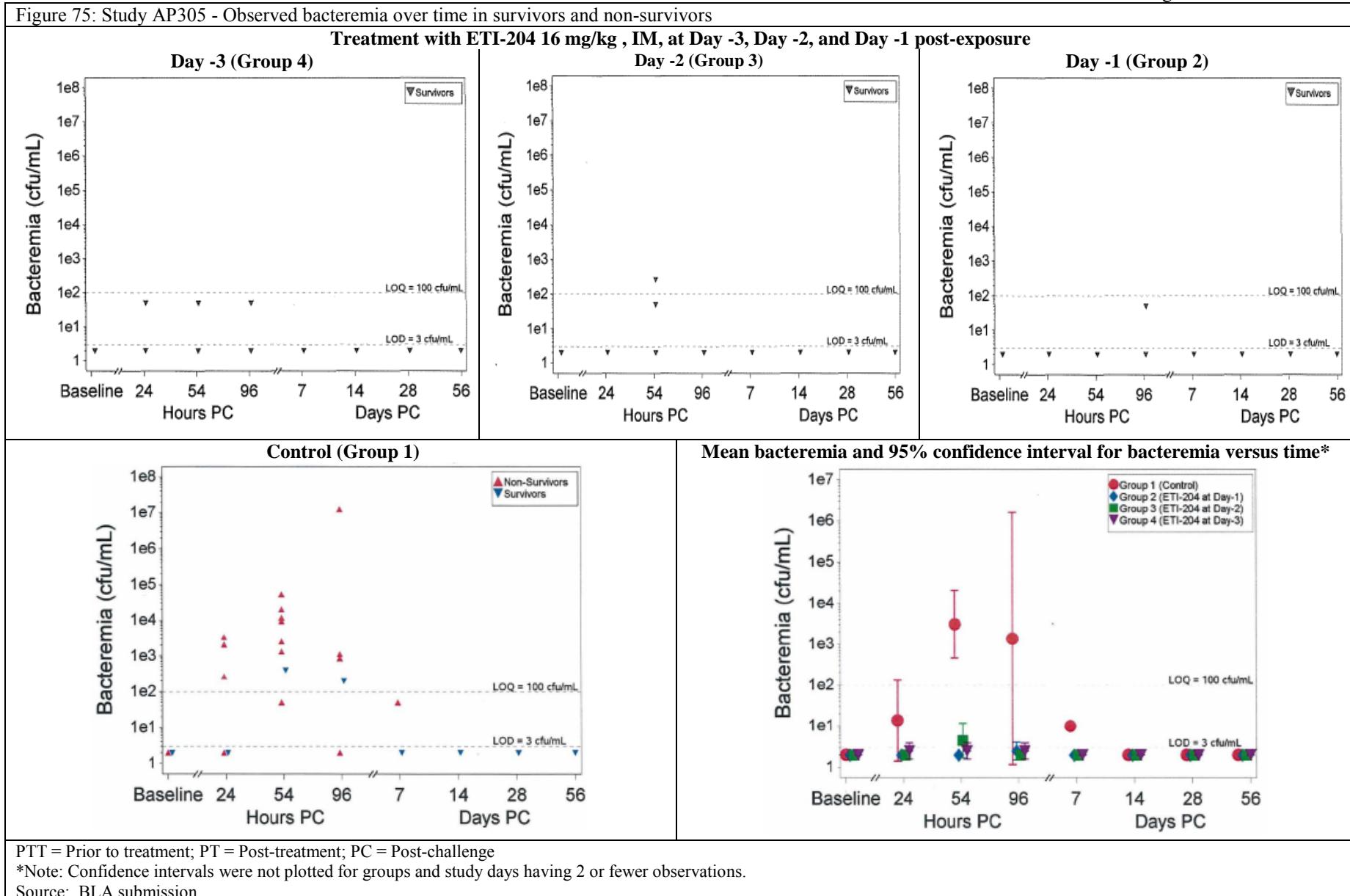
Figure constructed by Dr Xianbin Li, PhD (Statistics reviewer)

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Figure 75: Study AP305 - Observed bacteremia over time in survivors and non-survivors



