Journal of Veterinary Science & Animal Husbandry
Volume 5 | Issue 3
ISSN: 2348-9790

Research Article Open Access

Validation of an Anti-Protective Antigen ELISA for Quantitative IgG Evaluation in *B. anthracis* Immunized Horses

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Citation: Caldwell M, Hathcock T, Brock KV (2017) Validation of an Anti-Protective Antigen ELISA for Quantitative IgG Evaluation in *B. anthracis* Immunized Horses. J Vet Sci Ani Husb 5(3): 304

Received Date: August 01, 2017 **Accepted Date:** October 23, 2017 **Published Date:** October 25, 2017

Abstract

The potency test for anthrax vaccines has historically involved the challenge of actively or passively immunized laboratory animals with a fully virulent strain of Bacillus anthracis. Lethal challenge studies with the archetypal virulent strains such as *B. anthracis* Ames strain present considerable difficulties in laboratory management and handling and are too inefficient for the early evaluation of alternative preventative and therapeutic interventions. An ELISA for the evaluation of antibody response to protective antigen (PA) in horses immunized with the Sterne 34F2 strain spore vaccine was developed. The objective of this work was to study the performance of this assay in terms of the guidelines set forth by the International Conference on Harmonics (ICH) and the Center for Biologics Evaluation and Research (CBER) for analytical procedures. We have demonstrated a working range for this assay (73-1581 EU/ml) on the bases of the following parameters: linearity (25 and 1,662 EU/ml, $r^2 = 0.9988$, $p < 0.001$), accuracy (94.8 - 105.4 %, recovery within the range of 25 and 1,662 EU/ml), precision (≤ 17.6 % CV, repeatability; ≤ 15.7 and ≤ 13.1 % CV, intermediate precision per day and per analyst, respectively), limit of detection (2.25 EU/ml) and limit of quantitation (25 EU/ml). The assay was also demonstrated to be specific for the evaluation of anti-PA IgG antibodies. Based on the assay performance characteristics it was determined that the assay was adequate for use in *B. anthracis* immunogenicity testing in horses.

Keywords: Anti-PA IgG antibody; Bacillus anthracis; Sterne strain spore vaccine; ELISA; Horses

List of Abbreviations: ELISA: Enzyme Linked Immunosorbent Assay; PA: Protective antigen; rPA: Recombinant protective antigen; LF: Lethal factor; EF: Edema factor; EU: Experimental Unit; %CV: Percentage coefficient of variation; REF: Reference plasma; NHP: Normal horse plasma

Introduction

Bacillus anthracis is the causative agent of anthrax. One important aspect of virulence for *B. anthracis* is the production of three exotoxin proteins that are encoded on the pXO1 plasmid. These proteins are known as lethal factor (LF), edema factor (EF) and protective antigen (PA). These three subunits combine in binary fashion to form the active toxins, lethal toxin (PA + LF) and edema toxin (PA + EF). The common protein subunit in these toxins is the 83 kDa protein known as protective antigen. After binding to its cell surface receptor PA polymerizes into a heptamer pore and mediates cellular entry of the effector molecules, LF and EF [1]. Neutralizing PA is key to protection and has been demonstrated in several *B. anthracis* toxin and live virulent challenges [2-4].

The key application of this ELISA is in the evaluation of immunogenicity of vaccine lots and candidate vaccines in horses. In addition, this assay has also been used in a series experiments designed to assess the efficacy of equine source polyclonal hyper-immune plasma targeted against *B. anthracis*. A set of immune plasma samples was collected from horses immunized monthly with the veterinary Sterne strain spore vaccine for over 1 year. *B. anthracis* Sterne 34F2 strain is an attenuated fully toxigenic, acapsular (pXO1+, pXO2-) strain that is capable of producing all three toxin components. The immune response following exposure to Sterne-strain spores is reflective of natural infection, including an array of specific antibodies against antigens of the spore and each of the toxin components [5,6]. This vaccine is widely employed for the protection of livestock against anthrax, but has seen only limited application in the vaccination of humans stemming from concerns of the potential for a return to virulence [7].

