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Zeptomole per milliliter detection and quantification of edema factor in plasma by LC-MS/MS yields insights into toxemia and the progression of inhalation anthrax

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Abstract

Inhalation of *Bacillus anthracis* spores can cause a rapidly progressing fatal infection. *B. anthracis* secretes three protein toxins: lethal factor (LF), edema factor (EF), and protective antigen (PA). EF and LF may circulate as free or PA-bound forms. Both free EF (EF) and PA-bound-EF (ETx) have adenylyl cyclase activity converting ATP to cAMP. We developed an adenylyl cyclase activity-based method for detecting and quantifying total EF (EF+ETx) in plasma. The three-step method includes magnetic immunocapture with monoclonal antibodies, reaction with ATP generating cAMP, and quantification of cAMP by isotope-dilution HPLC-MS/MS. Total EF was quantified

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The study protocol was performed in 2006 and was approved by Battelle and the CDC Institutional Animal Care and Use Committees (IACUC) (CDC IACUC protocol no. 1459BOYMONX and Battelle MREF protocol no. 570).

Competing interests The authors declare that they have no competing interests.

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from 5PL regression of cAMP vs ETx concentration. The detection limit was 20 fg/mL (225 zeptomoles/mL for the 89 kDa protein). Relative standard deviations for controls with 0.3, 6.0, and 90 pg/mL were 11.7–16.6% with 91.2–99.5% accuracy. The method demonstrated 100% specificity in 238 human serum/plasma samples collected from unexposed healthy individuals, and 100% sensitivity in samples from 3 human and 5 rhesus macaques with inhalation anthrax. Analysis of EF in the rhesus macaques showed that it was detected earlier post-exposure than *B. anthracis* by culture and PCR. Similar to LF, the kinetics of EF over the course of infection were triphasic, with an initial rise (phase-1), decline (phase-2), and final rapid rise (phase-3). EF levels were ~ 2–4 orders of magnitude lower than LF during phase-1 and phase-2 and only ~ 6-fold lower at death/euthanasia. Analysis of EF improves early diagnosis and adds to our understanding of anthrax toxemia throughout infection. The LF/EF ratio may also indicate the stage of infection and need for advanced treatments.

Keywords

Mass spectrometry; Anthrax; Adenylyl cyclase; Edema toxin; HPLC; Cyclic AMP

Introduction

Anthrax is caused by the gram-positive spore-forming pathogenic bacterium *Bacillus anthracis*. The inhalation form of anthrax typically progresses rapidly and has high mortality rates. The lethal effects of infection are attributed to three protein toxins secreted by *B. anthracis*: lethal factor (LF) (90 kDa), edema factor (EF) (89 kDa), and protective antigen (PA) (83 kDa) [1]. LF, a zinc-dependent endoproteinase, cleaves the mitogen-activated protein kinase-kinase family of response regulators and affects a wide range of immune cells [2]. EF is a calcium-calmodulin-dependent adenylyl cyclase toxin [3], converting adenosine 5'-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [1, 4]. As a prototypic second messenger, cAMP supports diverse cellular responses. However, in mice, systemically delivered ETx generated higher levels of cAMP causing intestinal effusions, hemorrhage, tissue edema, damage, and death [5, 6]. Both EF and LF bind the truncated, 63 kDa form (PA63) of protective antigen (PA83), forming lethal toxin (LTx) and edema toxin (ETx), respectively. LTx and ETx are endocytosed and LF and EF are delivered into the cytosol by well-defined mechanisms [7]. There they employ their harmful enzymatic activities and synergistically disable cellular defenses against bacterial infection [8, 9]. Our laboratory previously reported the development of a rapid, selective, and sensitive mass spectrometry (MS) method to quantify LF and LTx [10, 11]. Sensitive detection of EF as well as LF would allow a better understanding of anthrax toxemia and the progression of infection.

Two EF assays have been previously described that directly react EF with ATP, then detect the cAMP generated by either a competitive enzyme immunoassay (EIA) [12] similar to commercially available competitive cAMP ELISA kits or a radio-metric assay [13]. These assays were not designed for EF selectivity and are potentially subject to interference from endogenous cAMP in serum or plasma. Many factors contribute to endogenous cAMP, as all mammals rely on cAMP for diverse cell signaling processes. The typical concentration

range of cAMP in the plasma of healthy adults is 4.3–8.6 ng/mL [14]. Additionally, other microorganisms produce adenylyl cyclase toxins as well, CyaA from *Bordetella pertussis* and ExoY from *Pseudomonas aeruginosa* [15, 16]. Because there is no additional specificity beyond cAMP detection for these methods, cross-reactivity from these microorganisms may be a concern for their application beyond selective experimentation. The EIA method reported a detection limit of 1 pg/mL for EF spiked in human plasma and 10 pg/mL spiked in animal plasma [12]. This method was used to measure EF in mice during the septic (blood borne) stage initiated by the direct injection of bacilli and spores of a capsule-negative, toxin-producing *B. anthracis* strain. No EF methods have yet been applied to experimental inhalation anthrax infections.

The focus of this report is the development and validation of a sensitive method for the quantification of total EF (EF+ ETx) in serum and plasma and application to a non-human primate model of inhalation anthrax for defining their levels and relationship to LF throughout the course of infection. The method combines EF selective magnetic immunopurification with EF catalysis of ATP to cAMP and isotope-dilution LC-MS/MS for accurate quantification. This is the first method to ensure signal (cAMP) selectivity with prior purification/concentration of EF and the first to employ LC-MS/MS for EF-dependent cAMP detection, gaining exquisite sensitivity (0.00002 ng/mL; 225 zeptomoles/mL) and specificity. This is also the first study to measure EF levels in rhesus macaques throughout the course of inhalation infection induced by aerosol exposure to the fully virulent *B. anthracis* Ames strain.

Material and methods

Chemicals and reagents

Superparamagnetic Dynabeads MyOne Tosyl-activated beads were from Life Technologies (Carlsbad, CA). Recombinant EF was produced as previously described [17]. Activated recombinant PA (PA63) was from List Biological Laboratories (Campbell, CA). Pertussis adenylyl cyclase toxin (CyaA) was from Sigma-Aldrich (St. Louis, MO). Adenosine 5'-triphosphate (ATP) disodium salt hydrate, adenosine-¹³C₁₀,¹⁵N₅ 5'-triphosphate sodium salt solution-labeled ATP (L-ATP), adenosine 3',5' cyclic monophosphate, Hepes buffer, CaCl₂, MgCl₂, calmodulin from bovine testes, and all other chemicals and reagents were from Sigma-Aldrich, except where indicated. A Kingfisher 96 magnetic purification system (ThermoFisher Scientific Inc., Waltham, MA) was used for automated sample preparation.