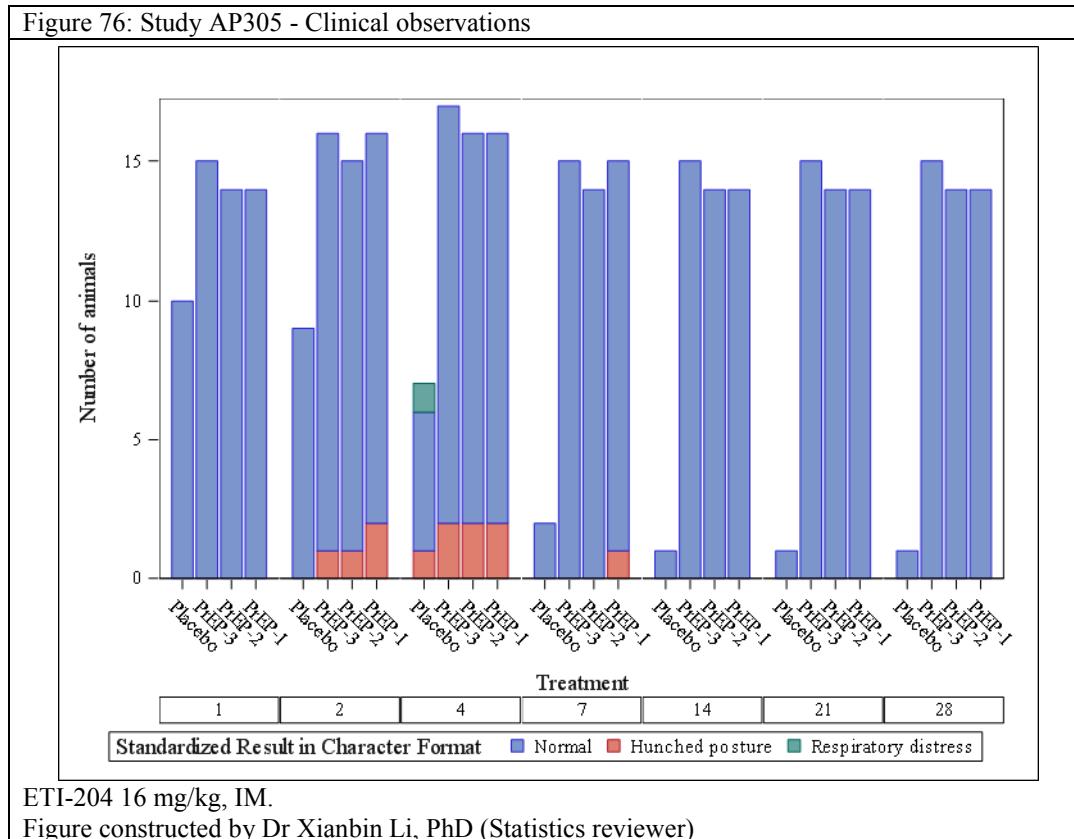
PTT = Prior to treatment; PT = Post-treatment; PC = Post-challenge

*Note: Confidence intervals were not plotted for groups and study days having 2 or fewer observations.

Source: BLA submission

Clinical Observations: Documentation of clinical observations, such as stool abnormalities, respiratory abnormalities, and hunched posture were noted. ETI-204 treated animals that survived to the end of the study, were reported as having hunched posture (8/43) or stool abnormalities within the first few days post-challenge but returned to normal within Day 7 post-challenge with only occasional diarrhea/ soft stool noted; these observations are common in laboratory housed monkeys. Overall, the animals in this pre-exposure study were less sick (Figure 76) compared to those in the treatment or post-exposure studies summarized above.

Figure 76: Study AP305 - Clinical observations



In all animals that survived to the end of the study, body weights on Day 56 were greater than or equal to Day 0 weights.

Necropsy and Histopathology: Gross lesions in animals, in the control group, found dead or euthanized due to moribund condition post-challenge included enlarged mesenteric lymph node, and thickening of the stomach as well as discoloration in the brain and liver; these observations are consistent with those summarized above for other studies.

Microscopic findings in the animals that died (control group) included acute lesions such as inflammation, hemorrhage, fibrin exudation, necrosis, hepatic sinusoidal leukocytosis, and/or the presence of large rod-shaped bacteria (consistent with *B. anthracis*) in multiple organs. The animals that survived (all treated animals) to scheduled termination, typically had lesions consistent with previous or ongoing inflammation that include hyperplasia (lymphoid follicles of the spleen, bronchial lymph nodes); hemosiderin pigment accumulation (bronchial), chronic inflammation (brain, lung, spleen, and/or kidneys); hematopoietic cell proliferation (liver),

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and/or sinusoidal leukocytosis (liver). These observations are similar to those summarized above for other studies.

Tissue bacterial assessments:

Histology: No bacteria were observed in any of the animals that survived; bacteria were observed in most of the tissues from all the placebo group animals that died (Table 151).

Cultures: No bacteria were reported in any of the tissues examined from animals either treated or untreated (animals C53558), that survived until Day 56 (Table 150). However, bacteria were observed in the majority of the tissues from the control animals that died (Table 151).

