096

Specific Detection of Anthrax Infection

N. Shine, K. Suryadi
List Biological Laboratories, Campbell, CA 95008 USA

Abstract:

Bacillus anthracis is regarded as a major biological warfare threat. Inhalation of *Bacillus anthracis* spores can quickly lead to a lethal blood infection. Antibiotic treatment can clear the bacterium from the host; however, this treatment must be initiated rapidly. By the time clinical symptoms are observed, toxin may already be present in lethal amounts. There is a critical need for an accurate, sensitive and simple assay to determine that infection has occurred, permitting immediate treatment. The presence early in infection of one of the virulence factors, anthrax lethal factor (LF), offers the opportunity for detection prior to decline of the patient.

This presentation will describe a fast, sensitive, specific and accurate detection method to determine active infection by *Bacillus anthracis* in plasma. LF is detected using a fluorescently labeled peptide substrate, MAPKKide® Plus (US Patent No. US 9,932,570 B2), which is not cleaved by plasma proteases and thus is specific for LF.

Several detection strategies have been evaluated. In the simplest method, the substrate is added directly to diluted plasma, and cleavage is monitored by the increase in fluorescence as a function of time. The limit of detection by this method is 25 ng lethal factor/ml of plasma in 15 minutes, 5 ng/ml after 45 minutes, and less than 1 ng lethal factor/ml of plasma after 5 hours. In recent developments the simplest method has been optimized to increase the sensitivity by including a sample preparation step. The most sensitive method involves the concentration of lethal factor using antibody-coated plates and HPLC with fluorescence detection. This method allows for detection of less than 5 pg lethal factor/ml of neat plasma after 2 hours of incubation.

In conclusion, data is presented demonstrating that MAPKKide® Plus is highly sensitive to cleavage by LF and resistant to cleavage by plasma proteases making it ideal for detection of early infections with *Bacillus anthracis*. This detection method has the potential to be used as a point-of-care method to monitor both the initial level of severity of the intoxication as well as the success of therapeutic interventions.

Introduction:

Anthrax is caused by the gram-positive spore-producing bacterium *Bacillus anthracis*. The principal virulence factors are a γ-linked poly-D-glutamic acid (PGA) capsule and a three-component exotoxin composed of protective antigen (PA), lethal factor (LF) and edema factor (EF).These proteins act in binary combinations. The complex of PA, the cell binding component, with the LF enzyme, is termed lethal toxin (LT). LT weakens the immune system and allows the dissemination of the bacteria leading to death. More recently several isolates of *Bacillus cereus* have been identified that cause anthrax-like disease and harbor the virulence plasmids that carry the genes for the anthrax toxin components, PA, LF and EF (1).

The most lethal manifestation of *Bacillus anthracis* infection is by inhalation. Due to the intentional release of anthrax spores in the bioterrorism attacks of 2001, *B. anthracis* was placed at the top of the CDC list of select agents. Once symptoms are severe and diagnosis using current methods is possible, the levels of toxins can be dangerously high. A quick, reliable test is needed to detect exposure early in the infection process, before the toxin and bacteremia become lethal. This test can also be used to detect LF produced by the anthrax toxin-expressing *Bacillus cereus* that also causes a life-threatening anthrax-like lung infection. *B. cereus*, in the event it is used for bioterrorism, may escape detection using current methods, while the LF produced early in infection is detectable.

There are a number of markers for inhalation anthrax infection, however, it has been shown that LF is present earliest in infection (2). The method described here takes advantage of the fact that anthrax LF is a zinc endoprotease which cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKK). We have produced a peptide mimic of these substrates and substantial signal amplification can be expected as a result of catalytic turnover.

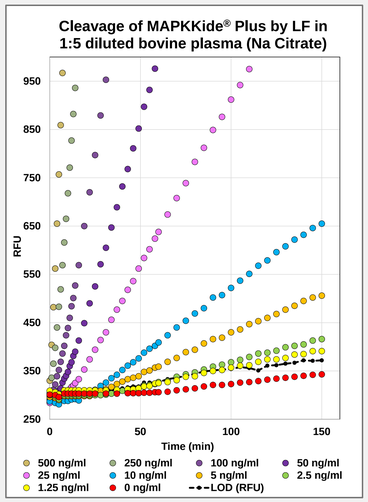
A series of fluorescently labeled peptide substrates, based on the native substrate sequence, were evaluated for specific cleavage by LF. One sequence, subsequently named **MAPKKide® Plus,** was shown to be specific for LF and resistant to cleavage by nonspecific proteases found in plasma. This newly designed substrate is highly sensitive to LF and may be used to detect early infections. Two methods for the detection of LF in plasma using this fluorogenic substrate, one HPLC-based and one a microplate assay have been described (3). Using the HPLC-based method allows for the detection of as low as 5 pg LF/ neat bovine plasma after 2 hours of incubation. Since significant levels of LF are present early in the infection cycle in plasma, a simpler more rapid microplate assay was evaluated using the direct addition of **MAPKKide® Plus** to diluted plasma.