Blood samples

A 10-donor Normal North American (NNA) plasma pool and 138 serum and 100 plasma samples from anonymous individual donors were from Tennessee Blood Bank (Memphis, TN), human subjects protocol number TBS-12-01, approved IRB# 201210385. Samples from individuals with acute *B. pertussis* and inhalation anthrax were from the sample archive of the Centers for Disease Control and Prevention (CDC) (CDC IRB Protocol no. 5343.0, Use of Residual Human Specimens for Laboratory Diagnostic Research).

Safety

All the procedures and sample handling followed the Biosafety in Microbiological and Biomedical Laboratories (BMBL) best practices recommendations for the safe conduct of work in biomedical and clinical laboratories. Work on sterile animal samples was conducted at CDC using all standard precautions in biosafety level 2 (BSL2) laboratory. The animal study with inhalation exposures conducted in 2006 were all performed at the Battelle facilities under BSL3 containment using all safety handling protocols required described below.

Animal ethics and study protocol

The inhalation anthrax study in rhesus macaques (*Macaca mulatta*) was performed at Battelle Biomedical Research Center (Columbus, OH). The study protocol was performed in 2006 and was approved by Battelle and the CDC Institutional Animal Care and Use Committees (IACUC) (CDC IACUC protocol no. 1459BOYMONX and Battelle MREF protocol no. 570). Methodological details and animal care were described previously [18]. In the interest of broadest use of this animal study, available samples were used for development and proof of concept for multiple anthrax toxin methods. Briefly, rhesus macaques were anesthetized and exposed to $\text{GMC} \pm \text{SE}$ of $378 \pm 8 \text{ LD}_{50}$ equivalents of *B. anthracis* Ames spores by head-only exposure in a class III biosafety cabinet. Serum and plasma samples were collected at 42 days pre-exposure (−42D) and post-exposure at 12, 24, 48, 72, 96, and 120 h. All samples were filter-sterilized and confirmed sterile by culture prior to shipment to CDC. In-house validated procedures at Battelle for *B. anthracis* inactivation of samples include filtration through 0.2- μm syringe filter followed by confirmation of sterility by inoculating 10% of the filtered sample into TSB-phenol red broth and incubation for 48 h at 37 °C. One hundred microliters of the incubated broth is plated onto 5% sheep blood agar and incubated for 48 h at 37 °C. Samples are sterile when no growth is seen in the broth or on the plate. Sterility is confirmed and reviewed before signing by safety officials and release of samples to other laboratories for testing.

Generation and purification of anti-EF and anti-PA monoclonal antibodies

Monoclonal anti-EF antibodies (EF-mAbs) were harvested from three 8–10-week-old BalbC mice immunized with recombinant EF. The antigen was prepared with Sigma Adjuvant System and 20 μg of the protein/adjuvant was injected subcutaneously followed by 10 μg boosts on days 21 and 35. Animals were sacrificed and spleens harvested on day 38. Primary splenocytes were washed with basal Gibco IMDM (Fisher Scientific, Pittsburgh, PA) and fused with the mouse myeloma cell line SP 2/0 (> 95% viability) at a 1:5 myeloma/spleen cell ratio) in the presence of PEG 4000. Cells were re-suspended in fusion medium (IMDM with additional supplements of 20% FBS) (Hyclone laboratories, Logan, Utah), amino acids, sodium pyruvate, and 50 units/mL murine recombinant IL-6 + HAT (Boehringer-Mannheim, Ingelheim am Rhein, Germany). Fused cells plated into five 48-well plates at 0.5 mL/well, were incubated for 48 h at 37 °C with 5% CO_2 , and then transferred into HT fusion media for 7 days. The cell culture supernatants were screened for antibody binding to EF using the activity assay as described below. Positive hybridomas were also sub-cloned by three successive rounds of limiting dilution with subsequent screening against EF. IgG from

hybridoma culture was purified by HiTrap Protein G sepharose affinity chromatography (GE Biosciences, Piscataway, NJ). Protein G-purified IgG1 kappa mAb was concentrated to 5 mg/mL in 50 mM HEPES/150 mM NaCl (pH 7.4) and frozen at -40°C . The IgG was analyzed by SDS-PAGE and Superdex-200 size-exclusion chromatography to assess purity and degradation/aggregation. Based on screening tests, the best EF-mAb for this assay was AVR3094 (EFG-4D1:3B11) (EF-mAb).

The generation, purification, and screening of PA-specific mAbs were described previously, resulting in the selection of AVR1046 (PA-mAb-I) and AVR1162 (PA-mAb-II) [18].

Antibody binding to the magnetic beads

EF-mAb, PA-mAb-I, and PA-mAb-II were each immobilized on magnetic tosyl-activated beads following the manufacturer's protocol. The PA-mAb-I and PA-mAb-II beads were mixed 1:1, then diluted 1:4 in phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBST). The EF-mAb and PA-mAb-I/II (PA-mAbs) beads were stored separately at 4°C until use. In the final optimized method, the EF-mAb and PA-mAb-I/II beads were mixed together to give a ratio of 1.5:1:1 of [EF-mAb]:[PA-mAb-I]:[PA-mAb-II] (EF/PA-mAbs).

Preparation of isotopically labeled cAMP for LC-MS/MS analysis

The labeled cAMP (L-cAMP) internal standard (IS) was prepared in house by incubation of stable isotope labeled ATP ($^{13}\text{C}_{10}\text{H}_{16}^{15}\text{N}_5\text{O}_{13}\text{P}_3 \cdot x\text{Na}$) with 200 ng EF captured on EF-mAb magnetic beads. After overnight incubation, the stable isotope labeled ATP completely converted to L-cAMP. The EF-mAb beads were separated from the L-cAMP containing reaction mixture by filtering through a 10-kDa spin column (Ultrafree MC, Bedford, MA) to eliminate carryover of EF-mAb beads. The L-cAMP mixture was diluted 1:1000 in 80% acetonitrile/ H_2O . To confirm the absence of residual EF activity, the diluted L-cAMP was mixed in reaction buffer containing unlabeled ATP, incubated overnight, and then analyzed by LC-MS/MS for native cAMP. The verified L-cAMP spiking solution was used for the final method, by addition of 20 μL directly to 10 μL of each reaction mixture after incubation.

Preparation of ETx spiked plasma pools

A primary ETx spiked plasma pool was prepared as follows: First, 10 μg recombinant EF (9.2 μL EF at 1.09 $\mu\text{g}/\mu\text{L}$) was mixed with 500 μg PA63 (500 μL PA63 at 1.0 $\mu\text{g}/\mu\text{L}$) for 60 min to allow binding and ETx formation. Then 490.8 μL of a 10-donor NNA plasma pool was added to obtain a 10 ng/ μL rETx complex containing plasma stock. The full formation of rETx without detectable free EF in the stock was verified by non-denaturing gel electrophoresis and silver staining (data not shown). The rETx 10 ng/ μL stock was further diluted in plasma, yielding five secondary stocks at 0.00001 to 1 ng/ μL rETx.