Table 151: Study AP305-Number of animals histologically and culture positive for *B. anthracis* in tissues

Tissue	Placebo		ETI-204 16mg/kg Day -3		ETI-204 16mg/kg Day -2		ETI-204 16mg/kg Day -1	
	Survivors (N=1)	Non Survivors (N=9)	Survivors (N=15)	Non Survivors (N=0)	Survivors (N=14)	Non Survivors (N=0)	Survivors (N=14)	Non Survivors (N=0)
Presence of bacteria by microscopy [1, 3]								
Brain	0/ 1	6/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Kidney	0/ 1	7/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Liver	0/ 1	7/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Lung	0/ 1	9/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Lymph Node, Bronchial	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Lymph Node, Mesenteric	0/ 1	0/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Site, Injection	0/ 1	4/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Skin	0/ 1	0/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Spleen	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Stomach	0/ 1	1/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Presence of bacteria by culture [1, 2]								
Brain	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Liver	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Lymph Node	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
Spleen	0/ 1	8/ 9	0/15	0/ 0	0/14	0/ 0	0/14	0/ 0
[1] All treated animals								
[2] Animal was considered positive if at least 1-5 colonies were present on plate								
[3] Histopathology was performed at ^{(b) (4)}								

Comments:

All animals treated with 16 mg/kg ETI-204, IM, between Day -3 and -1, prior to challenge, survived until Day 56. Only 14% of ETI-204-treated animals were bacteremic during the post-challenge period (1/14, 3/14, and 1/15 for ETI-204-treated animals on Day -1, Day -2, or Day -3, respectively); the positive cultures for the ETI-204-treated animals were transient and only present in low numbers. However, all vehicle control group animals were bacteremic for at least one time point post -challenge. No bacteria were observed in the tissues, by microscopy or culture, from ETI-204 treated animals as well as one control animal that survived. However, bacteria were observed in the tissues from all the control animals that died.

Clinical observations were consistent with *B. anthracis* infection within the first few days post-challenge; these abnormal observations resolved within the first week post-challenge. The animals in this pre-exposure study were less sick compared to those in the treatment or post-exposure studies summarized above. All animals surviving to scheduled termination had lesions consistent with anthrax infection as evidenced by chronic inflammatory changes. Among the control monkeys that died or were terminated in moribund condition, all had microscopic acute lesions consistent with *B. anthracis* infection.

7. Interpretive Criteria/Breakpoints

Not applicable.

8. The Labeling

8.1. Applicant's version of the microbiology section of the labeling

12.1 Mechanism of action

Obiltoxaximab is a monoclonal antibody that binds the PA of *B. anthracis*. [see (b) (4)
-Microbiology (12.4)]

12.4 Microbiology

Mechanism of Action

Obiltoxaximab is a monoclonal antibody that binds free PA with an affinity equilibrium dissociation constant (K_d) of 0.33 nM. Obiltoxaximab inhibits the binding of PA to its cellular receptors, preventing the intracellular entry of the anthrax lethal factor and edema factor, the enzymatic toxin components responsible for the pathogenic effects of anthrax toxin.

Activity In Vitro and In Vivo

Obiltoxaximab binds *in vitro* to PA from the Ames, Vollum, and Sterne strains of *B. anthracis*. Obiltoxaximab binds to an epitope on PA that is conserved across reported strains of *B. anthracis*.

In Vitro studies in a cell-based assay (b) (4)

combination of PA + lethal facto (b) (4)

(b) (4)

8.2. Comments

- The information stated in the 'Mechanism of action' section is appropriate. However, minor edits are recommended for clarity.
- Under the subheading 'Activity in vitro and in vivo' edits are recommended for accurate representation of data as well as clarity.

8.3. FDA's version of the labeling

12.1 Mechanism of action

Obiltoxaximab is a monoclonal antibody that binds the PA of *B. anthracis*. [see (b) (4)
Microbiology (12.4)]

12.4 Microbiology

Mechanism of Action

Obiltoxaximab is a monoclonal antibody that binds free PA with an affinity equilibrium dissociation constant (K_d) of 0.33 nM. Obiltoxaximab inhibits the binding of PA to its cellular receptors; such an effect appears to be concentration dependent. Binding of PA to the cell surface receptor prevents the intracellular entry of the anthrax lethal factor and edema factor, the enzymatic toxin components responsible for the pathogenic effects of anthrax toxin.

Activity In Vitro and In Vivo

Obiltoxaximab binds *in vitro* to PA from the Ames, Vollum, and Sterne strains of *B. anthracis*. Obiltoxaximab binds to an epitope on PA that is conserved across reported strains of *B. anthracis*.

