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Accurate and selective quantification of anthrax protective antigen in plasma by immunocapture and isotope dilution mass spectrometry†

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Abstract

Anthrax protective antigen (83 kDa, PA83) is an essential component of two major binary toxins produced by *Bacillus anthracis*, lethal toxin (LTx) and edema toxin (ETx). During infection, LTx and ETx contribute to immune collapse, endothelial dysfunction, hemorrhage and high mortality. Following protease cleavage on cell receptors or in circulation, the 20 kDa (PA20) N-terminus is released, activating the 63 kDa (PA63) form which binds lethal factor (LF) and edema factor (EF), facilitating their entry into their cellular targets. Several ELISA-based PA methods previously developed are primarily qualitative or semi-quantitative. Here, we combined protein immunocapture, tryptic digestion and isotope dilution liquid chromatography-mass spectrometry (LC-MS/MS), to develop a highly selective and sensitive method for detection and accurate quantification of total-PA (PA83 + PA63) and PA83. Two tryptic peptides in the 63 kDa region measure total-PA and three in the 20 kDa region measure PA83 alone. Detection limits range from 1.3–2.9 ng mL^{-1} PA in 100 μL of plasma. Spiked recovery experiments with combinations of PA83, PA63, LF and EF in plasma showed that PA63 and PA83 were quantified accurately against the PA83 standard and that LF and EF did not interfere with accuracy. Applied to a study of inhalation anthrax in rhesus macaques, total-PA suggested triphasic kinetics, similar to that previously observed for LF and EF. This study is the first to report circulating PA83 in inhalation anthrax, typically at less than 4% of the levels of PA63, providing the first evidence that activated PA63 is the primary form of PA throughout infection.

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Conflicts of interest

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names and commercial sources are for identification only and do not constitute endorsement by the U.S. Department of Health and Human Services, or the U.S. Centers for Disease Control and Prevention.
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Introduction

Bacillus anthracis, the causative agent of anthrax in humans and animals, is a Gram-positive spore-forming pathogen that is widely distributed in the environment.[1] Naturally occurring human anthrax manifests as cutaneous, gastrointestinal and inhalation forms, with cutaneous being the most common accounting for 95–98% of reported cases with a 20% mortality if untreated or <2% with antibiotic treatment.[1–3] Inhalation anthrax is the most deadly form, with a fatality rate of approximately 90% without treatment.[2] Recently, a new form of anthrax resulting from intravenous drug use was recognized, which was linked to heroin contaminated with *B. anthracis* spores.[4] The main virulence factors include three proteins, protective antigen (PA, a binding and translocation protein), lethal factor (LF, a zinc-dependent endoproteinase) and edema factor (EF, an adenylate cyclase). These proteins combine to form two toxins, lethal toxin (LTx; formed from PA and LF) and edema toxin (ETx; composed of PA and EF) that have synergistic effects in suppression of the host immune response.[5]

The mechanisms of action for PA, LF and EF have been studied extensively.[5,6] *B. anthracis* spores are phagocytized by macrophages where they germinate. The resulting vegetative cells express full-length PA (PA83; 83 kDa), LF (90 kDa) and EF (89 kDa). The individual proteins are relatively harmless on their own and PA83 does not appreciably bind either LF or EF.[7] It is well established[8] that domain 4 of PA83 binds to two known receptors on the host cell surface, tumor endothelium marker 8[8,9] and capillary morphogenesis protein 2.[10] There it is cleaved by furin-like protease activity[11] to a 63 kDa form (PA63) with loss of the 20 kDa N-terminal region (PA20). Recently, it has been reported that PA83 has calcium-dependent protease activity.[12] The PA63 self-associates into a heptamer[6] or an octamer,[13] which can then bind 3 or 4 molecules of LF and/or EF, thus forming LTx, ETx and possibly mixed toxin complexes. The PA63 oligomers form pores on the endosome membranes through which LF and EF are transported into the cytosol, where their combined enzyme activities exert potent synergistic toxic effects.[14]

It has been demonstrated that PA83 is not only cleaved and activated at cell surfaces, but is also cleaved to PA63 by the action of serum proteases and that functional LTx is found in blood in late stage infection.[7,14,15] The serum cleaved form is found bound to LF as LTx and circulating in serum throughout infection.[16] However, to date, no free PA83 has been detected in blood, although the PA20 fragment has been found.[17] The ratio of PA83 to PA63 throughout infection has not been described.

Diagnosis of inhalation anthrax can be challenging because initial symptoms are nondescript, yet the disease can rapidly progress without treatment.[1,18] Methods available for confirmation of anthrax infection include a wide range of antibody-based, nucleic acid-based, and phenotypic approaches[19,20] that encompass diverse targets including spore and vegetative cell markers, PA, LF, EF and their complexes, gamma-linked poly-D-glutamic acid (PGA) capsule, and bacterial mRNA and DNA. As part of the efforts to improve diagnosis of anthrax, a sensitive and specific mass spectrometry (MS) method for detection of LF in serum and plasma was developed and fully validated.[21,22] The LF MS method was recently included as a confirmatory anthrax diagnostic method in the updated Council of

State and Territorial Epidemiologists (CSTE) clinical case classification for human anthrax.[23] Its use in experimental anthrax revealed a triphasic progression of inhalation anthrax in rhesus macaques characterized by a rise-plateau-rise of LF over time.[24] The two phases of rapid toxin increases are typically separated by a period of variable length in which toxins plateau or decline (phase-2). This fits the clinical staging of inhalation anthrax described with a first, early prodromal phase, a second, intermediate/progressive stage, and a third, late-fulminant stage, with a low survival probability.[25] EF has recently been shown to have similar triphasic toxin kinetics but is typically present at much lower levels during anthrax infection compared to LF.[26] The levels and kinetics of PA at all stages of anthrax have not yet been reported and an understanding of these may tell us more about the course of disease and potential outcomes with inhalation anthrax. The importance of obtaining an overall picture of anthrax toxins has been stressed by others.[27,28]

A range of qualitative and quantitative immunocapture-based methods for PA developed for various clinical and research purposes were reviewed in 2006.[20] Some more recent immunoassay PA methods have used traditional ELISA,[29,30] electrochemiluminescence (ECL)[30,31] and time-resolved fluorescence (TRF).[32,33] Some alternative approaches have included fluorescence methods.[18,34] Taken overall, and given different approaches for determination of the limits of detection (LOD), the LODs appeared to range from 0.0001 to 83 ng mL^{-1} PA for these methods. However, many of the more sensitive measurements have not been achieved on relevant clinical samples such as plasma or serum. In general, lower method LOD's would provide the opportunity for earlier detection of anthrax infections (during phase-1 or phase-2), and therefore facilitate more successful treatment outcomes. Currently, an MS-based method for LF appears to provide the earliest time to detection, with LF levels in a non-human primate model ranging from 0.015–1.39 ng mL^{-1} from 18–24 hours after high spore dose exposures.[22] By 48 hours, the average time of symptom onset, LF levels ranged from 4.87–468 ng mL^{-1}. PA levels in New Zealand White rabbits measured by ECL ranged from approximately 2–200 ng mL^{-1} at 48 hours after high dose exposures.[35] In the context of an intentional release, index cases will typically present with symptoms and toxin levels above detection limits of most methods (i.e. around 1 ng mL^{-1}). Methods with lower detection limits, such as the MS-based LF method,[22] may be useful for diagnosing pre-symptom onset exposures from a known release.