Preparation of calibrators and quality control samples

Working plasma calibration standards (STD) and quality control (QC) samples were prepared in 50-mL volumetric flasks by pipetting 30 to 1000 μL volume of the five secondary rETx stocks, with no more than three 10-fold serial dilutions with the NNA

plasma pool yielding working calibration standards from 0.000006 to 12.5 ng/mL, and three QCs at 0.0003, 0.006, and 0.09 ng/mL.

Automated sample preparation

A 100 μ L aliquot of each STD, QC, blank, and unknown was added to 900 μ L of PBST in 2-mL 96-well plates. Then 43.5 μ L PBST, 20 μ L EF-mAb, and 11.5 μ L PA-mAb-I/II were added to each well of a separate plate. The automated KingFisher system mixed and processed the samples and mAbs. Briefly, mAb beads were transferred into the diluted samples and mixed for 1 h. Then EF/ETx-bound beads were washed twice in 1 mL PBST for 1 min, then twice in 200 μ L dH₂O for 0.5 min. Beads were transferred into 30 μ L optimized reaction buffer (described in results) and incubated for 4 h at 30 °C.

High-performance liquid chromatography

The HPLC separation was conducted on an Acquity UPLC H-Class system (Waters Corporation, Milford, MA) with a Javelin Guard Biobasic AX 5 μ m ion exchange 10 mm $\times$ 4 mm HPLC column (ThermoFisher Scientific, Waltham, MA). Flow rate was 300 μ L/min and the injection volume was 10 μ L. The optimal pH/organic eluent gradient was generated by programmed mixing of four mobile phases (MP): MP-A (100 mM CH₃OOH in H₂O^{lc}), MP-B (200 mM NH₄OH in H₂O^{lc}), MP-C (90% acetonitrile/10%–100 mM NH₄OAc), and MP-D was 100% H₂O^{lc}. Each elution run started at pH 6.5 and 80% MP-C followed by a step gradient program. During 0.0–0.9 min at constant pH 6.5, MP-C was decreased from 80 to 40%; during 0.9–6.0 min at constant 40% MP-C, the pH was increased from 6.5 to 8.5; during 6.0–6.5 min, the pH was increased from 8.5 to 9.5 while MP-C was decreased to 0%, during 6.5–11.0 min at constant pH 9.5 and 0% MP-C; and finally during 11.0–14.0 min, the system was equilibrated back to pH 6.5 and 80% MP-C. With this optimal step gradient program, cAMP eluted at 0.89 min, at pH ~ 6.5 and 80–40% MP-C, while ATP eluted at 8.15 min, at constant pH 9.5 and 0% MP-C. The actual quaternary mobile phase gradient program is available in Online Resource 1.

Tandem mass spectrometry

An API 4000 QTrap mass spectrometer (AB Sciex LLC, Framingham, MA) was operated in positive ion electrospray mode with a standard Turbo-V ion source. The settings were 35 psi curtain gas (N₂) flow, high collision gas flow, 45 psi nebulizer gas (air) flow, 500 °C source temperature, and + 4500 V ionization voltage. Unit resolution multiple reaction monitoring (MRM) was used with the following precursor/production *m/z* pairs used for quantification* and confirmation: for cAMP *m/z* 330→136*, 232, 312, for L-cAMP *m/z* 345→146*, 247, 327. ATP was monitored without fragmentation at *m/z* 508→508. Of note, cAMP and ATP were negative ions in the HPLC eluent, due to having less basicity than ammonium hydroxide. However, the method requires positive and not negative LC-MS detection mode, because in the gas phase, ammonia is less basic than cAMP and ATP and therefore enhances the positive ionization of cAMP and ATP.

Calculation of EF concentration

The calibration curve equations from which EF concentrations were calculated were generated by fitting the $\log_{10}$ transformed cAMP/L-cAMP LC MS/MS peak area ratios vs. $\log_{10}$ transformed EF concentrations with a five-parameter logistic regression (5PL) with robust weighting, using custom VB.Net software.

cAMP analysis by LC-MS/MS and ELISA

Three sets of EF were purified from plasma spiked at 2.5 ng/mL for a 2-fold serial titration in plasma down to 0.0000095 ng/mL for 19 concentrations and plasma blank, reacted with ATP in optimized buffer, then cAMP was analyzed by LC-MS/MS and competitive ELISA according to the manufacturers protocol using the Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY).

Performance validation

Quantitative performance characteristics were determined from 90 individual runs of standards and QCs over 90 days. The limit of detection (LOD) was determined for the 90 runs calculated as described previously using the slope and y-intercept from the linear regression of standard deviation (stdev) versus concentration for plasma blank and three low standards and mean of the blank for $\text{Conc}_{\text{LOD}} = (\text{mean}_b + 1.645(s_b + \text{int}))/1 - 1.645(\text{slope})$ with b (blank), s (stdev), and int (y-intercept) [11, 19, 20]. Precision as relative standard deviation (RSD) and accuracy were calculated for all spiked plasma standards and QCs for 90 runs. The accuracy expressed as % error was based on the mean of the observed concentration for 90 runs relative to the amount of spiked material in the plasma standards, QCs. According to FDA, precision with ligand binding assays, such as used in the first step here, is expected to be 25% RSD and 20% error, except at the upper and lower limits of quantitation, ULOQ and LLOQ, respectively, for which 25% error is accepted [21]. Accuracy over the range was also demonstrated with linearity of the observed mean and 95% confidence intervals ($\text{mean} \pm 1.96 \times \text{stdev}$) for 90 runs relative to the known spiked concentration in the plasma standards and QCs. Specificity was demonstrated by analysis of 138 serum and 100 plasma samples from healthy donors and 2 acute stage serum samples from *B. pertussis* infection which produces a similar adenylyl cyclase protein toxin. Sensitivity was evaluated by measuring EF in serum and plasma samples from three inhalation anthrax cases from unique events and *B. anthracis* strains [22–24] and experimentally exposed five rhesus macaques described previously [18]. Plasma standard material stability was tested at medium (0.012 ng/mL) and low (0.00076 ng/mL) levels in triplicate for 24-h bench-top stability, 3 freeze-thaw cycles, processed sample stability, and long-term stability (over 4.5 years).