In Vitro studies in a cell-based assay, using murine macrophages, suggest that obiltoxaximab neutralizes the toxic effects of (b) (4) lethal toxin. (b) (4)
(b) (4) a combination of PA + lethal facto (b) (4)

In vivo efficacy studies (b) (4) in NZW rabbits and cynomolgus (b) (4) challenged with the spores of *B. anthracis* by the inhalational route, showed a dose-dependent increase in survival (b) (4) following treatment with (b) (4). Exposure to *B. anthracis* spores resulted in increasing concentrations of (b) (4) PA in the serum of (b) (4) NZW rabbits and cynomolgus (b) (4). After (b) (4) treatment with obiltoxaximab there was a (b) (4) decrease in PA concentrations (b) (4)-in (b) (4) surviving (b) (4) animals. PA concentrations in placebo animals increased until they died. [see Clinical Studies (14.1)].

[See appended electronic signature page]

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Microbiologist, DAIP

CONCURRENCE:

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CC:

BLA# 125509

DAIP/PM/Jane Dean

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Appendix-1: Assays used for quantitative cultures

Study No.	SOP No.	LOD (cfu/mL)	<LOD as presented in the datasets	LLOQ (cfu/mL)	<LLOQ as presented in the datasets	Comments
Cynomolgus macaques						
AP202	(b) (4)	3	2	Not provided	Not provided	100 µL of different dilutions plated on TSA blood processed for culture. Value for LLOQ was not defined in study protocol and SAP. Section 4.2 of SAP specified that values less than the limit of detection (LOD) will be replaced with one-half the LOD and values less than the lower limit of quantification (LLOQ) will be replaced with one-half of the LLOQ for analysis.
AP203		3	2	100	50	The assay used was the same as for studies AP203 and AP204 and LLOQ should be considered as 100 cfu/mL. The results lower than LLOQ (reported as "+") were replaced with a 50 for statistical purposes in study report.
AP204						
AP201		33	17	1000	500	100 µL of different dilutions plated on TSA blood processed for culture.
AP307		3	2	100	50	
AP301		3	0	100	Not provided	Appendix K of study report (statistical report) defined treatment of values below limit of detection or quantitation as follows: The LOD for bacteremia was 3 cfu/mL and the LLOQ for bacteremia was 100 cfu/mL. Bacteremia levels less than the LOD, reported as "0" (negative for <i>B. anthracis</i>), or reported as "0,C" (negative for <i>B. anthracis</i> , contaminant) were replaced with one half of the LOD rounded to the nearest integer (i.e. 2 cfu/mL) for the statistical analysis, while values greater than the LOD but less than the LLOQ or reported as "+" (mean <i>B. anthracis</i> colony count or the majority of the colony counts for a given dilution set is less than 10) were replaced with one half of the LLOQ for the statistical analysis.
AP107						
AP305		3	2	100	50	
2469		3.33	1.67	250	50	Please note that units of limit of detection and quantitation are provided in Appendix S of study report as cfu/µL; the correct unit of measure is cfu/mL. Additionally, the results from plates with colonies between 10 and 24 colonies were reported in brackets due to differences in Client (NIAID) preferences regarding reporting.
1056		Not defined in report	50	Not defined	Not provided	Value of LOD was not specified in study protocol and report; Study report Appendix R (Statistical report) defined values below limit of detection and reported as "0" to be replaced with 50 cfu/mL.
New Zealand White Rabbit						
AR021	NM					
AR033	(b) (4) X-054-07	3	2	100	50	

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Study No.	SOP No.	LOD (cfu/mL)	<LOD as presented in the datasets	LLOQ (cfu/mL)	<LLOQ as presented in the datasets	Comments
AR034	(b) (4) X-054-07	3	2	100	50	
AR028	(b) (4) X-054-07	3	2	100	50	
1030	NM					
1045	NM					
AR004	NM					
AR007	NM					
AR012	NM					
AR0315	(b) (4) X-054-07	3	Not provided	100	Not provided	As stipulated in Appendix K of study report: Quantitative bacteremia values less than the limit of detection (LOD, 3 cfu/mL) were reported as "0," and values less than the limit of quantification (LOQ, 100 cfu/mL) were reported as "+." In the statistical analyses, quantitative bacteremia values reported as "0" were replaced with "2" (one half of the LOD rounded to the nearest integer), and the data reported as "+" were replaced with "50" (one half of the LOQ).
AR035 ¹	(b) (4)	3.33	Not provided	3.33	Not provided	As stipulated in study reports, the LLOQ was defined as 3.33 CFU/mL (1 CFU observed among triplicate spread culture plates each with 100 µL inoculum). Samples lacking any detectable bacterial burden (i.e., no colonies present) were classified as BLD or below limit of detection; by definition, bacterial concentrations were below the above-specified LLOQ these samples.
AR037 ¹	(b) (4)	3.33	Not provided	3.33	Not provided	For statistical analyses, values below limit of detection were replaced with 2.33 cfu/mL per study report
AR001	NM					
AR003	NM					