A traditional sandwich ELISA was developed with specific antibodies capable of distinguishing PA63 and PA83 to study cleavage and clearance of PA from blood.[15] A highly sensitive immunoassay using europium nanoparticles for detection has been reported.[32] The LOD was found to be about 0.02 ng mL^{-1} for PA in plasma, but it was only found to be suitable for qualitative or semiquantitative analysis. A PA ELISA using a europium anti-PA IgG for TRF was applied to inhalation anthrax samples.[33] Though not differentiating the two PA forms, it tested recovery of both PA83 and PA63 and achieved reliable detection limits of 0.551 and 1.58 ng mL^{-1}, respectively. However, the precision and accuracy achieved suggested the method was more appropriate for qualitative analysis of clinical samples. Other ECL methods have been utilized for PA83 quantitation in New Zealand white rabbits.[35] The authors described acceptable but limited assessment of accuracy and precision with a lower limit of quantitation of 0.5 ng mL^{-1} PA83 in plasma. However, the full method quantitative parameters and validation were not described and the method did not assess

recoveries of PA63.[35] Qualitative ECL and quantitative ELISA methods have been used to study inhalation anthrax in cynomolgus macaques.[36] Although an LOD of 2.4 ng mL^{-1} PA in serum using ELISA was reported, the form of PA was not defined and very limited details of the quantification are given.[36] To date, no methods have been applied to quantify and investigate the presence of both full length PA83 and active PA63 forms throughout the course of experimental inhalation anthrax.

Isotope dilution LC-MS/MS (IDMS) is ideally suited for accurate and sensitive quantitative methods and can provide a level of specificity that is generally not possible with fluorescence or luminescence tagged antibody detection systems. The MS methods developed in our laboratory for LF and EF employ antibody capture, enzymatic activity and IDMS strategies.[16,21,22,24,26,37] Here, we describe an antibody capture, on-bead tryptic digest, and IDMS method for the quantification of anthrax PA. This represents the first use of magnetic antibody bead capture for PA, combined with on-bead tryptic digestion, releasing PA peptides for LC-MS/MS quantification of targeted regions of PA. A combination of two antibodies were able to capture and purify both full length PA83 and active PA63, as they both bind to the PA63 region and not to the N-terminal PA20 region. Therefore, quantification of peptides within the 20 kDa region gave a measure of full length PA83 and peptides within the 63 kDa region gave a measure of both PA83 and PA63 (total-PA). The difference between the two provides a measure of active PA63 competent for cellular intoxication. We validated the method and applied it to differentiate and quantify the forms of PA in serum of five rhesus macaques (*Macaca mulatta*) with inhalation anthrax. These measurements were compared to previously published PA, LF, PCR and bacteremia results.[24]

Experimental procedures

Materials

Recombinant anthrax toxins PA83, PA63, LF and EF were from List Biological Laboratories (Campbell, CA). Normal North American (NNA) human ten-donor-pooled serum and plasma, and plasma (with EDTA anticoagulant) from 100 individual NNA donors were obtained from Interstate Blood Bank (Memphis, TN), Anti-PA mouse monoclonal antibodies (mAb) AVR1046 and AVR1162 were prepared at CDC by the Division of Scientific Resources,[21] Invitrogen™ DYNAL™ Dynabeads™ MyOne™ tosyl-activated magnetic beads (MB) were from ThermoFisher Scientific (Waltham, MA), 1× phosphate buffered saline with 0.05% Tween-20 (PBST) was from Growcells (Irvine, CA), 0.1% Formic Acid (FA) in water and 0.1% FA in Acetonitrile (ACN) were from ThermoFisher Scientific, RapiGest™ SF Surfactant (Waters Corporation, Milford, MA), Sequencing grade modified trypsin was from Promega, (Madison, WI). PA peptide 13C, 15N isotopically labeled internal standards (IS) included (I + 7)QYQR (for tryptic peptide T12), G(L + 7)DFK (for T14), EVISSDN(L + 7)QL (P + 6)ELK (T17), D(L + 7)NLVER (T54), and NN(I + 7) AVGADESVVK (T67) were custom made by MidWest Bio-Tech, Inc. (Fishers, IN). Unlabeled native forms of the same peptides were synthesized and quantified by amino acid analysis by New England Peptide (Gardner, MA). All chemicals were of the highest purity available and used without further purification.

Animal study protocol

Sample aliquots remaining from a previous approved protocol were used for this method.[24] Briefly, five female rhesus macaques obtained from Covance (Alice, TX) were anesthetized and challenged *via* head-only with *B. anthracis* Ames spores at a calculated aerosol dose of GMC ± SE of 378 LD_{50} ± 8% equivalents, higher than the target spore dose of 200 LD_{50} with exposure times ranging from 10 to 30 minutes.[24] Though the spore dose delivered was higher than the target, it corresponds to that used in previously reported studies designed to yield high infection rates.[38]

Animals were challenged at the Battelle Biomedical Research Center (Columbus, OH) and the study protocol was approved by Battelle (protocol no. 570, carried out 2006–2007) and the Centers for Disease Control and Prevention's (CDC) Institutional Care and Use Committees (IACUC; protocol no. 1459BOYMON) following National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Serum from the 5 macaques reported here was collected at 42 days pre-exposure and at 2, 4, 6, 12, 24, 48, 72, 96 and 120 hours post-exposure for the analysis of LF, PA and PGA. For the present study, only those samples with sufficient volume remaining were analyzed for PA.

Characterization of PA and peptide selection

Recombinant PA83 was the primary standard material used for quantification in this method. Relative amounts of PA63 and PA83 in different purchased lots of material over several years were confirmed to be within 20% of each other using the 'High-3' relative protein quantification method[39] on tryptic digests by nanoflow LC-MS/MS^E on a Synapt G2S (Waters Corporation) high resolution accurate mass system. Unambiguous tryptic peptide and MS/MS fragment identifications[40] were obtained by comparison with the expressed PA sequence in UniProtKB P13423. These findings formed the basis for selection of the 5-tryptic peptides used in this work, along with optimal internal standard isotopic label positions and multiple reaction monitoring (MRM) transitions, Table 1. Three peptides fall in the N-terminal 20 kDa region and two in the 63 kDa region.

Antibody capture optimization

The two anti-PA mAbs both capture PA83 and PA63 (total-PA). The AVR1046 epitope is within domain 4 (PA residues 625–764; P13423).[33,41] AVR1162 binds both PA83 and PA63.[33] Recoveries of PA83 and PA63 at 5 and 25 pmol mL^{-1} were evaluated, using AVR1046 and AVR1162 both individually and combined.