Results

We describe the development of a HPLC-MS/MS method for sensitive and accurate quantification of total EF in samples from anthrax infection. To improve sensitivity and selectivity, our method development included a step to isolate and concentrate EF from the plasma matrix (Fig. 1, step 1). This was combined with an EF reaction with ATP producing cAMP and isotope-dilution LC-MS/MS quantification of cAMP (Fig. 1, steps 2 and 3).

Method development required optimization of discrete steps described below. They are presented in reverse order of the final schematic based on the requirement of each step to optimize prior steps.

Optimization of the pH/salt/organic HPLC gradient

A challenging aspect of the LC-MS/MS detection was the separation of cAMP from high concentrations of unreacted ATP. This was achieved by careful optimization of a combined pH/ammonium acetate/acetonitrile solvent gradient, using the Auto-Blend Plus function of the Empower Waters Acquity software (Electronic Supplementary Material (ESM) Fig. S1). Hydrophilic interaction chromatography (HILIC) mode was used to elute cAMP at constant pH 6.5 and increasing 10 mM ammonium acetate while decreasing the acetonitrile content in the eluent. During the HILIC mode elution of cAMP, ATP bound to the protonated amino propyl group of the weak anion-exchange column by ionic interaction. Following the elution of cAMP, the pH was increased from 6.5 to 8.5 as acetonitrile content was decreased to 0%, allowing a switch from HILIC to weak anion-exchange (WAX) HPLC mode. ATP was eluted by further increasing the pH from 8.5 to 9.5, deprotonating the amino propyl group on the column, and releasing the ATP negative ions. The combination of HILIC and WAX modes eliminated the carryover of ATP from subsequent injections, thus eliminating an interference from the fragmentation of ATP to cAMP in the electrospray ionization LC-MS interface. A typical chromatogram of cAMP and ATP is shown from samples after a 4-h incubation of EF captured from plasma at 3.1 ng/mL (Fig. 1, step 3). With these conditions, cAMP eluted at 0.89 min and ATP at 8.15 min.

Optimization of the EF reaction

Optimal amounts of additives in the EF reaction buffer were critical to minimize background while maximizing sensitivity for EF-mediated cAMP production. Fractional factorial design of experiments using the JMP Statistical Discovery software (Cary, NC) was used to optimize the EF reaction buffer ingredients. EF reaction buffer was prepared with different levels of components and tested by incubation with 0–200 pg EF. The optimal composition was determined based on response surface modeling, maximizing the cAMP signal intensity per EF amount added, while minimizing background interferences. ATP, calmodulin, and CaCl_2 were essential ingredients as EF substrate and co-factors. BSA and EDTA were included because they reduced background interferences contributing to the cAMP signal. However, at higher than optimal concentrations they suppressed cAMP signal response (data not shown). Addition of MgCl_2 resulted in the most significant enhancement of cAMP signal response, consistent with the involvement of Mg^{2+} in the cyclization reaction at the EF active site [25]. The final reaction buffer composition included 20 mM HEPES, pH 7.3, 40 mM MgCl_2 , 1 mM EDTA, 1 mM ATP, 1 μM calmodulin, 10 μM CaCl_2 , and 0.1 mg/mL BSA.

EF activity and reaction time

Special method considerations were implemented to improve quantification by accounting for the high turnover rate of ATP to cAMP by EF. The manual addition of ATP reaction buffer to the first row or column on the 96-well plates would start reactions sooner than those in the last row or column, which would result in more variability in the quantitative

values. Therefore, transfer of beads to the reaction buffer was automated for simultaneous initiation of the reactions for the calibration standards and the unknowns. In addition, an incubation time of 4 h at 30 °C maximized cAMP accumulation and minimized differences across the 96-well plate. In the final steps for MS analysis, the beads and reaction buffer were mixed to homogeneity, then the plate placed on a 96-well magnet, pulling beads to the side of the wells away from the reaction buffer. Finally, rapid transfer of the reaction mix away from the EF beads and addition of IS in acetonitrile quenched any residual enzyme activity.

EF/ETx activity and immunocapture optimization

A series of experiments were performed to assess adenylyl cyclase activity and recovery of both forms of EF, free EF (EF), and PA63-bound EF (ETx) present during infection. Recoveries were determined by comparing EF activity after mAb bead capture to that for EF activity without capture. In all cases, the same starting sample and volumes were used for capture and non-capture control and the cAMP/L-cAMP peak area ratios represented differences in activity.

First, we tested recombinant EF activity in the presence of PA83 and PA63 using 3 pg EF premixed with PA83 and PA63 at w/w PA:EF ratios of 0:1 up to 64:1. EF activity was higher at all ratios of PA63:EF up to 64:1 where it was similar to EF alone (Fig. 2a). Activity with PA83:EF was similar to control up to 16:1 and was lower 32:1. The impact of full-length PA83 on EF activity is minimal in the context of infection during which PA83 levels are low or non-detectable compared to PA63 in all infection samples (Solano et al., in preparation).

Monoclonal antibodies (mAbs) can bind many exposed epitopes on EF. The mAbs may bind epitopes that neutralize the EF adenylyl cyclase activity or interactions with PA63, interfering with ETx recovery. A total of 26 EF-mAbs were screened for the ability to capture EF monomer while retaining its activity. The qualities of all EF-mAbs will be documented in detail in the future. One, AVR3094 (EF-mAb), was selected with good recovery and activity of recombinant EF and ETx. Recovery of EF for EF-mAb was > 100% over 3-orders of magnitude EF concentrations. A higher slope was also observed with EF-mAb capture, indicating enhanced EF activity (Fig. 2b). Recovery of ETx with EF-mAb was good but in a complex clinical infection sample with high ratios of ETx/total EF, two PA-mAbs (described above) in addition to EF-mAb were needed to supplement recovery (data not shown). Recovery with the three optimized EF/PA-mAbs was tested in two rhesus macaque (RM) infection samples with high ratios of PA and LF compared to EF. RM1 had 400× higher LF than EF, 570× PA63, no PA83, and RM2 had 18× higher LF, 65× PA63, 1.7× PA83. Recovery for PA-mAbs showed that ETx-alone was high in both samples, 71–77% of non-capture control and recovery with EF/PA-mAbs, gave 96% and 98% of control (Fig. 2c). The high (96–98%) recoveries for Ames inhalation anthrax samples demonstrated that the combined EF/PA-mAbs were excellent for total EF (EF+ ETx) recoveries in the presence of moderate and high PA and LF.

The recoveries in calibration materials with 100% ETx as complex were determined with optimized capture for total EF (EF/PA-mAbs) and ETx only (PA-mAbs). The calibration series was prepared with a high ratio of PA63:EF (50:1) in plasma, similar to those observed

in infection samples. The cAMP responses over the 6-orders of magnitude (0.000006 to 12.5 ng/mL) were essentially identical for total EF and ETx (Fig. 2d). This indicated that a high excess of PA63 did not affect the adenyl cyclase activity and recovery of ETx.

