A Novel Substrate for Specific Detection of Anthrax Infection

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ABSTRACT:

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

This report describes 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.

Three 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 <1 ng lethal factor/ml of plasma after 5 hours.

Two more sensitive methods include enrichment by capture from plasma using lethal factor antibody-coated microtiter plates or similarly coated immuno-tubes. The captured lethal factor is exposed to the MAPKKide Plus, and the amount of cleavage is determined either by HPLC or microplate reader. Concentration of lethal factor using the antibody-coated plates and HPLC allows for detection of less than 5 pg lethal factor/ml of neat plasma after 2 hours of incubation. Using antibody-coated immuno-tubes, 20 pg lethal factor/ml plasma can be detected in 5 hours by a simple end point read of fluorescence in a microplate reader.

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*.

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 and can cause death.

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 amount of toxin becomes lethal.

There are a number of markers for inhalation anthrax infection including PA, LF and PGA, however, it has been shown that LF is present earliest in the 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). Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. Generally, peptide substrates based on the native sequences can be cleaved nonspecifically by other proteases in complex matrices such as plasma and serum. A series of fluorescently labeled peptide substrates, based on the native substrate sequence, were evaluated for specific cleavage by LF.

This presentation focuses on one sequence subsequently named **MAPKKide® Plus** which was shown to be specific for LF and resistant to cleavage by nonspecific proteases found in plasma. Significantly, 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 (1). Using the HPLC- based method allows for the detection of as low as 5 pg LF/ neat bovine plasma after 2 hours of incubation. The limit of detection using the simpler microplate assay is 1 ng LF/ ml bovine plasma after 5 hours of incubation. The increased sensitivity of the HPLC-based assay is due in part to the separation of the fluorescence of the cleaved fluorophore from the significant fluorescence remaining from the fluorophore that is still attached to the uncleaved substrate.

The objective of the current study was to determine how rapidly higher levels of LF could be detected using a simple microplate assay since it has been shown that significant levels of LF can be detected in plasma of infected individuals early on in the infection cycle, (2, 3, 4.).

MATERIALS AND METHODS:

Anthrax lethal factor (Product #172 or #169), are products of List Biological Laboratories, Inc. The 96-well, black, flat bottom, non binding plates used for the fluorescent plate assay were from Corning (cat # 3991). Bovine plasma (cat # 7310806) was purchased from Lampire Biological Laboratories.

Sample Preparation: Stock solutions of the fluorogenic substrates, **MAPKKide® Plus** (± biotin), were made 2.5 mM in DMSO based on the peptide content determined by elemental analysis. The substrates were diluted in assay buffer: 20 mM HEPES, pH 8.0 containing 0.1% Tween-20.

LF Activity Assays:

A rapid microplate assay method was evaluated for two ranges of LF: 10 to 1000 pg LF/ml 1:10 diluted bovine plasma and 5 to 250 ng/ml 1:5 diluted bovine plasma. Dilution of the bovine plasma was necessary in order to minimize background.

For the method detecting higher levels of LF (5 to 250 ng/ml 1:5 diluted bovine plasma), 10 μM MAPKKide Plus was added directly to the 1:5 diluted bovine plasma, and the assays were run at 37°C using the kinetic mode of the plate reader with readings at 1 or 3 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 = 9).

For the range 10 and 1000 pg/ml 1:10 diluted plasma, 1.25 μM MAPKKide Plus was added directly to the diluted bovine plasma and the time-dependent increase in fluorescence was monitored at 37°C hourly for 5 hours. For the blank samples containing no LF there were 3 sets of quadruplicates and the standard deviation was calculated from these three sets. At each time point, the plate was read 5 times to increase the precision of the fluorescence readings. The standard curve was analyzed using a linear regression fit forcing the intercept through the mean value of the blanks. The limit of detection was calculated from the normal distribution of the blank samples (mean + 3 stdev; n = 3 sets of quadruplicates) and calculated as pg LF/ml plasma using the standard curve. Subsequently, 6 data sets were evaluated, 2 data sets per day for 3 consecutive days. The data is presented as the average of these 6 data sets, each with 4 replicate samples and 12 replicate blanks. At each time point, the plate was read 3 times to increase the precision of the fluorescence readings.

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. An excitation wavelength between 360 to 368 nm can be used.

RESULTS:

A. MAPKKide® Plus Specificity

The change in fluorescence for 2.5 μM **MAPKKide® Plus** in the presence of 1:10 diluted plasma without LF is compared to a peptide with the same sequence but lacking the unnatural amino acids (Control Peptide). The results demonstrate that the Control Peptide is non-specifically cleaved by plasma proteases while **MAPKKide® Plus** is not (**Table 1, Figure 1**). The cleavage of **MAPKKide® Plus** in plasma is only observed in the presence of LF.

