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Validated MALDI-TOF-MS method for anthrax lethal factor provides early diagnosis and evaluation of therapeutics

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ABSTRACT

Anthrax lethal factor (LF) is a zinc-dependent endoprotease and a critical virulence factor for *Bacillus anthracis*, the causative agent of anthrax. The mass spectrometry (MS) method for total-LF quantification includes three steps; 1) LF specific antibody capture/concentration, 2) LF-specific hydrolysis of a peptide substrate, and 3) detection and quantification of LF-cleaved peptides by isotope-dilution MALDI-TOF/MS. Recombinant LF spiked plasma was used for calibration and quality control (QC) materials. Specificity was 100% from analysis of serum and plasma from 383 non-infected humans, 31 rabbits, and 24 rhesus macaques. Sensitivity was 100% from 32 human clinical anthrax cases including infections by inhalation, ingestion, cutaneous and injection exposures and experimental infections for 29 rabbits and 24 rhesus macaques with inhalation anthrax. Robustness evaluation included sample storage, serum and plasma, antimicrobial and antitoxin effects and long-term performance. Data from 100 independent runs gave detection limits 0.01 ng/mL (111 amol/mL) for the 4-h method and 0.0027 ng/mL (30 amol/mL) for an alternate 20-h method. QC precision ranged from 7.7 to 14.8% coefficient of variation and accuracy from 0.2 to 9.8% error. The validated LF MS method provides sensitive quantification of anthrax total-LF using a robust high throughput platform for early diagnosis and evaluation of therapeutics during an anthrax emergency.

Introduction

Anthrax is a bacterial infection caused by the Gram-positive, spore-forming organism *Bacillus anthracis*. Cutaneous, ingestion or inhalation anthrax occur when spores enter the body via pre-existing lesions in the dermal or intestinal integument, or by inhalation, respectively [1]. *B. anthracis* may also cause a serious soft-tissue infection from injection of spore-contaminated heroin [2]. Though *B. anthracis* is a Category A select agent with high potential for terrorist use, naturally-occurring anthrax is reported frequently around the world (http://www.promedmail.org). As a zoonotic disease, most human anthrax outbreaks are associated with close contact with infected domestic animals and their products. The majority of naturally-occurring cases consist of cutaneous anthrax. Inhalation anthrax has the highest case:fatality ratio; as high as 94% in the absence of early antimicrobial intervention and 45% with early treatment and aggressive supportive care [3,4].

B. anthracis virulence is dependent on the production of the two binary protein exotoxins and a gamma-linked poly-D-glutamic acid (PGA) capsule [5]. The binary toxins are composed of three distinct proteins; protective antigen (PA), lethal factor (LF), and edema factor (EF). PA, which is responsible for toxin internalization, combines with EF, an adenylyl cyclase, or LF, a zinc-dependent endoproteinase, forming edema toxin (ETx) and lethal toxin (LTx), respectively [6]. LF is a 90,000 Da protein that hydrolyzes and inactivates members of the mitogen-activated protein kinase kinase (MAPKK) family of response regulators that are central to inflammatory signaling pathways [7–9]. EF is an 89,000 Da protein adenylyl cyclase that converts ATP to cyclic-adenosine monophosphate (cAMP). It causes the classical edema of anthrax and also has immune-suppressive properties [10]. Together, the anthrax toxins synergize to disrupt immune responses and promote the pathogenesis and proliferation of *B. anthracis in vivo* during infection [1].

Early diagnosis and prompt antimicrobial interventions are key to reducing mortality rates. Specialized anti-toxin therapeutic medical countermeasures (MCM) may also help to reduce mortality rates, especially with advanced disease. Validated, rapid, high throughput

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technologies for detection and quantification of *B. anthracis* virulence factors, especially the toxin components, are of high public health impact for early disease detection. They are also useful for evaluating the effectiveness of anthrax MCM by measuring toxin clearance during treatment [11]. A quantitative, high analytical sensitivity assay that detects specific proteins secreted by the organism, rather than the organism itself meets these objectives.

As the universal protein responsible for intoxication by both lethal and edema toxins and the target of most therapeutics, many methods for PA have been developed [12–15]. For the more sensitive of these assays, precision and/or accuracy were limited in practice, and they were determined to be better for qualitative detection, rather than quantitation of PA [14,15]. While PA is an attractive target, the enzymatic activity of LF provides a unique opportunity for detection and quantification. Instead of detection of the LF molecule itself, its cleavage of a peptide substrate at specific residues and detection of its products amplifies its presence many-fold. Methods utilizing LF activity have been developed using Förster resonance energy transfer (FRET) and electrochemiluminescence (ECL) modified LF-substrates [16–18]. Both strategies rely on changes in their respective signal with cleavage of the target substrate by LF. These methods have been developed primarily to screen inhibitors of LF, using relatively high LF levels, 2–10 nM, equivalent to 180–899 ng/mL [16,17], and 5000 ng/mL LF [18]. None have been used to quantify LF or evaluate the limits of LF detection which is needed for early diagnosis.

We previously described development of a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS method for quantification of total-LF activity [19,20]. Here we report the optimized, validated method including performance characteristics and long-term stability. The method uses the LF zinc-endoproteinase activity to amplify detection via its cleavage of a peptide substrate. In contrast to diagnostic methods that detect the organism, MS detection of LF was not subject to interference from antimicrobials which clear bacilli or immunotherapeutic intervention [11,20]. Application of the method to track toxin levels in inhalation anthrax during the first clinical use of the anti-toxin anthrax immune globulin intravenous (Human) (AIGIV, Anthrasil™) demonstrated its utility for tracking toxin clearance [11]. LF measurements were required as part of the investigational new drug (IND) protocol for the use of AIGIV. The LF MS method has been validated to characterize its quantitative and diagnostic performance.