Recombinant PA83 standard purity assessment

It was necessary to verify the purity and quantify the amount of PA83 in the recombinant material used in the method. Therefore, the recombinant PA83 was independently quantified by isotope dilution LC-MS/MS using peptide-based calibration curves. Briefly, five-point peptide standard calibration curves for the 5 native peptides were prepared based on concentrations obtained by amino acid analyses. Aliquots of recombinant PA83 in 50 mM ammonium bicarbonate were subjected to tryptic digestion following a procedure similar to that described below but without the antibody capture. Internal standard peptides were added and calibration curves over the range 60–300 pmol mL^{-1} final concentration were fitted by

linear regression. Results of this peptide-based quantification of the PA83 material confirmed that the amounts were correct, within the experimental error of the method.

Sample preparation

Appropriate safety control measures, including engineering, administrative, and personal protective equipment, were used for all procedures based on a site-specific risk assessment that identified physical, health and procedural hazards.

Recombinant PA83 was spiked into plasma pools to produce a calibration curve with 10 standards concentrations spanning between 0.017 to 333 pmol mL^{-1} along with quality control (QC) pools at 2.75 (QCL) and 60 (QCH) pmol mL^{-1}. A plasma blank was also included. Standards and QC pools were aliquoted and kept frozen at −70 °C until further use. Anti-PA mouse monoclonal antibodies (mAb) AVR1046 and AVR1162 were prepared as described previously,[33] and then individually cross-linked to magnetic antibody beads following manufacturer's instructions. The resulting antibody coated beads solutions were mixed 1 : 1 (v/v) to allow for optimal dual antibody binding to the tosyl-activated MBs.

Three steps are followed for PA analysis. In step 1, PA is bound to dual anti-PA magnetic beads (PA-MB). Except for plate preparation, all events were carried out using a KingFisher Flex purification system 96 plate (ThermoFisher Scientific, Waltham, MA). Blanks, standards, QCs and unknown samples were spiked at 100 μL of each, with 900 μL of PBST buffer in a deep 96-well plate. 20 μL PA-MB per sample were resuspended in 70 μL of PBST buffer in a separate 200 μL 96-well plate. PA-MB were mixed with the samples, for 1 hour PA binding, then transferred and washed twice in 1 mL PBST for 1.5 minutes each, then two times in 200 μL dH_20 for 1.5 minutes after which, the beads were released and collected. The beads were manually transferred with multi-channel pipette from the final plate to a 24 or 48-well PCR plate from Molecular Products, Inc. (San Diego, CA) on a DynaMag™ 96 side magnet plate (ThermoFisher Scientific) and dH_20 was removed from the PA-MB's.

In Step 2, PA is hydrolyzed by trypsin with an on-bead tryptic digest. All events were carried out in a GeneAmp® PCR system 9700 thermal cycler (ThermoFisher Scientific). In general, plates were capped and mixed at 1500 rpm for 2 minutes using an Eppendorf MixMate mixer (Eppendorf, Hauppauge, NY) after the addition and/or incubation steps. Briefly, after water removal, 10 μL of 0.2% RapiGest in 50 mM ammonium bicarbonate was added to each sample, heated for 10 minutes at 99 °C, then cooled to 4 °C. Then 10 μL with 1.3 μg of sequencing grade modified trypsin (pre-diluted 1 part trypsin to 2 parts 50 mM ammonium bicarbonate) was added, samples incubated at 42 °C for 2 hours, then cooled at 4 °C. Lastly, a PA internal standard (IS) solution was prepared prior to addition by adding 200 μL of the PA IS mix containing the PA isotopically labeled peptides to 400 μL of 0.45 M HCL. 10 μL of this PA IS solution was added, then samples were incubated for 1 hour at 37 °C, then cooled at 4 °C. The sample plate was mixed, then centrifuged for 30 minutes at 4 °C at 2204 rcf, placed on the magnet and 25 μL of the digested sample mixture was placed in individual LC vials for LC-MS/MS analysis.

Step 3 includes isotope-dilution LC-MS/MS for analysis of specific tryptic peptides over the length of PA, including three in the 20 kDa and two in the 63 kDa regions. Details of LC-MS/MS are described below.

LC system

A Waters Acquity UPLC® system (Waters Corporation) was used for all LC-MS/MS analyses. An Acquity UPLC® BEH reverse phase C18 1.0 × 100 mm ID, 1.7 μm particle size analytical column (Waters) was used for all separations. The column was kept at 50 °C and the autosampler at 6 °C throughout all analyses, and the sample injection volume was 10 μL. The aqueous mobile phase (A) and the organic mobile phase (B) consisted of 0.1% FA in water and 0.1% FA in acetonitrile respectively. Peptides were separated using a linear gradient from 2.5% (v/v) B to 35% (v/v) B in 13 minutes at 200 μL min^{-1}, and the cycle time was 16.5 minutes including a high-organic wash and reequilibration to initial conditions.

Mass spectrometer

A QTRAP® 6500 triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, MA) with an IonDrive Turbo-V ion source operating in standard positive ion electrospray ionization (ESI) mode and multiple reaction monitoring (MRM) at unit resolution was used for all LC-MS/MS analyses. PA tryptic peptide data were acquired using MRM transitions as listed in Table 1 for 5 native and 5 corresponding isotopically labeled PA tryptic peptides. The isotopic labels confer identical chemical properties, such as retention time, whilst making these internal standard peptides fully distinguishable based on their mass differences (7 or 13 Da, depending on the peptide) compared to the native forms. In each case, the *m/z* values were chosen based on theoretical models of the peak tops of the isotopic envelopes at 80% height at the 0.7 full width half-maximum quadrupole resolution used. Two MRM transitions were included for each tryptic peptide, with expected signal ratios being used to confirm correct peak identifications. Fixed instrument parameters included curtain gas 35, collision gas High, ion spray voltage 5500 V, source temperature 350 °C, ion source gas 1 (GS1) 50, and ion source gas 2 (GS2) 50. Declustering potential (DP) and collision energy (CE) voltages were optimized for each individual peptide. Instrument control and data acquisition were performed using Analyst software v1.6.2.

Data processing and quantification of PA

Analyst Quantification Results tables were built for each analytical run, and then raw chromatograms and sample information (but not peak areas) were exported using custom VB. Net automation programs. All peak integration, quantitation and reporting functions were fully automated by a custom VB. Net program, with minimal manual intervention. Performance of the custom software for quantification purposes was confirmed to be identical to that of Analyst v1.6.2 (data not shown), whilst providing a wider range of capabilities and improved ease of use. The custom software included functions for least-squares fitting of calibration curves based on equally-weighted $\log_{10}$ peak area ratios (native/internal standard areas) and $\log_{10}$ given amounts using 3rd-order (cubic) polynomials as required for the PA method, and for reporting quantitative measurements at the peptide and protein levels after combining results for groups of MRM transitions.