Table 1: Fluorescence observed for substrates in 1:10 diluted plasma without LF.

A: Fluorescence observed for 2.5 μM substrates in 1:10 diluted bovine plasma, No LF*.						
Temperature (°C)	MAPKKide® Plus	Standard Deviation	%CV	Control Peptide	Standard Deviation	%CV
37	**109**	*8.10*	*7.4*	**102**	*2.05*	*2.0*
37	**123**	*3.58*	*2.9*	**353**	*11.85*	*3.4*
37	**134**	*4.37*	*3.3*	**860**	*21.41*	*2.5*
37	**142**	*5.95*	*4.2*	**1420**	*38.26*	*2.7*
37	**144**	*5.27*	*3.7*	**1854**	*52.87*	*2.9*
37	**151**	*6.15*	*4.1*	**2251**	*62.75*	*2.8*
37	**147**	*6.13*	*4.2*	**2571**	*74.02*	*2.9*
ambient	**163**	*7.79*	*4.8*	**4074**	*121.89*	*3.0*
ambient	**169**	*7.37*	*4.4*	**3920**	*118.87*	*3.0*
B: Fluorescence observed for 25 μM substrates in 1:5 diluted bovine plasma, No LF**						
37	**622**	*65*	*10.5*	**558**	*45*	*8.1*
37	**572**	*16*	*2.8*	**646**	*54*	*8.4*
37	**585**	*18*	*3.0*	**1284**	*70*	*5.5*
37	**586**	*28*	*4.7*	**1890**	*149*	*7.9*
37	**601**	*28*	*4.7*	**2806**	*157*	*5.6*
37	**589**	*22*	*3.8*	**3632**	*149*	*4.1*
37	**588**	*17*	*2.9*	**4465**	*170*	*3.8*
ambient	**760**	*51*	*6.7*	**26027**	*949*	*3.6*

*Statistics are based on 12 replicates; 3 reads per time point.
**Statistics are based on 6 replicates; excitation/emission wavelengths set to 360nm/460nm with no cutoff.

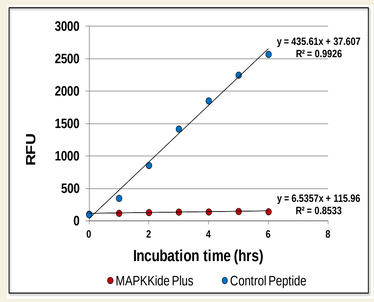

Figure 1: Fluorescence (RFU) as a function of time for **MAPKKide® Plus** (**red**) and Control Peptide (**blue**) in 1:10 plasma at 37 °C in the absence of LF.

B. Detection of LF using MAPKKide® Plus using a Rapid Microplate Assay

1. The increase in fluorescence with time on exposure of **MAPKKide® Plus** to a series of LF concentrations in bovine plasma is shown in **Fig 2**. Samples were prepared in plasma and diluted 1:5 with reaction buffer. Samples without LF in 1:5 diluted plasma are represented by the gray circles, and the LODs at each time point are shown by a black dashed line. After correction for dilution, samples with high levels of LF, 250 and 100 ng LF/ml plasma are detected in 2 and 5 minutes, respectively. Samples of LF in bovine plasma containing 50 ng LF/ml plasma are detected in 10 minutes while samples containing 25 and 5 ng/ml are detected in 15 and 45 minutes, respectively.

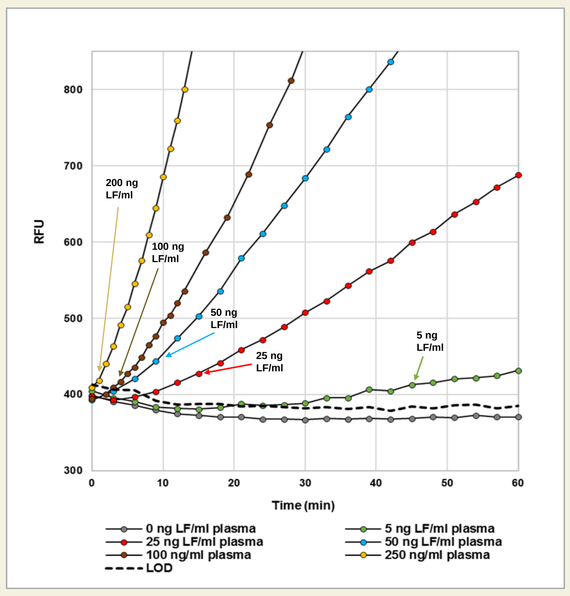

Fig 2: Cleavage of 10 μM MAPKKide Plus by a series of concentrations of LF (ng/ml) in bovine plasma as a function of time.