Material and methods

Chemicals, reagents, equipment and safety

Chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO) except where indicated. Dynabeads® MyOne™ Tosyl-activated magnetic beads were purchased from Invitrogen Co. (Carlsbad, CA). Recombinant LF (rLF) was purchased from List Biological Laboratories (Campbell, CA). Two non-neutralizing monoclonal anti-LF antibodies (LF-mAb) were prepared in the Division of Scientific Resources of CDC as previously described [19]. AIGIV was obtained from the Division of Strategic National Stockpile, CDC (Atlanta, GA). Pooled human donor plasma (n = 10 individuals per pool) (PH-plasma) and individual normal North American (NNA) donor serum and plasma samples were obtained from Interstate Blood Bank (Memphis, TN). Equipment included a Kingfisher 96 magnetic particle processor (Thermo Fisher Scientific., Waltham, MA), 4800 Plus Proteomics Analyzer (MALDI-TOF MS) (AB Sciex, Foster City, CA) and Gene Amp@ PCR thermocycler (ThermoFisher, Waltham, MA). All procedures and sample handling were conducted at Biosafety Level 2 containment as recommended and described [21]. Additional safety measures are described in each section as needed.

Peptides

The peptide substrate (LF-S), SKARRKKVY**PY**PXENFPPSTARPT (2804.2 m/z) (Midwest Biotechnology, Indianapolis, Indiana), is hydrolyzed by LF between the proline and tyrosine (bold font) yieldings two smaller peptides, an amino-terminal product (NTP), SKARRKKVY**P** (1232.8 *m/z*) and carboxy-terminal product (CTP), **Y**PXENFPPSTARPT (where X = norleucine) (1589.8 *m/z*). The norleucine included in the optimized substrate was substituted for a methionine in the original published LF method [19]. The change prevented oxidation of methionine and two potential mass forms and peaks in the substrate and more importantly the cleaved CTP, as described previously [22]. Internal standard (IS) peptides identical to the NTP and CTP products included a single heavy alanine ($^{13}C_3H_4D_3{}^{15}NO_2$) in each peptide. The NTP and CTP IS were each +7 mass units relative to the native peptides, NTIS, SK(A+7)RRKKVYP (1239.8 *m/z*) and CTIS, YPXENFPPST (A+7)RPT (1596.8 *m/z*) (Los Alamos National Laboratory, Los Alamos, NM).

Calibration standards and quality control material

The original standards, quality control (QC) and test samples, were prepared in 2007 by spiking rLF (1 μg/μL) in 0.45 μm filtered PH-plasma, constructing a 13 level standard series from 100 to 0.0125 ng/mL and three QCs at 6.3, 1.25 and 0.14 ng/mL. New LF standards from 11.8 to 0.003 ng/mL and three QC's at 0.722, 0.203, and 0.044 ng/mL were prepared in PH-plasma. Both standard sets were prepared using a non-serial strategy with multiple diluted stock pools. The new standards were adjusted to match the original 2007 standards for consistency. Equivalence was demonstrated by cross quantitation of standards, QCs and 66 clinical and animal infection samples.

LF MS method

Step 1) Magnetic immunopurification (1-h)

LF specific magnetic antibody coated beads (LF-MAB's) were prepared by cross linking two anti-LF monoclonal antibodies, AVR1674 (LF-MAB-1) and AVR1675 (LF-MAB-2), to tosyl-activated magnetic beads at 2 mg each per mL of beads as directed by the manufacturer. Automated sample preparation (Kingfisher 96) moved the beads through multiple steps using 100 μL samples/standards in 900 μL phosphate buffered saline (PBS) with 0.05% Tween 20 and processed as described previously [23].

Step 2) LF peptide cleavage reaction (2-h and 18-h)

LF immobilized on LF-MABs were transferred to 30 μL of LF reaction buffer containing 50 μM LF-S and protease inhibitor described previously [19]. The beads and substrate were incubated in a thermocycler at 37 °C for 2-h, then 5 μL was removed and analyzed by MS (Step 3 below). The remaining 25 μL continued to react at 37 °C in a PCR thermocycler for at least 18-h or overnight (ON).

Step 3) MALDI-TOF MS analysis and quantification

At 2-h and 18-h reaction times, 5 μL of the LF reaction mix was added to 45 μL of MALDI matrix, α-cyano-4-hydroxycinnamic acid (CHCA) at 3.125 mg/mL, containing 9.45 pmol NTIS and 6.25 pmol CTIS (CHCA-IS), then spotted at 1 μL per spot in quadruplicate onto an Opti-TOF 384-spot stainless steel MALDI plate (AB Sciex, Foster City, CA). Mass spectra were acquired and processed as described previously [23]. The CTP/CTIS peak area ratio (CT-response) were plotted versus LF concentration with dual $\log_{10}$ transformation and 5-parameter logistic (5 PL) regression with robust weighting as described previously for lethal toxin complex [24]. The total time for the primary LF method is 4-h (1-h sample preparation, 2-h reaction, 1-h MS analysis) and for the ON method is 20-h (1-h sample prep, ≥18-h reaction, 1-h MS).