Limits of detection (LOD) were calculated for individual MRM pairs (for the native and corresponding internal standards) using statistical methods similar to those described previously[16] based on the first 22 analytical runs. LOD's were in the region of 0.018 to 0.035 pmol mL^{-1} for the most sensitive MRM transition from each peptide (equivalent to 1.3 to 2.9 ng mL^{-1} for the PA83 mass), Table 1. Total-PA (PA63 + PA83), was quantified by averaging signals above the LOD's from peptides T54 and T67 (Fig. 1). Similarly, full length PA83 was quantified by averaged signals from peptides T12, T14 and T17 (Fig. 1). To ensure quality, signals from a minimum of two MRM transitions and two peptides were necessary for a positive result in all cases. To be included in the quantitative measurements, each MRM signal was also required to be within the range of the standard curve, and to have a peak area ratio that was 3-fold or more above that of the plasma blank sample in the same analytical run. The application of these filters along with multiple MRM transitions and multiple peptides conferred both robustness and specificity on the quantitative measurements. These calculations were carried out in units of pmol mL^{-1}, after which the reported amounts of total-PA, PA83 and PA63 (obtained by subtracting PA83 from total-PA) could be converted to ng mL^{-1}.

Method validation

Data from 22 analytical runs acquired over a 10-week period by one analyst using one LC-MS/MS instrument were collected. Quantitative measurements were obtained for total-PA and PA83 alone as described above for the two QC materials at 2.75 pmol mL^{-1} and 60 pmol mL^{-1}. The 2.75 pmol mL^{-1} QC pool yielded combined MRM means and coefficients of variation (%CV's) of 2.69 (5.3%) and 2.70 pmol mL^{-1} (5.3%) for total-PA and PA83 respectively, both with errors of 1.0%. The means and CVs for the 60 pmol mL^{-1} QC pool were 59.0 (5.7%) and 59.0 pmol mL^{-1} (6.3%) for total-PA and PA83 respectively, both with errors of 1.7%. Quality control procedures met the requirements for quantitative analytical LC-MS/MS methods.[42]

Results and discussion

Method development and performance

The binary anthrax toxins affect the outcome of infection in various ways, exerting deleterious effects on different tissues.[43] LF, LTx and EF levels have been quantified[16,22,26] but less is known about PA levels and its forms during the course of infection. This knowledge is needed to understand the progression of anthrax and which PA forms are the most appropriate targets for therapeutic interventions. The PA mass spectrometry method described here complements the LF and EF methods in use in our laboratory for the comprehensive study of anthrax toxin levels during infection and treatment. The organism secretes full-length PA83 monomer, which is known to be processed to the active PA63 form. Therefore, both forms are anticipated to be found *in vivo*. The method is different from most others developed for PA,[15,32,33] in that it is the first to (1) combine quantification of both PA83 and PA63, (2) employ targeted MS-based peptide specificity, (3) include full validation and (4) be applied to measure both forms throughout the all stages of inhalation anthrax in a non-human primate model.

The overall strategy for analyzing both PA forms was to extract total PA by targeting the carboxy-terminal domains for magnetic antibody bead capture purifying both PA83 and PA63 (total-PA) (Fig. 1). Tryptic digest on the beads would release peptides over the length of all PA forms extracted. PA20 peptides would only be present in full length PA83 (PA83), whereas PA63 peptides would be present in both PA83 and PA63 (total-PA). As measured by LC-MS/MS, the difference between total-PA and PA83 would give the amount of PA that is active PA63. Following PA-targeted mAb capture, tryptic digestion yields the peptide targets indicated (Table 1 and Fig. 1). The peptides identified were unique and two, T54 and T67, present in both PA forms, quantify total-PA, whereas T12, T14, and T17, present only in PA83, quantify PA83.

Typical LC-MS/MS performance for a high-level PA83 plasma standards sample is shown in Fig. 2, with chromatograms of the 5 native PA peptides and their associated internal standard peptides. Signal over the range of calibration standards from 0.017 to 333 pmol mL^{-1} were best modeled for all peptides with equally weighted $\log_{10}$ peak area ratios (native/internal standard areas) and $\log_{10}$ given amounts using 3rd-order (cubic) polynomial fits (Fig. 3). In addition to the 22 analytical runs used for method validation, a further 49 analytical runs have been obtained to date (most of which were for additional inhalation anthrax studies not described here). The 71 analytical runs gave precision <7.0% CV for the QC materials, providing confidence of the general suitability and robustness of this method.

Antibodies were selected with the strategy to target domains 2–4 and pull out both full length PA83 and active PA63. AVR1046 was selected since the epitope was previously mapped to domain 4 (PA residues 625–764; P13423).[33,41] AVR1162 was also selected which was shown to bind both PA83 and PA63 but not compete with AVR1046.[33] Assessment of recoveries for AVR1046 and AVR1162 both alone and combined (2 mAbs) showed that 2 mAbs improved recovery of PA83 up to 16% (to a total of 89%) over that of AVR1046 alone at 5 and 25 pmol mL^{-1} PA83 protein concentrations in plasma. However, recovery of PA63 with AVR1046 alone was high (87%) and no further enhancement was gained by addition of AVR1162. Overall, AVR1046 was sufficient for recovery of PA63, and AVR1162 improved recovery of PA83 indicating that the combination of the two anti-PA mAbs was beneficial for total-PA recoveries. The use of full-length PA83 as the standard for quantification has the advantage that all samples (standards, QCs, blanks and unknowns) are subject to the same antibody extraction procedure and all following steps (digestion, LC-MS/MS). The use of standards and QC's on the same plate therefore largely corrects for the recovery of the antibody extraction and the digestion. Accuracy of the LC-MS/MS analysis is based in the use of isotope labeled peptide internal standards.

Recombinant PA83 used as a primary standard material in our method was quantified using five amino acid-based synthetic peptide standard curves (Table 1). A mean recovery of 95.3% of the expected level of recombinant PA83 with standard deviation of 13.2% ($N = 20$) was obtained. This confirmed that the assigned level of PA83 was correct within an acceptable experimental error for this type of measurement.

The reports that PA83 is activated to PA63 by serum proteases *in vivo*[7,14] are relevant as to whether the recombinant PA83 spiked in EDTA-plasma in our primary standards would also

be cleaved to PA63. This could hinder accurate quantification of the peptides in the PA20 region (T12, T14 & T17). Confirmation of stability in plasma was obtained by comparisons of peptide signals from the PA83 in the plasma standards (stored at −70 °C for 3 years) with peptide signals from the same batch of recombinant PA83 prepared in buffer alone. The plasma standards were subjected to antibody capture and tryptic digest, but the PA83 in buffer only required tryptic digestion for this purpose. The ratios of absolute signals for the target peptides representing the total-PA and PA83 measurements differed by 5% (within experimental error), indicating minimal conversion of PA83 to PA63 in our spiked primary plasma standards.

The spiked plasma QC materials were further assessed for stability using conditions that might occur during sample handling procedures (ESI, Tables S1–S3†). The results for total-PA and PA83 were all within the FDA-recommended 15% of the established QC values for each QC material.[44]

Interferences in the context of complex infection samples were also assessed. Plasma spiked with combinations of recombinant PA63, PA83, LF and EF was evaluated for anthrax toxin recovery and accuracy, Table 2. A total of 24 individual samples were prepared, which were then analyzed in 3 or 4 analytical runs performed on different days to provide technical replicates. Three levels of PA63 and PA83 were chosen spanning the working range of the PA method, along with levels of LF and EF chosen to model possible ratios in inhalation anthrax samples. Accuracy and precision of the total-PA, PA63 and PA83 measurements were acceptable alone and in all combinations with other toxin components. In samples spiked with both PA63 and PA83, total-PA measurements were doubled as expected. These results confirm that the combined Ab capture and trypsin hydrolysis are equally effective for PA63 and PA83, and that the presence of either LF or EF has no adverse effect on the performance of the method.

