Sensitive Detection of Anthrax Lethal Factor in Plasma Using a Specific Biotinylated Fluorogenic Substrate.

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

ABSTRACT:

PURPOSE OF STUDY: A fast, sensitive, specific and accurate detection method to determine active infection by *Bacillus anthracis* in complex matrices has been developed using a fluorescently labeled peptide substrate, **MAPKKide® Plus***. Two methods for the detection of lethal factor (LF) in plasma using this fluorogenic substrate were demonstrated: one HPLC-based and the other a microplate assay. The goal of this study was to improve the sensitivity of the microplate assay.

METHODS: The **MAPKKide® Plus*** substrate was biotinylated on the N-terminal. Several strategies were evaluated to determine the sensitivity that could be achieved.

1. The biotinylated **MAPKKide® Plus*** substrate was attached to streptavidin-coated beads prior to exposure to a solution of LF in plasma or to LF enriched by antibody capture from plasma.

2. The biotinylated **MAPKKide® Plus*** substrate was added directly to a solution of LF in plasma, or to LF enriched by capture from plasma. Streptavidin-coated beads were then used to remove uncleaved substrate from the solution to minimize background fluorescence.

RESULTS: The results obtained from the strategies outlined above are compared to those obtained using both the HPLC-based method which allows for the detection of less than 5 pg LF/ml of neat bovine plasma after 2 hours of incubation; and the simpler microplate assay where the limit of detection is 1 ng LF/ml of bovine plasma after 5 hours of digestion.

CONCLUSIONS: **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*.

*Patent Pending

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.

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 (Boyer AE, et al, Infect Immun. **77**:3432-3441). 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. Previously two methods for the detection of LF in plasma using this fluorogenic substrate, one HPLC-based and one a microplate assay have been described. 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 improve the sensitivity of the simpler microplate assay. **MAPKKide® Plus** with the N-terminal biotinylated was synthesized. The idea was to pull out the uncleaved substrate using streptavidin-coated beads so that the excitation wavelength could be set to optimize the cleaved fluorophore's fluorescence.

MATERIALS AND METHODS:

Anthrax lethal factor (Product #172), and the chicken IgY polyclonal anti-LF antibody (Product # 769A) are products of List Biological Laboratories, Inc. The Nunc-Immuno Tubes, Maxisorp (cat# 444202) used for LF antibody coating and the dimethyl sulfoxide (DMSO) (cat # TS-20684) were purchased from ThermoScientific. 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. The LF was added to neat bovine plasma.

LF Activity Assays:

Antibody Capture: The Nunc-Immuno Maxisorp Tubes were coated with 1 mL of a 10 µg/ml solution of a chicken affinity purified polyclonal IgY antibody to anthrax lethal factor (List Prod # 769A). The immuno-tubes were incubated with the IgY overnight at 2-8°C. After 5 washes with 1.5 mL each of PBS containing 0.05% TWEEN-20 (PBST), the anti-LF coated tubes were exposed to 1 mL of a series of LF concentrations in neat plasma. The tubes were incubated at 22°C for 1 hour. Tubes were then washed 3 times with 1.5 ml PBST and 1.0 ml of 10 µM **MAPKKide® Plus** (± biotin) was added. The reaction was allowed to proceed for 3, 5 and 22 hours at 37°C. At each time point 250 µl of the reaction mixture was removed from triplicate tubes and placed in a 96 -well black plate. In the case of the biotinylated **MAPKKide® Plus** a 0.5 ml aliquot of each triplicate was added to streptavidin-coated beads after 22 hours of exposure to LF. The mixture was gently rotated at ambient room temperature for 1 hour and then read in the platereader.

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.

Time (hrs)	Temperature (C)	RFU	
		MAPKKide® Plus	Control Peptide
0	37	108	102
1	37	123	353
2	37	134	860
3	37	142	1420
4	37	144	1854
5	37	151	2251
6	37	147	2571
21	ambient	163	4074
39	ambient	169	3920

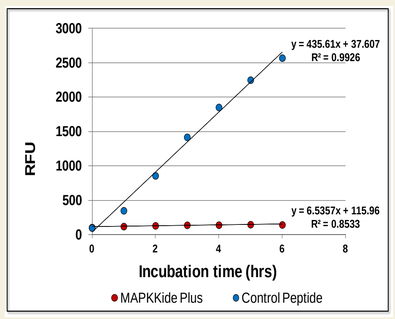

Figure 1: Fluorescence (RFU) as a function of time for **MAPKKide® Plus** (**red**) and Control Peptide (**blue**) in 1:10 plasma at 37 °C in the absence of LF.

B. Optimization of the Microplate Assay Method to Improve Sensitivity

Previous efforts to detect LF in plasma by monitoring the increase in fluorescence using a fluorescent microplate reader had several limitations.

1. First of all, while the fluorescence of the released fluorophore after cleavage is greater than the fluorescence for the substrate-attached fluorophore there is some background fluorescence prior to cleavage, limiting detection of low levels of LF.

2. Secondly, the plasma had to be diluted 1:10 in order to minimize the background fluorescence from the plasma. This changed the sensitivity by a factor of 10. The minimum concentration of LF that could be detected was 0.1 ng LF/ml of 1:10 diluted plasma or 1 ng LF/ml plasma.