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Appendix-2: Assays used for quantitating PA by ELISA

Study No.	Assay Validation Report No.	PA by ELISA		Comments*
		LLOQ (ng/mL)	<LLOQ as presented in data sets	
Cynomolgus macaques				
AP202	14027IPVRDW_(b)(4) Free PA	5	2.5	Earlier studies indicated that PA levels decrease below the LLOQ of the VP2012-257 assay at first time-point post treatment. PA assay was optimized at new facility to decrease LLOQ to enable PA quantification 15 minute post ETI-204 treatment for AP202.
AP201	QD-210*(b)(4) Total PA	LOD: 2.4 LLOQ: 2.4	Not provided	Limit of detection (LOD) defined as 2.4 ng/mL; LLOQ also set at 2.4 ng/mL. Per study report Appendix H, values that were less than the LOD (reported as "BD") were replaced with 1.2 ng/mL (one half of the LOD of 2.4 ng/mL). Studies AP201, 1056 and 2469 referenced the same SOP (X-180). The in life portions of AP201 and 1056 was completed before the assay validation was complete. Therefore, LOD and LOQ (2.4 for both) determined during qualification was reported.
AP203	VP2012-257(b)(4) Free PA	9.68	4.84	Elusys developed and validated a more specific assay that utilizes a monoclonal capture and detected PA83 and PA63 only.
AP204				
AP307	NM			
AP301				
AP305	NM			
2469				
1056	VP2008-200	LLOD:0.87 LLOQ: 4.2	< LLOD: 0.435	The assay was developed and validated by NIAID. The assay utilized a polyclonal capture and detected PA20 in addition to PA63 and PA83. Limit of detection defined as LLOD of 0.87 ng/mL; LLOQ defined as 4.2 ng/mL (Study report Appendix I). Per study report, PA-ELISA values less than the LLOD were replaced with 0.435 ng/mL (one half of the LLOD). Results greater than LOD were reported as actual values.
	QD-210*(b)(4) Total PA	LOD: 2.4 LLOQ: 2.4	1.2	Studies AP201, 1056 and 2469 referenced the same SOP (X-180). The in life portions of AP201 and 1056 were completed before the assay validation was complete. Therefore, the LOD and LOQ (2.4 for both) determined during qualification was reported. Limit of detection defined as LOD of 2.4 ng/mL; LLOQ also defined as 2.4 ng/mL.

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Study No.	Assay Validation Report No.	PA by ELISA		Comments*
		LLOQ (ng/mL)	<LLOQ as presented in data sets	
New Zealand White Rabbit				
AR021	NM			
AR033	VP2012-256	9.68	Not provided	Elusys developed and validated a more specific assay that utilizes a monoclonal capture and detected PA83 and PA63 only. The numeric value for <LOQ is not provided in SEND; as specified in study protocol section 9.2, values less than LOQ will be replaced with ½ the LOQ for analyses. Therefore, values were replaced with 4.84 ng/mL for statistical analyses.
AR034	VP2012-256	9.68	4.84	
AR028	VP2012-256	9.68	4.84	
1030	QD-186*	LOD: 2 LLOQ: 4.9	Not provided	The in life portion was completed before the assay validation was complete. Therefore, the LOD and LOQ determined during qualification were reported. Limit of detection defined as LOD of 2 ng/mL; LLOQ defined as 4.9 ng/mL (study report Appendix I).
1045	QD-186*	LOD: 2	1	The in life portions was completed before the assay validation was complete. Therefore, the LOD determined during qualification was reported. Limit of detection defined as LOD of 2 ng/mL;
AR004	NM			
AR007	NM			
AR012	NM			
AR0315	NM			
AR035	NM			
AR037	VP2012-256	9.68	1	Values below LLOQ were represented as 1 in accordance with study report
AR001	NM			
AR003	NM			

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Appendix-3: Assays used for Anti-PA IgG antibodies

Different assays were used for measurement of antibody levels by ELISA or ECL assay.