Our strategies for combining multiple peptide MRM results per PA region improves assurance of the correct result and reporting high-quality protein-level measurements. This also improves selectivity with the requirement for two or more peptide results. Selectivity was evaluated by analyses of known blank samples (10 human plasma, 10 human serum), and 5 pre-exposure rhesus macaque plasma samples. All yielded negative results for all 5 peptides and MRM's. The ng mL^{-1} equivalent LODs of 1.3 to 2.9 ng mL^{-1} obtained during validation were confirmed in practice with the comprehensive analysis of 1350 positive and negative anthrax infection samples, for which overall detection limits for both total-PA and PA83 were found to be in the region of 2.2 ng mL^{-1} (data not shown). While these detection limits are not better than some reported previously, this is the first method to do so with good accuracy and precision over a range of concentrations and conditions relevant to those seen during inhalation anthrax (Table 2).[33]

Inhalation anthrax toxin levels in a rhesus macaque model

Serum samples from five rhesus macaques exposed to high doses of *B. anthracis* Ames strain spores by inhalation were previously analyzed by PA ELISA, LF, *pagA* (the protective

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c8an02479k

antigen gene) PCR and blood culture (bacteremia).[24] Subsequently, the same samples were analyzed for PA using this new immunocapture, trypsin digest and IDMS method. Table 3 and Fig. 4 show selected results from the original study along with total-PA, PA83 and PA63 measurements using the new MS method. The PA MS measurements update and replace the earlier PA ELISA measurements (retained in Fig. 4 for comparison). Table 3 also includes two updated values for LF, using an improved dual-antibody method developed in our laboratory.[22]

All pre-exposure samples (collected at −42 days) were negative for all methods, total-PA, LF, PCR and culture (data not shown). Analysis of the samples using MS showed that total-PA was first detected in all 5 animals at 48 hours, when culture and *pagA* PCR were first positive (Table 3). Total-PA was consistently detected and quantified at all subsequent time points. As with LF, total-PA was consistently positive at 72 hours when cultures were negative in some animals. PA83 was only detected in four samples, of which three were at death/euthanasia. In these four samples, ratios of PA83 compared to total-PA were low, from 0.6 to 3.9%. The theoretical w/w ratio of PA63 : LF in complex is 1.4 (PA63 octamer/4 LF)[6] or 1.6 (PA63 heptamer/3 LF).[13] We showed previously that the amount of LF complexed with PA63 (LTx) ranged from 5% in early infection up to 100% in late stages.[16] The observed ratios of PA to LF here range from 1.6–15.8 with a mean of 6.6. This suggests that there is excess active PA63 circulating providing a reservoir for continued toxin formation. The results clearly show that PA63 is the primary form of PA present in serum throughout infection.

PA63 levels appeared to be consistent with the triphasic progression reported for LF[24] and EF[26] previously. In the presented study on rhesus macaques, the detection limits and granularity of sample collection did not show the full triphasic profiles, however we have observed this pattern in PA63 in other animal inhalation anthrax studies not reported here. PA63 was not detected at 24 hours for animals A, C, and D. That is because the lowest LF levels reported in animals A, C and D at 24 hours (Fig. 4) were in the 0.006–0.200 ng mL^{-1} range, suggesting that PA levels were below the PA MS method LOD. But by 48 hours the LF levels increased more than 100-fold at the end of phase-1 into easily detectable ranges for PA MS. At phase-1 (48 hours), PA63 ranged from 84.3–310 ng mL^{-1}, then at phase-2 (72 hours) declined or plateaued to 74–163 ng mL^{-1}, then at phase-3 (96–120 hours, except for animal D), increased to 236–42 435 ng mL^{-1} (Table 3). PA results found for animal D were the lowest among all animals and the kinetic profiles were similar to LF with phase-2 declines lasting two days (from 48–96 hours) before starting to rise at 120 hours.[24] This animal did not reach terminal stage until 9 days after exposure but samples later than 5 days were not available because of the study design and IAUAC protocol.

This work supports previous studies that found PA63 in late stages of infection in various animal models.[7,15] It is important to highlight that the detection of PA83 observed in the serum of animals A, B and E represents the first report of this form during inhalation infection. As discussed, detection of full-length PA83 was based on the measured target peptides T12, T14 and T17 in the PA20 region of the protein. PA83 was measured in only one animal at an early time point, at 4.4 ng mL^{-1} at 48 hours (animal A). This animal had the highest total-PA levels, 314 ng mL^{-1}, at that early time point. PA83 was also positive at

terminal time points in animals A, B, and E, in samples that also had the highest total-PA levels. That PA83 is only found transiently and at low ratios compared to PA63 and in these animals only when PA was higher, suggests that its hydrolysis is ongoing and that PA83 is detectable when organism and toxin burdens exceed certain levels. The processes of ongoing cellular toxin binding and uptake, intoxication and receptor turnover, along with the ongoing progression of infection, bacteremia and continued release of PA83, LF and EF, complicate the dynamics.

Conclusions

In this study, a precise, accurate and highly selective method quantifying total-PA and PA83 was developed, validated and applied. We showed that PA63 and full-length PA83 were both present and successfully quantified *in vivo* in sera of rhesus macaques with inhalation anthrax. The significance of the amounts of PA63 and PA83 found during anthrax infection warrants further analysis. The work presented here was developed to provide a better understanding of anthrax toxemia and to elucidate the degree of PA processing during the different stages of anthrax infection. These findings complement the existing LF, LTx, EF and ETx measurements by mass spectrometry in our laboratory in these samples and can potentially improve our understanding of anthrax.