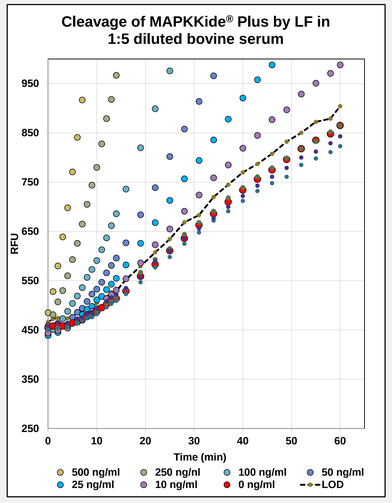
Initial studies were performed using bovine plasma formed using Na citrate as the anticoagulant. A number of anticoagulants are routinely used to form plasma and it has been shown that the levels of plasma components including proteins are dependent on both the anticoagulant used and on the species of animal. The objective of the current study was to compare the detection of LF using **MAPKKide® Plus** in bovine serum and plasmas formed using 5 different anticoagulants.

Results:

A. Detection of LF in Bovine Plasma and Serum using MAPKKide® Plus in a Simple, Rapid Microplate Assay

The cleavage of 10 µM **MAPKKide® Plus** by a series of concentrations of LF (ng/ml) in bovine plasma (**Fig 1**) is compared to cleavage in bovine serum (**Fig 2**). Samples were prepared in the plasma or serum and diluted 1:5 with assay buffer. Samples without LF in 1:5 diluted plasma are represented by the red circles, and the LODs in RFUs at each time point are shown by a black dashed line. The concentrations of LF in the matrix are corrected for the 1:5 dilution.





B. Table 1: Fluorescence observed for **MAPKKide® Plus** in five different 1:5 diluted bovine plasmas and bovine serum after incubation with LF. The concentrations of LF (ng/ml) are corrected for the 1:5 dilution.

Amount of LF (ng/ml)	Reaction time required[1] (min)					
	Anticoagulant in plasma					Serum
	Na-Citrate	K2-EDTA	K3-EDTA	Li-Heparin	Na-Heparin	
500	1	1	1	1	2	1
250	1	2	2	7	5	1
100	3	7	6	19	13	4
50	6	14	12	37	25	7
25	13	28	22	58	34	10
10	28	46	40	125	85	14
5	34	70	49	ND[2]	ND[2]	ND[2]
2.5	70	80	ND[2]	ND[2]	ND[2]	ND[2]
1.25	100	ND[2]	ND[2]	ND[2]	ND[2]	ND[2]

[1]Time to obtain an RFU above the LOD (RFU). The limit of detection was calculated from the normal distribution of the blank samples (mean + 3 stdev; n = 3 triplicates).
[2]No response detected in the total time of the reaction (2.5 hrs).

C. In prior studies, the level of LF in plasma of intoxicated animals and several humans was determined using mass spectroscopy (4, 5, 6). The source of the plasma sample, time post challenge that the measurement was made, and the average LF concentrations are listed in **Table 2**. The time to detect these same levels of LF using our microplate assay and **MAPKKide® Plus** is also given in **Table 2**.

Table 2: Detection of LF in plasma

Source of plasma sample	Time post challenge (hr)	Average LF (ng/ml)	Time to detect using MAPKKide® Plus* (min)
Six Rhesus Macaques (reference #4)	30	3.84	<70
	36	36.05	<15
	48	118.8	<3
Human Cases (references #5 and #6)	3 days	294	<1
	2 days	58	<6

***** Data from citrated plasma (**Table 1**)

Materials and Methods:

Anthrax lethal factor, Product #169 and MAPKKide® Plus, Prod #532 are from List Biological Laboratories, Inc. The 96-well, black, flat bottom, nonbinding plates used for the fluorescent plate assay were from Corning (cat # 3991). The filtered adult bovine plasmas: Na-Citrate, cat #7310806; K2-EDTA, cat #7310807; K3-EDTA, cat #7310808; Li-Heparin, cat #7310810 ; Na-Heparin, cat #7310811; and the filtered adult bovine serum cat #7330800 were purchased from Lampire Biological Laboratories.