To summarize, the best recoveries were obtained with a triple combination of mAbs for total EF, EF/PA-mAbs (AVR3094+AVR1046+AVR1162). Excellent recoveries were obtained for both complex infection samples and recombinant materials. Analyzing the spiked plasma calibrators in an identical way alongside QC and unknowns ensured standardization, precision, and accuracy as described below. In addition, performing comparative measurements with the PA-mAbs alone may be useful for selective recovery of PA-bound EF and measuring the relative amounts of ETx during infection.

Method validation

With the recovery and activity optimized, the ETx plasma standards, blanks, and QCs were analyzed 90 times on 90 days for over 4 years. Due to the wide concentration range and the heterogeneous nature of the mAb binding and enzyme kinetics, the calibration curves were sigmoidal similar to ELISA methods. The best curve fit was obtained using dual log₁₀ transformation and 5PL curve fitting with robust weighting similar to methods validated for total-LF MS [20] (Fig. 3a). Precision (RSD) and accuracy (% error) over the full range of standards covering 6-orders of magnitude are included in Table 1. The acceptable quantitative performance was observed in the ranges in bold font, for values with RSD 25% and error 20%. RSD ranged from 7 to 21% RSD, which, when combined with 0.51–16.9% error at those levels, yielded acceptable confidence. RSD was above the acceptable range at Std-01. Unknowns near Std-02 and higher are diluted and reanalyzed in duplicate. RSD was also higher in the lowest two standards, below the calculated LOD. The LOD was calculated as described above for the 90 runs at 0.000020 ng/mL (20 fg/mL) in plasma [19]. This falls between Std-10 and Std-11. With only 10.1% error and 21% RSD at Std-10 above and 22.7% error and 38% RSD at Std-11 below the LOD, these parameters allow estimates of 19.9% error and 34% RSD at the LOD. Therefore, we have set the LLOQ equal to the LOD because the accuracy and precision of measurements between Std-10 at 0.000048 ng/mL and the LOD at 0.00002 ng/mL is sufficient and fit for purpose at these low levels. The cAMP peak signal near the LOD can be easily visualized in the chromatograms for low-level plasma standards and blank which shows the cAMP peaks visible between STD-10 (0.000048 ng/mL) and STD-11 (0.000012 ng/mL) and an arrow approximating where the calculated LOD falls (Fig. 3b). Means and stdev were graphed versus EF concentration demonstrating good linearity with a slope of 1.09 and R^2 of 0.9997 (Fig. 3c). The repeated quantification of QCs covering the range (spiked at 0.0003, 0.006, and 0.090 ng/mL) gave RSD of 16.6, 11.7, and 15.8% and accuracy of 91.2, 99.5, and 96.4%, respectively (Table 1).

Stability of the plasma standards material was tested at two concentrations, medium (0.012 ng/mL) and low (0.00076 ng/mL). Samples under all conditions were analyzed in triplicate (Table 2). After three additional freeze-thaw cycles, the difference from typical analysis conditions (once-thawed controls), was – 4.0% and – 4.8% for the medium and low standards, respectively. Stability testing for a bench sample held at room temperature for 24 h was slightly higher than control + 1.0% and + 4.2%, for the medium and low EF standards,

respectively. Sufficient volumes of each standard were prepared in late 2012 and stored at -80°C for long-term analysis. The same material analyzed in September 2018 was $+1.0\%$ and $+1.6\%$ higher than the same standard material measured in February 2013, over 4.5 years earlier, for medium and low EF standards, respectively. All of these differences are less than the typical relative standard deviation of QCs in those quantitative ranges (Table 1).

Specificity was demonstrated by obtaining consistent non-detectable results for 238 samples from healthy donors, 138 serum and 100 plasma, as well as non-detectable results for sera from two individuals with acute stage whooping cough, indicating the infection with *B. pertussis* that produces the CyaA adenyl cyclase did not cross-react. Samples from *P. aeruginosa* infection were not available. Inhalation anthrax is rare. For sensitivity, positive results were obtained for EF in serum, plasma, and pleural fluid from three known human inhalation anthrax cases [22–24]. In experimental inhalation anthrax, EF was positive early post-spore exposure (described below) and throughout inhalation infection in five infected rhesus macaques (Table 3).

As mentioned above, Duriez et al. described a method with direct analysis of EF-generated cAMP from complex infection samples by competitive EIA [12]. We also considered whether a similar competitive ELISA would accurately quantify EF and compared LC-MS/MS and ELISA for cAMP detection from three reaction sets of EF captured from plasma over 5-orders of magnitude. It showed that LC-MS/MS area ratios were easily discriminated with good reproducibility at each level over the full range from 2.5–0.0000095 ng/mL whereas ELISA was limited to about 2-orders of magnitude EF concentrations (Online Resource 2). There was also greater variability for ELISA except at four concentrations. In addition, detection limits with the ELISA by the prescribed analytical convention, signal at three times the blank, inverse for the competitive ELISA OD at three times lower, was near 0.00015 ng/mL EF (2.8 times the blank), whereas for LC-MS/MS was at 0.000019 ng/mL EF (4.0 times the blank), near the calculated LOD for 90 standard runs. Detection by LC-MS/MS gave better detection limits, precision, accuracy, and flexibility for a broader range of concentrations.

Total EF measurements in rhesus macaques with inhalation anthrax

Five rhesus macaques described previously with inhalation anthrax resulting from high-dose exposures, succumbed to infection at 96 h (RM1–3), 120 h (RM4), and 9 days (RM5) [18]. Serum samples collected pre-challenge and daily out to 120 h were analyzed for total EF using the optimized activity assay with a triple combination of EF/PA-mAbs. EF levels were compared to LF levels, PCR, and culture results reported previously [18, 20] (Table 3). EF was first detected at 24 h post-challenge in RM1 and RM3 and at 48 h in RM2, RM4, and RM5 and was positive at all subsequent time points and remained detectable over the course of infection. In RM1, EF was the only anthrax biomarker positive at 24 h. However, in RM2 and RM5, only LF was positive at the 24-h time point. Both LF and EF were positive at 24 h in RM3. The two methods combined resulted in 4 of 5 animals (80%) positive at 24 h post-exposure, whereas no animals were positive for organism-dependent methods, culture, and *pagA* PCR, at 24 h. As described previously, culture and/or PCR were first positive at 48 h, then culture reverted from positive at 48 h to negative or $\pm$ at 72 h post-challenge (during

phase 2) in 4/5 animals (80%) [18]. PCR was positive at 72 h in three of the four culture negative/ $\pm$, suggesting that the organism was present but was below culture detection limits. The culture was positive again in all animals at final/terminal time points.