In comparison to traditional and previously reported methods for the detection of anthrax PA, mass spectrometry in combination with antibody immunocapture and tryptic digestion provides the necessary analytical selectivity for the accurate identification and quantification of different PA species in serum or plasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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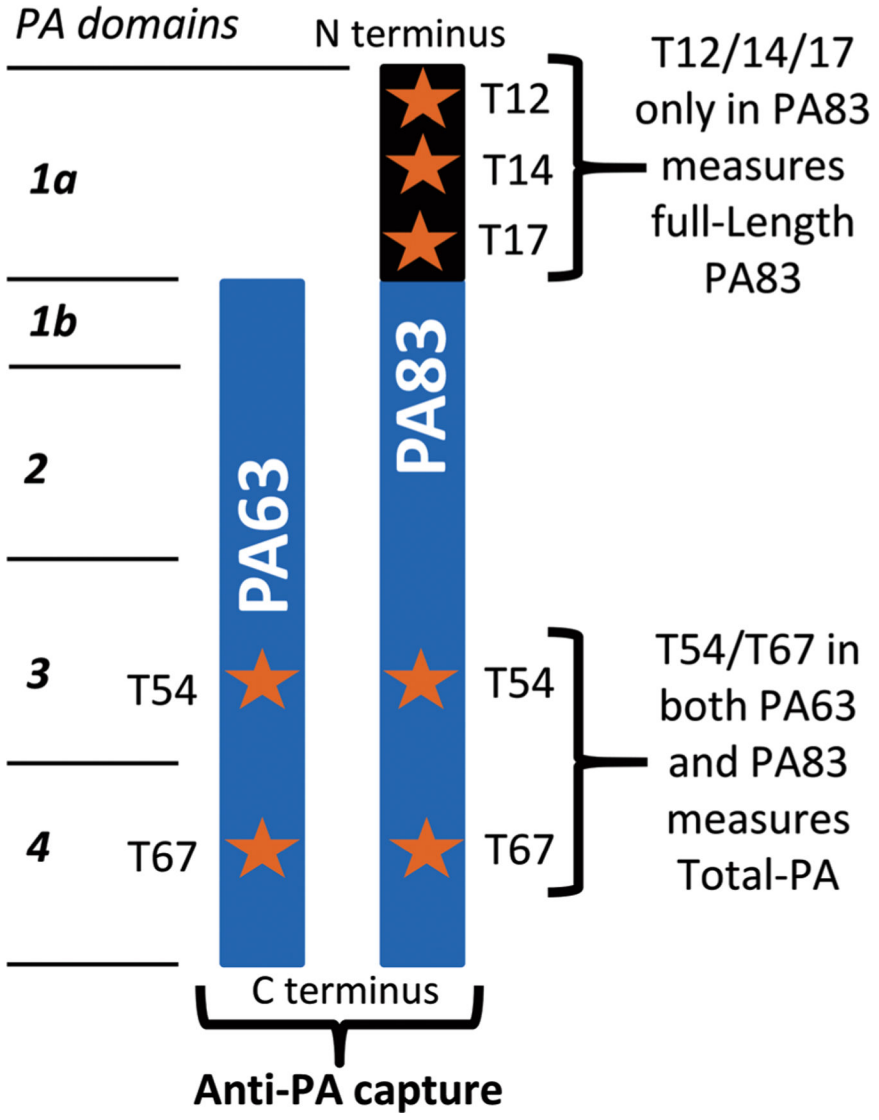

Fig. 1.

Quantification strategy using select PA tryptic peptides. Epitopes for both anti-PA mAbs are within PA domains 1b through 4, such that both full-length PA83 and truncated PA63 are captured. Free PA20 (not shown) is not captured. Proteotypic peptides are indicated by orange stars. Signals from peptides T54 and T67 are representative of total-PA (PA63 + PA83), and signals from peptides T12, T14 and T17 are representative of PA83.

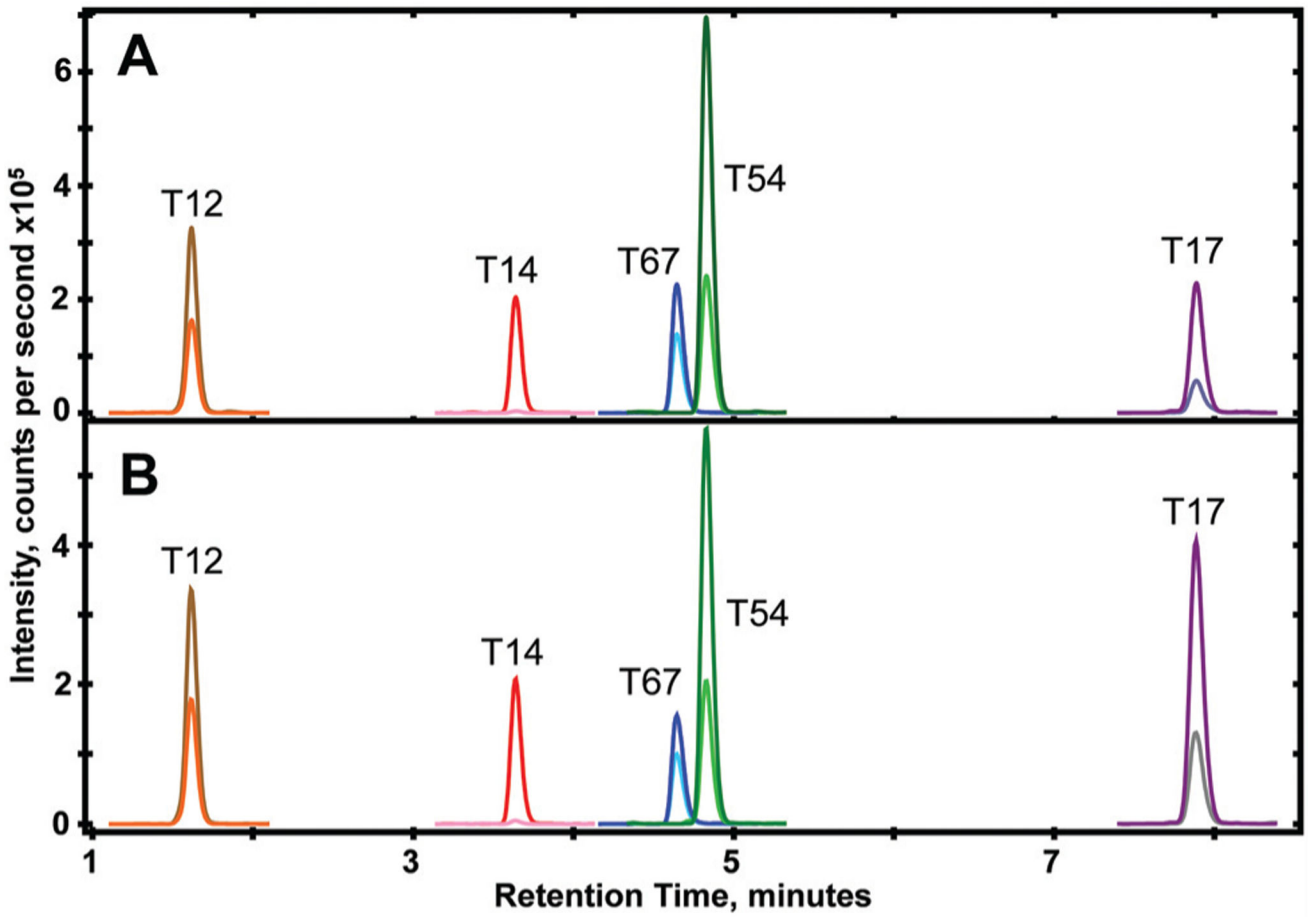

Fig. 2.

Typical chromatographic performance of the anthrax protective antigen method, for a high standard (12.34 pmol mL^{-1} PA83). (A) Individual signals from 10 analyte MRM transitions for the 5 native target peptides T12–T67, and (B) corresponding signals from the 10 internal standard MRM transitions (Table 1) for isotope dilution quantification. In this PA83 standard sample, the levels of peptides in the internal standard mixture gave approximately the same signal intensities as the native peptides. These internal standard levels gave optimal quantitative performance using area ratios, without any observed LC column carryover or isotopic interferences.