The data shown are from the microplate reader using the kinetic mode.

Table 2: Fluorescence observed for **MAPKKide® Plus** in 1:5 diluted plasma after incubation with LF.

Amount of LF (ng /ml plasma)	time (min)	RFU	Mean blank (RFU)	Standard deviation	%CV	LOD* (RFU)
5	45	413	369	5.09	1.4	384
25	15	428	373	4.86	1.3	388
50	10	444	380	4.08	1.1	392
100	5	428	372	6.52	1.8	392
250	2	441	378	5.58	1.5	395

The limit of detection was calculated from the normal distribution of the blank samples (mean + 3 stdev; n = 9).

Plots of the cleavage of **MAPKKide® Plus** as a function of time for LF concentrations ≤ 1 ng are shown in **Fig 3**, each concentration yielding a unique slope. These slopes are then plotted as a function of concentration (**Fig 4**), which can be used to determine the amount of LF present in unknown samples. The limit of detection, calculated from the normal distribution of the slopes (mean + 3 stdev; n = 6), was 80 pg/ml of 1:10 diluted bovine plasma. This is equivalent to 800 pg LF/ml in neat bovine plasma.

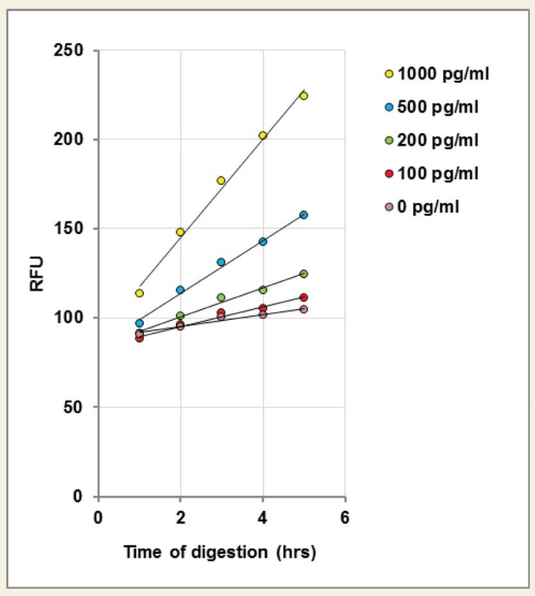

Fig 3: Kinetic analysis of microplate assay data.

Cleavage of MAPKKide Plus by a series of concentrations of LF(pg/ml) in 1:10 diluted bovine plasma as a function of time.

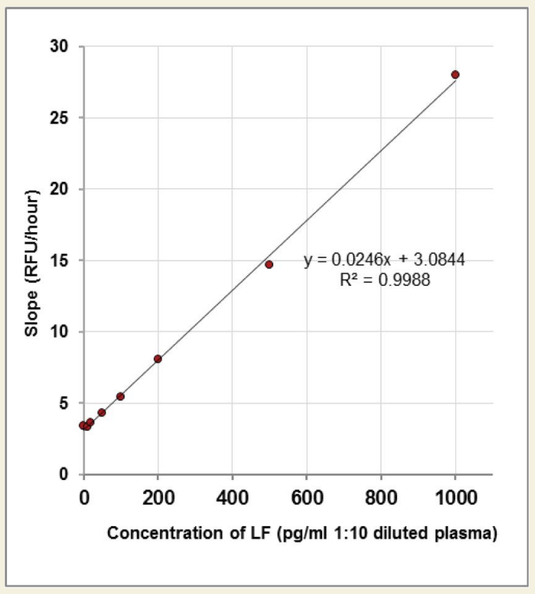

Fig 4: Rate of cleavage of MAPKKide Plus as a function of LF concentration.

Slope in RFU/hour as a function of concentration of LF in 1:10 diluted bovine plasma.

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CONCLUSIONS:

This report describes a fast, sensitive, specific and accurate simplified method to detect active infection by *Bacillus anthracis* in bovine plasma at very early stages of intoxication. The data indicate that higher levels of LF, i.e. 250, 100, 50, 25 and 5 ng LF/ml plasma can be detected in 2, 5, 10, 15 and 45 minutes, respectively, from a 1:5 dilution of plasma, direct addition of MAPKKide® Plus, and kinetic readout from a microplate reader. These levels of LF are in the range found in animals and in one case, an intoxicated human. In rhesus macques, 40 ng/ml LF was detected 48 hours post infection by inhalation (2). In another rhesus macque study, levels of LF ranging from 7 to over 100 ng/ml plasma were detected 36 hours after exposure (3). In one case of inhalation anthrax in a human, over 200 ng/ml was detected 2-3 days after onset of symptoms (4). Here it is shown that a single microtiter plate assay can detect these levels in less than 1 hour.