LF levels were previously shown to be triphasic over the course of infection [18] similar to the clinical course of infection described in human inhalation anthrax [26]. EF kinetics were also triphasic, mirroring LF, but with lower levels for EF (Fig. 4). Both toxins increased to 48 h (phase-1) with high LF compared to EF, then both declined at 72 h with EF declining much faster (phase-2), showing a large divergence in LF and EF levels. At terminal time points both LF and EF increased, with EF rising more rapidly at 96 h in RM1–3 and 120 h in RM4 (phase-3), showing a convergence of toxin levels. LF in RM5 declined further from 72 to 96 h whereas EF increased slightly from 72 to 96 and again at 120 h, but EF levels remained largely divergent from LF (765-fold lower) and did not exceed phase-1 (48 h) EF levels (Table 3, Fig. 4). This animal survived to 9 days and at the last measurement (120 h) was still likely in phase-2 (clinically intermediate progressive) [26], 4 days from death. No additional samples could be taken during the last 4 days due to the IACUC protocol used for this study. Overall, EF levels were much lower than LF at phase-1, declined more than LF at phase-2, then rose more rapidly than LF in phase-3. LF/EF ratios were also an important quantitative measure for comparison. In the 4 animals with all three phases, we observed that $\text{GMC} \pm \text{SE}$ LF/EF ratio was high at 94 ± 0.17 at the end of phase-1 (48 h), then even higher at the end of phase-2 to 692 ± 0.27 (72 h), then was much lower at terminal phase-3 at 6.2 ± 0.44 (Table 3). This shows that low LF/EF ratios represent a discriminating feature of terminal (clinically late fulminant) anthrax.

Discussion

We have previously described strategies that allow the catalytic activity of protein toxins produced by microorganisms to be harnessed, amplifying their detection and the ability to diagnose important infections [27]. The resulting reaction products correlate very well with starting toxin concentration providing an amplified quantifiable relationship. When combined with prior selective purification/concentration and detection/quantification by isotope-dilution mass spectrometry, the three steps each provide additive layers of specificity and sensitivity. Here, we combined selective antibody magnetic bead capture to purify and concentrate total EF (EF+ETx) with an optimized reaction buffer allowing enhanced catalysis of ATP to cAMP and LC-MS/MS detection for a highly sensitive method for measuring EF levels during anthrax infection. EF reactions with cAMP detection have been described previously but their applications are limited [12, 13]. The advancements included targeted antibody extraction, optimized buffer conditions for EF reactivity and LC-MS/MS for detection. The advantage of magnetic antibody bead capture is the extraction of EF/ETx from serum or plasma, which removes it from potential endogenous cAMP in the sample for which the reference range in humans is 4.3–8.6 ng/mL [14], well above the direct cAMP detection limits for the Enzo kit at 0.037 pmol/mL (0.012 ng/mL cAMP). The capture step avoids possible false positives from naturally occurring cAMP in a sample. This is even more relevant because cAMP has been found to be abnormally elevated in certain stages of pregnancy [28], hyperthyroidism [29], renal failure [30, 31], and eczema [32] among others. For methods with potential clinical application, the specificity for the source of cAMP is

essential. In addition, this new step concentrates EF, improving detection limits and reducing variability from potential matrix interferences that might influence activity. The capture step combined with adenylyl cyclase activity may be applied to other infections with known adenylyl cyclase toxins such as *B. pertussis* [33], *P. aeruginosa* [34], and potentially *Leptospira interrogans* for which adenylyl cyclases have been identified [35, 36]. It would simply require the development of unique toxin-specific antibodies. This first implementation of isotope-dilution LC-MS/MS for detection and quantification of EF-generated cAMP also provides greater dynamic range, sensitivity, specificity, and quantitative accuracy compared to ELISA methods.

The combination of EF/PA-mAbs allowed measurement of total EF (EF+ETx) and PA-mAbs alone provides the option for measuring PA-specific ETx activity as shown for 2 RM samples (Fig. 2c). Insufficient volumes of these much-studied RM samples precluded measurement of ETx here, but it will be evaluated in future studies. For total EF, detection was 24 h earlier than culture/PCR and remained measurable during sharp declines in toxin levels when culture reverted to negative. The low EF levels early and during phase-2 demonstrate the requirement for the exceptional sensitivity achieved here with the LOD at 0.00002 ng/mL (20 fg/mL and 225 zeptomoles/mL for the 89 kDa protein). Comprehensive method validation also showed good precision, accuracy, stability, and 100% sensitivity and specificity. The performance assessments support its utility for EF detection and quantitation.

The previously described method by Duriez et al. used a competitive EIA, to analyze cAMP from direct reactions of EF spiked plasma and homogenized ear supernatants and plasma collected from septicemic mice [12]. Their sample preparation included a 20-fold dilution of plasma sample in reaction buffer with ATP followed directly by EIA analysis. Their detection limits spiked in human and animal plasma were 0.001 and 0.01 ng/mL EF, respectively. Our detection limit of 0.00002 ng/mL total EF was 50- and 500-fold lower than the direct EIA, for human and animal plasma, respectively. Our comparison to the similar competitive cAMP ELISA showed high precision over 5-orders of magnitude for LC-MS/MS and overall, less precision for ELISA for only about 2-orders of magnitude EF concentrations. As analyzed on samples prepared with our purified/concentrated EF and optimized reaction, these comparative results produce a more favorable outcome for the ELISA than would have resulted using the diluted/non-purified samples for the EIA method. All steps in the optimized EF method, especially LC-MS/MS analysis, were essential to the final precision, accuracy, sensitivity, and specificity achieved over a broad range of concentrations.

This study is also the first to measure EF in rhesus macaques with Ames strain inhalation infection. The use of a non-human primate (NHP) model and inhalation infection most closely matches that occurring in human inhalation anthrax. The method detected EF early and throughout the course of infection with triphasic kinetics, an initial rise (phase-1), decline (phase-2), and final rapid rise leading to high terminal measurements (phase-3), similar to LF and LTx [11, 18]. Compared to LF, EF levels were very low throughout phase-1 and phase-2. The low EF levels and high LF/EF ratios in phase-1 and phase-2 correspond to early prodromal and intermediate progressive stages. The rapid decline in EF

at phase-2 is similar to the rapid decline in *B. anthracis* poly- γ -D-glutamic acid capsule antigen, PGA, reported previously for these animals and coincides with reversion of culture from positive to negative [18]. This indicates that EF is more responsive to culture status than LF. Phase-2 is thought to be due to the innate immune response attempt to clear the infection. Decreased bacteremia, decreased toxin production, ongoing toxin cellular uptake, and many-fold lower starting EF levels may collectively be responsible for the more rapid declines observed for EF. The phase-3 convergence of EF and LF to high toxemia and low LF/EF ratios corresponds with late fulminant stage where survival is less likely [26]. These novel findings in a non-human primate (NHP) model using inhalation exposures are expected to model that observed in human inhalation anthrax.