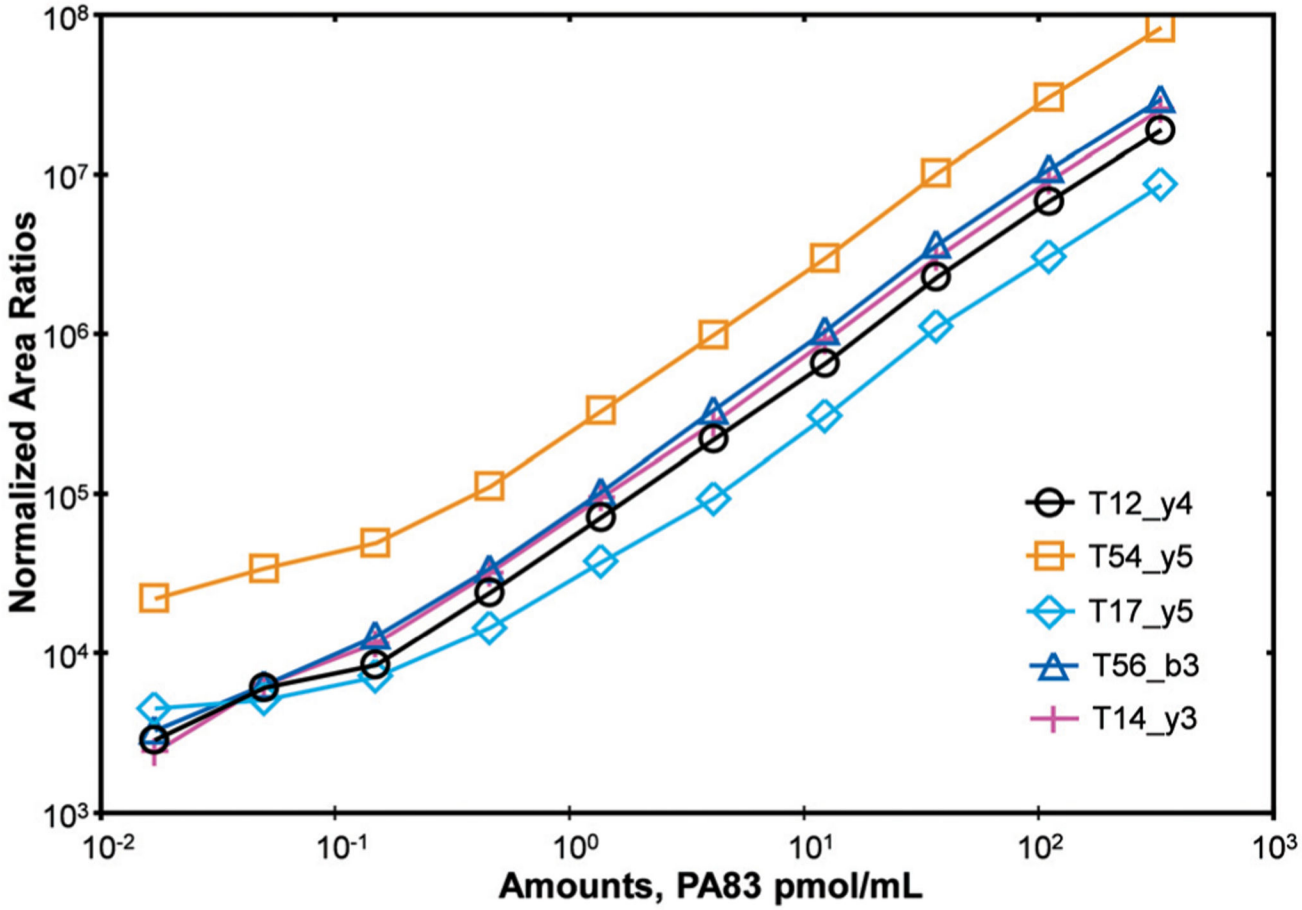

Fig. 3.

Normalized analyte/internal standard peak area ratios from the set of 10 standards in one analytical run, for 5 out of the 10 MRM pairs listed in Table 1. The standards points for each MRM are joined by straight lines to highlight the slight curvature, in support of 3rd-order polynomial curve fitting (not shown). The standards concentrations range between 0.017 and 333 pmol mL^{-1} of PA83 in human plasma.

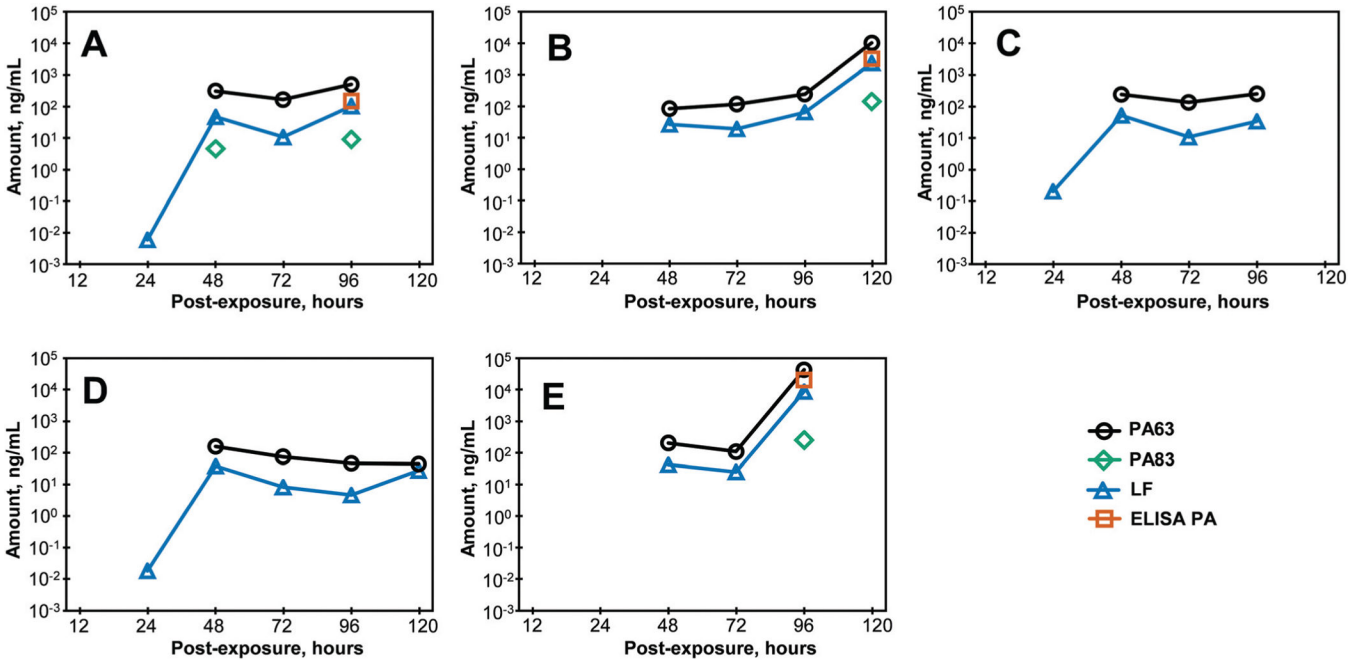

Fig. 4.