Study no.	Site	Analysis report no.	Assay method ID	Validation report no.	LLOD	LLOQ	ULOQ	Detects ETI-204
NZW rabbits								
AR028	(b) (4)	(b) (4) 2-059	(b) (4)	(b) (4) 1-010	NA	50 ng/ml	6000 ng/ml	Yes
AR034		12-128		1-010	NA	50 ng/ml	6000 ng/ml	Yes
AR035		12-053		1-010	NA	50 ng/ml	6000 ng/ml	Yes
AR037		12-135		1-010	NA	50 ng/ml	6000 ng/ml	Yes
AR0315	AR0315		VP2004-108	1 µg/mL	5 µg/mL	NA (samples diluted into linear range)		Unknown
AR033 ¹	(b) (4) 11-191		(b) (4) 11-010	NA	50 ng/ml	6000 ng/ml	Yes	
AP202	13-223		12-117	NA	100 ng/mL	5000 ng/ml	Yes	
2469	NIAID 2469		VP 2008-221	1.6 µg/mL	5.42 µg/mL	256 µg/mL (included in validation for informational purposes only)		Unknown
Surviving animals ² from Studies AP201, AP203, AP204	11-103 (AP201) 12-085 (AP203) 11-105 (AP204)		11-035	NA	100 ng/ml	5000 ng/ml	Yes Does not detect endogenous anti-PA IgG	

¹Testing for anti-PA IgG was not conducted for study AR033. PK analysis was conducted with non-specific Protein A/G reagent that does not differentiate between endogenous anti PA IgG and ETI-204. PK analysis was later repeated with a selective reagent and added as an addendum.

²Testing for anti-PA IgG was not conducted for studies AP201, AP203, and AP204. Testing for ETI-204 (also anti-PA IgG) was conducted as part of PK analyses and included surviving animals. Assay utilized sheep anti human IgG detection reagent and endogenous anti-PA IgG is not detected in the assay.

Note:

- The assay used for testing of antibodies in sera from animals in Study AR028 was an ECL assay. For other studies an ELISA assay was used.
- The assay used for testing of antibodies in sera from animals in Studies AR0315 and 2469 was based on anti-rabbit IgG or anti-monkey IgG polyclonal antibodies, respectively, as the capture reagent; these assays were designed to measure endogenous anti-PA IgG antibodies. For other studies, the assay was based on PA and non-specific Protein A/G as the capture reagents; although these assays were designed to measure concentrations of ETI-204, endogenous anti-PA IgG antibodies are detected as well.

Appendix-4: ETI-204 product used in different studies

Table: ETI-204 product in different studies

Study number	Product Source	Lot number
New Zealand White rabbits		
ETI-204 - treatment studies		
AR021	Baxter	Baxter (b) (4) lot 103B20-X109-TRO6
AR033	Baxter	Baxter 103B20-X109-TR06 (PBR-0024-001)
ETI-204 + antibacterial drug combination studies		
NIAID 1030	Baxter	Lot 103B20-X109-TR06
NIAID 1045	Baxter	Lot 103B20-X109-TR06
AR028	Baxter	Baxter PBR-0024-001 (BDS Lot# 103B20-X109-TR06)
AR034	Lonza	Lonza Biologics 250241
AR007	(b) (4) (b) (4) MoAb)*	Lot number FIL031COI
AP-10-055	Baxter	103B20-X109-TR06
ETI-204 post-exposure prophylaxis studies		
AR004	(b) (4) (b) (4) MoAb)*	Lot Number ET 258-125
AR012	Baxter	(b) (4) Lot ET403-188
AR0315	Baxter	Lot# 103B20
AR035	Lonza	Lot Number 250241 (Product 103B (b) (4) Lot # 103B20-X109-TR06- this is from appendix E Certificate of analysis)
AR037	Lonza	Lot Number 250241
ETI-204 pre-exposure prophylaxis studies		
AR001	(b) (4) (b) (4) MoAb)*	Lot Number ETI 227-181
AR003	(b) (4) (b) (4) MoAb)*	Lot Number ET 258-184
Cynomolgus monkeys		
ETI-204 - treatment studies		
AP201	Baxter	103B20-X109-TR06 (Elusys Lot number ET 472-084)
AP202	Lonza	Lot No: 3-FIN-1514
	Baxter	Lot No: PBR0024001
AP203	Lonza	Lot No: 250241
AP204	Baxter	103B20-X109-TR06 (Elusys Lot number ET 472-084)
ETI-204 + antibacterial drug combination studies		
NIAID 1056	Baxter	Lot 103B20-X109-TR06
NIAID 2469	Baxter*	Lot PBR-024-001
ETI-204 post-exposure prophylaxis studies		
AP301	Lonza	Lonza Biologics/250241
AP307	Lonza	Lonza Biologics 250241
AP107	Baxter	Baxter (b) (4) lot 103B20-X109-TRO6
ETI-204 pre-exposure prophylaxis studies		
AP305	Lonza	Lonza Biologics/3-NFF-0220