Sample Preparation: Stock solutions of the fluorogenic substrate, **MAPKKide® Plus,** were made 1.25 mM in DMSO. The substrate was then diluted 5-fold to 250 µM in assay buffer: 20 mM HEPES, pH 8.0 containing 0.1% Tween-20.

LF Activity Assay: LF was spiked into each plasma or serum sample and then each was diluted 1:5 using assay buffer. The reaction was initiated by addition of 10 µM **MAPKKide® Plus** directly to the 1:5 diluted plasma or serum, and the assays were run at 37°C using the kinetic mode of the plate reader with readings at 1-minute intervals. The samples were run in triplicate with 9 replicate blanks. The limit of detection was calculated from the normal distribution of the blank samples (mean + 3 stdev; n = 3 triplicates).

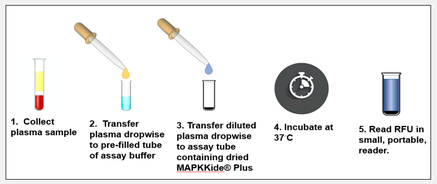
Microplate reader: Assays were performed on a SPECTRAmax GEMINI XPS fluorescence microplate reader (Molecular Devices). The excitation wavelength was set to 368 nm and emission to 452 nm with a cutoff filter at 435 nm.

Conclusions:

This report describes a fast, sensitive, specific and accurate simplified method to detect active infection by *Bacillus anthracis* in bovine plasma formed using 5 different anti-coagulants. Bovine serum is also evaluated. Levels of LF between 1.25 to 500 ng/ml were tested from a 1:5 dilution of plasma or serum by direct addition of **MAPKKide® Plus**, and a kinetic readout using a microplate reader. The data indicate that plasma formed using Na-citrate as the anticoagulant allows the most sensitive detection of LF. As shown in **Table 1**, 1.25ng/ml LF can be detected in less than 2 hours. The data indicate the detection limit for LF in plasma is dependent on the anticoagulant used. For bovine serum, the limit of detection was 10 ng/ml and this level of LF was detected in 14 minutes; more rapidly than in any of the plasmas evaluated. Most importantly, the levels of LF detected in plasma using **MAPKKide® Plus** are in the range found in animals and in several cases, intoxicated humans and thus these LF levels are clinically relevant (**Table 2**). This assay can also be used to detect LF produced during intoxication with anthrax toxin-expressing isolates of *B. cereus*, an emerging bioterrorism threat.

Future Goals:

The concept for the development of a point-of-care device is shown below.



References:

1. Brezillion C, Haustant M, Dupke S, Corre JP, Lander A, Franz T (2015) Capsules, toxins and AtxA as virulence factors of emerging *Bacillus cereus* Biovar *anthracis*. PLoS ONE Neglected Tropical Diseases 9(4): e0003455. https://doi.org/10.1371/journal.pntd.0003455.

2. Boyer AE, Quinn CP, Hoffmaster AR, Kozel TR, Saile E, Marston CK, et al. Kinetics of lethal factor and poly-D-glutamic acid antigenemia during inhalation anthrax in rhesus macaques. Infect Immun. 2009;77:3432-3441.

3. Suryadi K, Shine N (2018) Design and use of a novel substrate for simple, rapid, and specific early detection of anthrax infection. PLoS ONE 13(11): e0207084. https://doi.org/10.1371/journal.pone.0207084.

4. Gallegos-Candela M, Boyer AE, Woolfit AR, Brumlow JO, Lins RC, Quinn CP, et al. Validated MALDI-TOF method for anthrax lethal factor provides early diagnosis and evaluation of therapeutics. Anal Biochem. 2018;543:97-107.

5. Walsh JJ, Pesik N, Quinn CP, Urdaneta V, Dykewicz CA, Boyer AE, et al. A case of naturally acquired inhalation anthrax: Clinical care and analyses of anti-protective antigen immunoglobulin G and lethal factor. Clin Infect Dis. 2013;44:968-971.

6. Mark D. Sprenkle et al. Emerging Infectious Diseases 2014, 20:310-314.