Progression of inhalation anthrax in rhesus macaques. PA63 and PA83 profiles for inhalation anthrax in 5 macaques (A–E) obtained by immunocapture, tryptic digest and IDMS are shown in black and green respectively. PA results are plotted here on a $\log_{10}$ scale and are compared to LF values (in blue) and PA ELISA (orange) previously reported by Boyer *et al.* [24]

Table 1

Anthrax protective antigen tryptic peptides quantified by LC-MS/MS. MRM names comprise the tryptic peptide ID followed by the MS/MS fragment ion designation according to Roepstorff *et al.*[40] Amino acid residues and domains are numbered based on UniProtKB entry P13423. Heavy isotope labeled amino acids only present in the internal standards are marked by asterisks; Ile and Leu are +7 Da and Pro is +6 Da, using universal ^{13}C and ^{15}N labels. All Q1 *m/z* values are for $(M + 2H)^{2+}$ precursors, and all Q3 *m/z* values are for singly-charged fragment ions. The *m/z* values were optimized for the peak tops of the isotopic envelopes at the MS instrument resolution, and differ somewhat from monoisotopic *m/z* values

MRM name	Peptide sequence	Amino acid residues	Domain (region)	Analyte Q1 > Q3 *m/z*	Internal standard Q1 > Q3 *m/z*
T12_y4	I*QYQR	147–151	1 (PA20)	354.30 > 594.30	357.76 > 594.30
T12_y3	I*QYQR	147–151	1 (PA20)	354.30 > 466.24	357.76 > 466.24
T14_y4	GL*DFK	158–162	1 (PA20)	290.23 > 522.30	293.71 > 529.31
T14_y3	GL*DFK	158–162	1 (PA20)	290.23 > 409.21	293.71 > 409.21
T17_y4	EVISSDNL*QLP*ELK	173–186	1 (PA20)	793.19 > 486.30	800.13 > 493.31
T17_y5	EVISSDNL*QLP*ELK	173–186	1 (PA20)	793.19 > 599.38	800.13 > 606.40
T54_y5	DL*NLVER	526–532	3 (PA63)	429.82 > 630.36	433.32 > 630.36
T54_y4	DL*NLVER	526–532	3 (PA63)	429.82 > 516.32	433.32 > 516.32
T67_b3	NNI*AVGADESVVK	630–642	4 (PA63)	658.53 > 342.18	662.01 > 349.19
T67_b4	NNI*AVGADESVVK	630–642	4 (PA63)	658.53 > 413.22	662.01 > 420.23

Table 2

Specificity and selectivity for the PA method. A total of 24 blank plasma samples were spiked with PA63, PA83, LF and EF materials as shown (corresponding to 2000, 400 and 100 ng mL^{-1} for LF and EF), and were then analyzed for total-PA (PA63 + PA83) and PA83 only using the IDMS PA method in 3 or 4 separate analytical runs. PA63 was obtained by subtraction of PA83 from total-PA. Results are presented as means with 1 standard deviation. —, less than LOD

Sample ID	PA63 Spike, pmol mL^{-1}	PA83 Spike, pmol mL^{-1}	LF Spike, pmol mL^{-1}	EF Spike, pmol mL^{-1}	PA63 Mean (SD), pmol mL^{-1}	PA83 Mean (SD), pmol mL^{-1}	Replicate runs
1		25			—	26.5 (1.04)	4
2	25	25			24.1 (2.49)	27.0 (1.23)	3
3		25	22.2		—	26.3 (1.84)	3
4		25		22.5	—	27.4 (1.66)	3
5		5			—	5.40 (0.33)	4
6	5	5			4.65 (0.32)	5.63 (0.13)	3
7		5	4.4		—	5.50 (0.36)	3
8		5		4.5	—	5.80 (0.37)	3
9		1			—	1.09 (0.06)	4
10	1	1			0.86 (0.05)	1.10 (0.03)	3
11		1	1.1		—	1.06 (0.05)	3
12		1		1.1	—	1.04 (0.03)	3
13	25				26.8 (4.66)	—	4
14	25	25			25.8 (3.23)	27.9 (2.06)	3
15	25		22.2		30.2 (1.22)	—	3
16	25			22.5	26.1 (1.51)	—	3
17	5				4.94 (0.22)	—	4
18	5	5			4.42 (0.19)	5.76 (0.18)	3
19	5		4.4		5.52 (0.23)	—	3
20	5			4.5	5.28 (0.24)	—	3
21	1				0.97 (0.08)	—	4
22	1	1			0.90 (0.09)	1.11 (0.13)	3
23	1		1.1		1.07 (0.04)	—	3
24	1			1.1	1.00 (0.06)	—	3

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Table 3

Values for PA found in an inhalation anthrax study in rhesus macaques. Animal exposure was done at the Battelle Biomedical Research center (Columbus, OH) reported by Boyer *et al.*[24] Pre- (pre-exp) and post-exposure serum samples for all five animal subjects (A–E) were analyzed for PA by mass spectrometry according to sample availability. —, less than LOD. LF values and qualitative results for PCR of pagA and blood culture (bacteremia) reproduced here for comparative purposes were those as reported by Boyer *et al.*,[24] with two exceptions. The LF samples marked (*) had a higher recovery when reanalyzed using an improved method.[22]

Animal ID	Hours Post-exposure	PA63, ng mL^{-1}	PA83, ng mL^{-1}	LF, ng mL^{-1}	PA63/LF ratio	PCR/bacteremia status
A	Pre-exp	—	—	—		−/−
A	24	—	—	0.006		−/−
A	48	310	4.4	45.2	6.9	+/+
A	72	163	—	10.3	15.8	+/−
A	96	504	9.0	103	4.9	+/+
B	Pre-exp	—	—	—		−/−
B	24	—	—	—		−/−
B	48	84.3	—	25.9	3.3	−/+
B	72	112	—	19.0	5.9	+/±
B	96	237	—	63.6	3.7	NS
B	120	9781	143	2262*	4.3	+/+
C	Pre-exp	—	—	—		−/−
C	24	—	—	0.200		−/−
C	48	238	—	51.7	4.6	+/+
C	72	136	—	10.6	12.8	+/−
C	96	247	—	34.1	7.3	+/+
D	Pre-exp	—	—	—		−/−
D	24	—	—	0.018		−/−
D	48	155	—	38.1	4.1	+/+
D	72	74.3	—	8.06	9.2	−/−
D	96	46.1	—	4.48	10.3	NS
D	120	44.1	—	27.7	1.6	NS
E	Pre-exp	—	—	—		−/−
E	24	—	—	—		−/−

Animal ID	Hours Post-exposure	PA63, ng mL^{-1}	PA83, ng mL^{-1}	LF, ng mL^{-1}	PA63/LF ratio	PCR/bacteremia status
E	48	204	—	42	4.9	+/+
E	72	110	—	25.1	4.4	+/+
E	96	42 435	247	8669*	4.9	+/+