In order to address both limitations, two strategies were evaluated.

1. In order to remove the uncleaved **MAPKKide® Plus** from the reaction, the substrate was modified to contain a biotin at the N-terminal. The cleavage of the biotinylated **MAPKKide® Plus** by LF was not effected by this modification. Subsequently, the substrate was attached to streptavidin coated magnetic beads which were then added to the plasma containing a series of LF concentrations. The results indicated that the cleavage of **MAPKKide® Plus** from the coated beads was significantly inhibited.

   Alternately, streptavidin coated beads were added after cleavage of **MAPKKide® Plus** by LF to remove uncleaved substrate. This did not significantly improve the sensitivity of the assay.

2. The sensitivity of this assay can be significantly improved by enrichment of the LF from plasma using antibody-coated microtiter plates. The amounts of LF captured depends on the surface area coated with antibody. The 96-well C8 Starwell Maxi Nunc-Immuno Module Plates used for HPLC detection do not allow detection in a microplate reader. In order to increase the amount of antibody coated on the surface, Maxisorp, immuno-tubes were evaluated. Two antibodies were tested and only an affinity purified polyclonal antibody (see Materials) allowed sufficient capture of the LF. In addition to enrichment of the LF, the method allowed capture of small amounts of LF from plasma without dilution. After addition of the **MAPKKide® Plus**, samples were monitored at 3, 5, and 22 hrs of incubation at 37°C using a fluorescent microplate reader .

C. Microplate Assay Method Using Antibody Capture to Concentrate the LF and Increase Sensitivity.

1. The data obtained for each time point (3, 5, and 22 hours) for 0, 20, 50, 100, 500, and 1000 pg LF/ml neat plasma are presented in **Table 2**. Analysis for each response curve (**see Figure 2**) is given in **Table 3**. The response at each time point, as a function of concentration of LF, is linear.

Table 2: Fluorescence observed for the cleavage of biotinylated **MAPKKide® Plus** after exposure to LF captured from plasma for 3, 5, and 22 hours.

LF (pg/ml plasma)	Average RFU*		
	3 hour digest	5 hour digest	22 hour digest
0	54	56	64
20	55	58	78
50	58	63	96
100	64	72	130
500	99	128	362
1000	147	205	700

*All standards and blanks are run in triplicate. For the blanks there were three sets of triplicates and the average RFU was calculated for 3 triplicates.

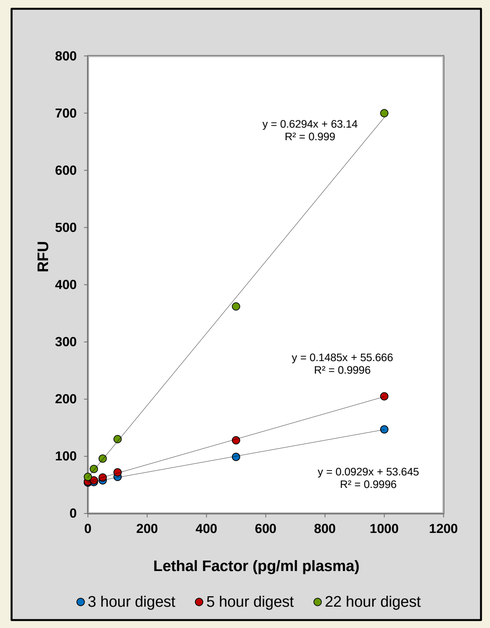

Figure 2: Plot of RFU versus concentration of LF (pg/ml bovine plasma) for 3 hour (blue), 5 hour (red), and 22 hour (green) digestion of biotinylated **MAPKKide® Plus**.

Table 3: Slope, intercept, and correlation coefficients obtained for each incubation time.

Measurement	Incubation Time (hr)		
	3	5	22
Slope (RFU/pg LF)	0.093	0.149	0.630
Intercept (Peak area)	53.6	55.7	65.1
R^2	0.999	0.999	0.999
Std deviation of the blanks*	0.4	0.6	0.9
3x standard deviation + the average	55	58	67
LOD (pg LF/ ml plasma)	20	20	<20

*For the blanks there were three sets of triplicates and the standard deviation was calculated for 3 triplicates.

2. The limit of detection for each incubation time was estimated from the average of the triplicate blanks plus 3 standard deviations of the blanks (99% confidence). This data is shown in **Table 3**. The results indicate that the limit of detection at the 3 and 5 hour time points is 20 pg LF/ml of neat plasma and for the 22 hour incubation the limit of detection is < 20 pg LF/ml neat plasma.

CONCLUSIONS:

This report describes a fast, sensitive, specific and accurate method to detect active infection by *Bacillus anthracis* in bovine plasma at very early stages of intoxication. The method has been simplified to use a fluorescent microplate reader with capture of the LF from plasma using antibody coated immuno-tubes. The response at each time point (3, 5, and 22 hours), as a function of concentration of LF, is linear. The limit of detection is 20 pg LF/ml plasma after 3 or 5 hours of incubation and < 20 pg LF/ml plasma after 22 hours. Both the biotinylated and the unmodified **MAPKKide® Plus** were evaluated. Similar results were obtained for both substrates.