LF and EF were compared recently using less selective and less sensitive methods and in mice with cutaneous infections of a bioluminescent wild-type strain [37]. Progression was tracked by the appearance of bioluminescence (BLI) movement from the injection site (ear) to other organs with 5 stages; non-detect (stage 1), first detectable BLI in the ear (stage 2), in cervical lymph nodes (stage 3), spread to spleen (stage 4), and to lung and septicemic (stage 5). Though the route of exposure and animal are different, the progression related to toxemia may be comparable. This cutaneous mouse (CM) study reported high plasma LF/EF ratios that consistently decreased out to stage 4; LF/EF of 1479, 490, and 81, and 3.2 in stages 1, 2, 3, and 4, respectively, then increased to 50 in terminal stage 5 (ratios calculated as the inverse of plasma LF/EF mean $\log_{10}$ in Table 3 from [37]). The high LF/EF ratios in CM stage 1–3 are similar to phase-2 in RM. The low LF/EF ratios in stage 4 CM are similar to terminal phase-3 in RM. However, this CM model showed an increase and divergence of LF/EF again in stage 5, not observed in inhalation anthrax in RM. The steadily decreasing LF/EF ratios in CM are also in contrast to the RM which increases from phase-1 to phase-2 by 5–40-fold before declining (EF convergence with LF) in phase-3. The differences in LF/EF ratios may be due to differences in the route of exposure, the animal model and host defenses, or a combination. The similarities in early stages support the findings in both models.

Three RM had terminal LF/EF ratios at 3.6, 3.9, and 6.1 which was similar to those found at 5:1 in both transcriptional analysis of *lef*(LF) and *cya*(EF) in *B. anthracis* cultures [38] and in the late stages of rabbits infected subcutaneously with 20 LD₅₀ spores [39]. The similarity of LF/EF in the late stages of two infection routes for two animal models and during independent culture suggests that any host influences, whether by anthrax receptor saturation or immunological responses, have been overcome and are irrelevant. Here, we have shown that LF and EF measurements combined can provide important information about the stage of infection with high LF/EF ratios indicative of early and low LF/EF ratios of late-stage infection. Though EF levels are much lower than LF in early stages of infection, the sensitivity allowed detection of EF in one animal at 24 h that was negative for LF, for a combined positive toxin result in 80% of animals. EF may be an additional test available to improve diagnostic coverage in the early stages of an event. Additional studies will assess early detection further.

In the early stages of a natural or intentional exposure event the *B. anthracis* strain will be unknown. The toxin genes of *B. anthracis* are relatively stable with few changes resulting in

amino acid substitutions (non-published data). The positive results for EF in three unrelated inhalation anthrax infections [22–24] with distinct strains [40] indicate any differences, if present, did not impact detection of EF-mAb-specific adenylyl cyclase activity.

Conclusions

We reported the development, validation, and application of a highly sensitive and selective mass spectrometric method for anthrax edema factor activity. Three novel features were combined for this method: (1) triple mAb magnetic immunopurification/concentration, (2) optimized EF catalytic reaction, and (3) isotope-dilution LC-MS/MS detection, allowing synergistic enhancement of assay sensitivity and specificity over methods based on detection of cAMP alone. This study provides the first report of total EF measured over the full course of Ames strain inhalation anthrax in the non-human primate model. Because EF and LF synergize to depress immunity and produce lethal effects, the measurement of both anthrax toxins may help to shed light on why treatments sometimes fail. It may also allow additional diagnostic coverage in early stage anthrax. Future application of LF and EF assays during antimicrobial or antitoxin treatment may provide a better understanding of anthrax pathogenesis and strategies for improving survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EF	Edema factor
ETx	Edema toxin
LF	Lethal factor
LTx	Lethal toxin
PA	Protective antigen
STD	Standard
QC	Quality control
stdev	Standard deviation
mAb	Monoclonal antibody

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Fig. 1. Edema factor three-step method schematic. Step 1: EF/ETx is purified from anthrax samples by EF- and PA-mAbs on magnetic beads. Step 2: adenylyl cyclase (AC) reaction where purified EF is incubated with ATP, calcium, and calmodulin producing cAMP. Step 3: cAMP from processed plasma standard 2 at 3.1 ng/mL total EF detected and measured by LC-MS/MS

Fig. 2.

EF/ETx recovery and activity from recombinant materials and infection samples. EF/ETx generated cAMP area ratios for **a** activity of recombinant EF (rEF) in the presence of PA63 and PA83 at the ratios indicated, **b** recovery of rEF monomer by EF-mAb magnetic beads over a range of concentrations compared to no-capture control, **c** recovery of EF/ETx from two samples from rhesus macaques with inhalation anthrax diluted 1:10 (RM1) and 1:100 (RM2) for total EF (EF/PA-mAbs) and ETx (PA-mAbs) compared to no-capture control, and **d** recovery of rETx from standards containing 50:1 PA63:EF for total EF (EF/PA-mAbs) and ETx (PA-mAbs)

Fig. 3.

Detection and quantification of total EF adenylate cyclase activity in plasma. **a** Dual log₁₀ transformed plot of total EF concentration versus the area ratio of the cAMP analyte ion generated by the custom VB.Net software for 5-PL curve fits with robust weighting. The cutoff for detection is determined by both the calculated limit of detection (LOD) (gray vertical line) and the signal at 3 three times the area ratio generated from the plasma blank (yellow horizontal line). **b** Chromatogram of the cAMP confirmation ion trace for the five lowest standards and the plasma blank. The black arrow indicates the calculated LOD. Focused y-axis shows more chromatographic detail of signal near the LOD. **c** Plot of spiked edema factor concentration vs the calculated mean from 90 analytical runs demonstrate linearity and 95% confidence intervals (CI) over the range. Black symbols represent calculated upper and lower 95% CI for standards S2–S11 in Table 1. The equation and R^2 are from the linear fit

Fig. 4.