*MoAb - monoclonal anti-PA antibody

*Drug Product manufactured at (b) (4) generated from Baxter drug substance

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

SHUKAL BALA
12/16/2015

KERRY SNOW
12/16/2015



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Center for Drug Evaluation and Research
WO Bldg 51
10903 New Hampshire Ave.
Silver Spring, MD 20993

Date: 11/20/2015
To: Administrative File, STN 125509/0
From: Bo Chi, Ph.D., CDER/OPQ/OPF/DMA/Branch IV
Endorsement: Patricia Hughes, Ph.D., Acting Branch Chief, CDER/OPQ/OPF/DMA/Branch IV
Subject: New Biologic License Applications (BLA)
Applicant: Elusys Therapeutics, Inc.
US License: 1907
Facility: Lonza Biologics, Incorporated
Portsmouth, NH
FEI: 3001451441
Product: Anthim (Obiltoxaximab)
Dosage: 600mg/6 mL single use vial, Intravenous Infusion
Indication: Adult and pediatric patients with inhalational anthrax due to *Bacillus anthracis* in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate
PDUFA date: March 20, 2016

Recommendation: The recommendation for approval of the drug substance part of this BLA from product quality microbiology perspective is pending until the [REDACTED] (b) (4) have been submitted and reviewed. Rabbit pyrogen test may be used to release drug product if the endotoxin [REDACTED] (b) (4) demonstrate low endotoxin recovery.

Review Summary

Elusys has submitted this Biologics License Application (BLA) for obiltoxaximab for treatment and prophylaxis of inhalational anthrax. The drug substance (DS) is manufactured at Lonza, Portsmouth, NH. The drug product (DP) is manufactured at [REDACTED] (b) (4). The application contains CMC information in an eCTD format.

This review contains the assessments of the manufacturing process of obiltoxaximab drug substance from microbiology perspective.

Assessment

Drug Substance (3.2.S)

General Information (3.2.S.1)

35 Page(s) have been Withheld in Full as b4 (CCI/TS)
immediately following this page

(b) (4)

Container Closure System (3.2.S.6)

The bulk drug substance is filled into [REDACTED]

(b) (4)

Satisfactory

Stability (3.2.S.7)

A [REDACTED] (b) (4)-months expiry period has been proposed for the bulk drug substance stored at the recommended storage condition of [REDACTED] (b) (4)°C. No bioburden or endotoxin test is conducted on the [REDACTED] (b) (4) on the stability program, which is acceptable.

The stability program and data should be further reviewed by the OBP reviewer.

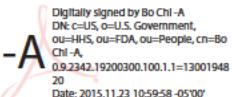
Satisfactory

Conclusion

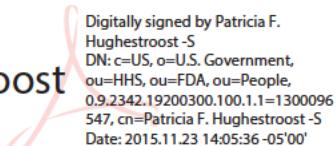
- I. The recommendation for approval of the drug substance part of this BLA from product quality microbiology perspective is pending until the [REDACTED] (b) (4) have been submitted and reviewed. Rabbit pyrogen test may be used to release drug product if the endotoxin [REDACTED] (b) (4) demonstrate low endotoxin recovery.
- II. Information and data in this submission not related to microbial control of the drug substance should be reviewed by the OBP reviewer.
- III. See Panorama for the compliance status of the facilities.

Cc: Chi
Hughes
Dean

Primary reviewer signature

Bo Chi -A
Digitally signed by Bo Chi -A
DN: c=US, o=U.S. Government,
ou=HHS, ou=FDA, ou=People, cn=Bo
Chi -A
0.9.2342.19200300.100.1.1=13001948
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Date: 2015.11.23 10:59:58 -05'00'

Secondary reviewer signature

Patricia F.
Hughestroost
-S
Digitally signed by Patricia F.
Hughestroost -S
DN: c=US, o=U.S. Government,
ou=HHS, ou=FDA, ou=People,
0.9.2342.19200300.100.1.1=1300096
547, cn=Patricia F. Hughestroost -S
Date: 2015.11.23 14:05:36 -05'00'



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Center for Drug Evaluation and Research
10903 New Hampshire Avenue
Silver Spring, MD 20993

Date: 23 November 2015
To: Administrative File, STN 125509/0
From: John W. Metcalfe, Ph.D., CDER/OPQ/OPF/DMA
Endorsed: Patricia Hughes, Ph.D. CDER/OPQ/OPF/DMA
Subject: Original Biologic License Application
US License: 1907
Applicant: Elusys Therapeutics, Inc.
Facility: (b) (4)
Product: Anthim (Obiltoxaximab)
Dosage: Sterile solution (600 mg/6 mL) in a single dose vial for IV infusion
Indication: The drug product is indicated for the treatment of adult and pediatric patients with inhalation anthrax in combination with antibacterials
Sub Dates: Original Application: 20 MAR 2015; Micro Info Amendments: 15 SEP 2015, 27 OCT 2015 and 13 NOV 2015
Due date: 23 November 2015

Recommendation: BLA 125509 was reviewed for microbial control of the drug product manufacturing process and for drug product sterility assurance. Although no deficiencies have been found thus far that would preclude approval, additional information is needed in order to provide an approvability recommendation.