Comparison of total LF and total EF levels over the course of infection in five rhesus macaques with inhalation anthrax. Samples collected pre-exposure and every 24 h out to 120 h in five rhesus macaques (RM) exposed by inhalation to high dose *B. anthracis* Ames spores. RM1, RM2, and RM3 died/ethanized at 96 h, RM4 at 120 h, and RM5 after 9 days. EF as well as LF exhibited a triphasic kinetics of toxemia [18]. LF and EF increased in phase-1 (P1), LF declined/plateaued and EF levels declined rapidly diverging from LF in phase-2 (P2), then both increased, with EF more rapidly, converging with LF at terminal stages in phase-3 (P3). LF/EF ratios represent an indicator of anthrax progression. The arrow demonstrates the divergent LF/EF levels during an extended phase-2 for RM5

Table 1

Performance evaluation of spiked standards and quality control (QC) materials. ETx spiked in plasma forms calibration standards and QC materials that were analyzed 90 independent times over a period of 4 years. Means, standard deviation (Stdev), precision (relative standard deviation—RSD), and accuracy (% error and % accuracy) of all results were included. Standards and QCs with acceptable precision ($\leq 21\%$ RSD) and accuracy ($\leq 20\%$ error) were noted with bold font. The calculated limit of detection (Calc LOD) at 0.00002 ng/mL (italic) falls between Std-10 and Std-11.

	Expected (ng/mL)	Observed mean (ng/mL)	Std Dev	RSD	% Error	% Acc
Std-01	12.5	9.1	2.83	31.0 *	27.2 *	72.8
Std-02	3.12	3.4	0.70	20.4	9.6	90.4
Std-03	0.78	0.91	0.164	18.0	16.9	83.1
Std-04	0.19	0.21	0.030	14.0	12.1	87.9
Std-05	0.049	0.05	0.0040	8.8	6.9	93.1
Std-06	0.012	0.012	0.00084	7.0	0.53	99.5
Std-07	0.0031	0.0029	0.00025	8.7	5.7	94.3
Std-08	0.00076	0.00088	0.00011	13.0	15.3	84.7
Std-09	0.00019	0.00020	0.000033	16.6	3.4	96.6
Std-10	0.000048	0.000043	0.000009	21.0	10.1	89.9
Calc LOD	NA	<i>0.000020</i>	NA	NA	NA	NA
Std-11	0.000012	0.000015	0.0000056	38.0 *	22.7	77.3
Std-12	0.000006	0.0000062	0.0000041	65.6 *	3.8	96.2
Blank	0.000000	–0.000000010	0.0000049	NA	NA	NA
QC-Low	0.00030	0.00033	0.000054	16.6	8.8	91.2
QC-Med	0.0060	0.0060	0.00070	11.7	0.51	99.5
QC-High	0.090	0.093	0.015	15.8	3.6	96.4

* Standards above the 21% RSD and 25% upper and lower limit of quantification

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Stability of plasma standards material was assessed for a medium level at 0.012 ng/mL and low level at 0.00076 ng/mL concentrations. The control was thawed and analyzed in September 2018, alongside identical aliquots that were frozen and thawed and frozen-thawed an additional three times (– 80 °C) and an aliquot left on the bench at room temperature for 24 h (bench-top). Freeze-thaw (FT) and bench-top stability samples were compared to the control as the percent (%) difference of the means. Current control results were also used as long-term stability test (LTS) as compared to results from the same material analyzed in February 2013. All samples were analyzed in triplicate, replicates 1–3 (Rep)

Table 2

	Control and LTS Sept 2018	3 FT cycles	Bench-top stability	Original Feb 2013
Medium plasma ETx standard material (0.012 ng/mL)				
Med Rep 1	0.0114	0.0107	0.0124	0.0120
Med Rep 2	0.0123	0.0109	0.0121	0.0117
Med Rep 3	0.0130	0.0135	0.0125	0.0126
Mean	0.0122	0.0117	0.0123	0.0121
% difference	+ 1.0	– 4.0	1.0	–
Low ETx plasma standard material (0.00076)				
Low Rep 1	0.000894	0.000780	0.000976	0.000813
Low Rep 2	0.000892	0.000838	0.000949	0.000858
Low Rep 3	0.000957	0.000994	0.000931	0.001028
Mean	0.000914	0.000870	0.000952	0.000900
% difference	+ 1.6	– 4.8	4.2	–

Table 3

Quantitative values of EF and LF in ng/mL and qualitative values for PCR (*pagA*) and culture in samples collected from 5 rhesus macaques infected by inhalation of *B. anthracis* Ames strain spores. RM1, RM2, and RM3 died/euthanized at 96 h (4D), RM4 at 120 h (5D), and RM5 at 9D (no terminal sample collected). Serum samples were only collected out to 120 h. Terminal whole blood collected for RM-05 was culture positive on day 9 (not shown). EF levels were low (0.66 ng/mL) until terminal samples. LF/EF ratios shown were high early in phase-1 (48 h), even higher in phase-2 (72 h), and decrease in late stages phase-3 (96–120 h) (bold font). Phase-3 samples were not collected for RM-05. Culture results were classified as positive (+) when there were more than five colonies, negative (–) if there were no colonies and ± if there are 1–5 colonies. h = hours and D = days. Time points are relative to inhalation spore exposure time. LF levels were measured previously and reanalyzed with improved methods [18, 20]. The detection limit for this study is 0.00002 ng/mL (0.02 pg/mL) allowing detection of several animal samples (italic) lower than or close to the LOD of previous methods for spiked animal samples at 10 pg/mL, respectively [12]

	Time point	– 42D	24 h	48 h	72 h	96 h	120 h
RM1	EF	<LOD	<i>0.00016</i>	0.51	0.060	2220	
	LF	<LOD	<LOD	42.0	25.1	8670	
	PCR	–	–	+	+	+	
	Culture	–	–	+	+	+	
RM2	LF/EF			82.1	418	3.9	
	EF	<LOD	<LOD	0.66	0.019	5.88	
	LF	<LOD	0.006	45.2	10.3	103	
	PCR	–	–	+	+	+	
RM3	Culture	–	–	+	–	+	
	LF/EF			68.4	535	17.5	
	EF	<LOD	<i>0.00042</i>	0.53	<i>0.0086</i>	9.40	
	LF	<LOD	0.20	51.7	10.6	34.1	
RM4	PCR	–	–	–	+	+	
	Culture	–	–	+	–	+	
	LF/EF		475	97.8	1231	3.6	
	EF	<LOD	<LOD	0.18	0.023	0.47	369
	LF	<LOD	<LOD	25.9	19.0	63.6	2262
	PCR	–	–	–	+	NA	+
	Culture	–	–	+	±	NA	+
	LF/EF			144.9	836.4	136.4	6.1

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Time point		- 42D	24 h	48 h	72 h	96 h	120 h
RM5	EF	<LOD	<LOD	0.115	0.00061	0.0025	0.036
	LF	<LOD	0.018	38.1	8.06	4.48	27.7
	PCR	-	-	+	-	NS	NS
	Culture	-	-	+	-	NS	NS
	LF/EF			332	13,100	1775	765