INTRODUCTION

The subject BLA was submitted on 20 March 2015 for Anthim (obiltoxoximab); an affinity enhanced IgG1 monoclonal antibody produced in GS-NSO myeloma cells that targets *B. anthracis* protective antigen. The application is submitted in eCTD format. The drug substance is manufactured at Lonza Biologics (101 International Dr., Portsmouth, NH).

This review covers issues pertaining to the microbiological quality of the drug product. The microbiological quality review of the drug substance was performed by Dr. Bo Chi in a separate review memorandum.

Microbiology Information Requests were forwarded to the applicant on 27 August 2015, 06 October 2015, 05 November 2015 and 23 November 2015. The applicant responses to these

requests were submitted on 15 September 2015, 27 October 2015 and 13 November 2015. The questions and applicant responses are summarized in appropriate sections of this review.

ASSESSMENT

P DRUG PRODUCT

P.1 Description of the Composition of the Drug Product

- Description of drug product

The applicant describes the subject drug product as a “clear to opalescent, colorless to pale yellow-pale brownish-yellow solution” (module 3.2.P.1 of the subject submission).

- Drug product composition

The drug product composition is provided in table 1 which is copied from table 1 of module 3.2.P.1.

Table 1. Drug Product Composition

Component	Function	Quality Standard	Concentration	Amount per Vial (b) (4)mL)
ETI-204	Active ingredient (b) (4)	Elusys (see 3.2.S.4.1)	100 mg/mL	(b) (4)
L-Histidine		USP/NF	40 mM	
Sorbitol		USP/NF	200 mM	
Polysorbate 80 (b) (4)		USP/NF	0.01%	
		USP/NF	As needed	
		USP/NF	As needed	

- Description of container closure system

The container closure system is comprised of a (b) (4)mL Type 1 glass vial (b) (4), a grey (b) (4) rubber stopper (b) (4) and an aluminum (b) (4) flip-off seal (b) (4)

Satisfactory

Reviewer's Comment

The applicant has provided an adequate description of the drug product for the reviewer to assess the manufacturing process, controls and testing with regard to the microbiological quality of the drug product.

P.2 Pharmaceutical Development

P.2.5 Microbiological Attributes

- Container-Closure and Package integrity

The applicant demonstrated container closure integrity by performing a microbial immersion test in media filled vials. The applicant states that they have determined the worst case capping limits to be compression forces (b) (4)

(b) (4)

. The units used for container closure integrity testing were filled with 2.5 mL of TSB. A total of 1377 units were filled, half of which were capped using each of the above worst case compression forces.

A contract testing facility [REDACTED] performed (b) (4) the CCI testing.

[REDACTED] To demonstrate growth promotion of the media, 2 units each were inoculated with:

- *Aspergillus brasiliensis*
- *Bacillus subtilis* subsp. *spizizenii*
- *Candida albicans*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*

(b) (4)

Data from this container closure integrity test are described in section 5.8.2 of module 3.2.P.3. Growth was observed in all of the positive controls and growth promotion controls. No microbial growth was observed in either the challenge units or the negative controls.

- Preservative Effectiveness

The subject drug product is labeled as a single dose product.

Satisfactory

Reviewer's Comments

1. The application also describes a helium leak test performed on 40 filled units by [REDACTED] (b) (4). This information is not reviewed here since the microbial ingress data are acceptable.
2. The applicant's verification of container closure integrity is consistent with regulatory expectations for a sterile pharmaceutical product.
3. Demonstration of preservative effectiveness is not necessary for a single dose product.

P.3 Manufacture
P.3.1 Manufacturers

(b) (4)

P.3.3 Description of the Manufacturing Process and Process Controls

The drug product manufacturing process begins by

(b) (4)
(b) (4)

IR for Applicant on 23 November 2015

1. Reference is made to table 4 of module 3.2.P.3.4 which identifies the controls for manufacture of the drug product. We note that the volume of the bioburden sample is not provided.
 - Amend table 4 of module 3.2.P.3.4 with the bioburden sample volume. If the volume is less than 100 mL, provide a rationale for the size of the sample.

Summary of Response Pending Receipt

(b) (4) **MANUFACTURING PROCESS**

-
-

(b) (4)

(b) (4)

9.

10.

11.

END