Historically, potency testing for *B. anthracis* vaccines and passive immunotherapy involved the lethal challenge of passively or actively immunized guinea pigs [8]. These bioassays are expensive and handling fully virulent *B. anthracis* requires specialized

containment facilities. A well-defined immunogenicity assay was needed to provide a mechanism to accurately quantify the anti-PA antibody titer early in the development of the hyper-immune plasma during the during the immunization process. Anti-PA IgG enzyme-linked immunosorbent assays (ELISA) have been extensively evaluated in several species for the evaluation of immunogenicity following vaccination against *B. anthracis* [9-13]. Immunogenicity assays are designed to assess antibody response against a set dose of vaccine. Two types of immunogenicity assays have been developed for the assessment of anti-PA IgG antibodies in other species; antigen-specific ELISA and toxin neutralization assays [14,15].

Equine-derived immune products continue to be important therapeutic modalities for toxin based diseases such tetanus, botulism, and snake envenomation, as well as some viral disease such rabies [16-18]. The use of equine-derived antiserum as passive immunotherapeutics for the treatment of anthrax has a long history and was common place prior to the advent of antimicrobial drugs. Currently, there are two US Food and Drug Administration approved passive immunotherapeutics for use as adjunctive treatment of anthrax; raxibacumab (GlaxoSmithKline, London, UK) a human IgG1λ monoclonal antibody specific for PA and produced in a murine cell culture system, and Anthrax Immune Globulin Intravenous (AVIG, Anthrasil, Cangene Corporation, Winnipeg Manitoba, Canada), a human polyclonal antiserum derived from the plasma of persons immunized with anthrax vaccine adsorbed (AVA, BioThrax, Emergent BioSolutions Inc., Rockville, MD) [19]. These products have been subjected to extensive efficacy testing in multiple animal models of anthrax infection. However, their manufacture is costly and dependent upon an established biomedical infrastructure. Due to the low manufacturing cost, simplified logistics, high antibody yield and low risk of transfusion infections, the use of a well-characterized and fully developed equine-derived immunotherapeutic against anthrax represents a compelling alternative for resource limited nations. Equine-derived immunotherapeutics could provide a parallel pathway toward access to these types of therapies. The goal of this work is to describe and characterize an *in vitro* assay that would be useful in the immunogenicity testing of equine-derived immune products along this pathway of development.

The objective of validation of an analytical procedure consists of defining performance characteristics and determining if the assay consistently meets its intended purpose and pre-determined specifications and quality attributes. The International Conference on Harmonics (ICH) and the Center for Biologics Evaluation and Research (CBER) have set forth guidelines for validating assays, which are summarized in the validation of compendial procedures [15,20]. We conducted a series of evaluations designed to examine the assays capacity for specificity, linearity, limit of detection, accuracy, precision and limit of quantitation.

Materials and Methods

Equine Immunization

All procedures described within this study involving the use of animals were approved by Auburn University Institutional Animal Care and Use Committee (protocol #2012-2105). Four apparently healthy Percheron draft mares were obtained and were housed and cared for in compliance with the above IACUC guidelines.

The *B. anthracis* Sterne strain spore vaccine used was the commercially available Anthrax Spore Vaccine, licensed for use in horses (Colorado Serum Company, Denver, CO). Each vial was maintained at 40 °C protected from light until ready to administer. Each horse was immunized with 1.0 ml of *B. anthracis* Sterne strain spore vaccine administered subcutaneously (SC) in the neck. A booster dose was administered SC on Day 21. Thereafter, each horse was immunized approximately once monthly for one year. Whole blood samples were collected in sodium citrate from each horse on Days 0, 21, 56 and monthly thereafter for titer analysis. The whole blood was centrifuged at 5,000 x g for 20 minutes, plasma separated, and stored at -80 °C until ready for analysis. Plasma used for negative controls were obtained from two horses with no known exposure or history of immunization against *B. anthracis* (Normal horse plasma, NHP). Samples were collected and processed as described for principal samples.

Antigens

Recombinant protective antigen (rPA) was obtained from List Biological Laboratories (Campbell, CA). Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 μg/μl and stored at -80 °C until ready to use. The ovalbumin used as a control in the specificity experiments was obtained from Thermo Scientific (Imject Ovalbumin, Thermo Scientific, Rockford, IL). Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 μg/μl and stored at -80 °C until ready to use.

Plasma Samples

Plasma was obtained from horses hyper-immunized with *Bacillus anthracis* Sterne strain 34F2 spore vaccine (Anthrax Spore Vaccine, Colorado Serum Company, Denver Colorado) at monthly intervals for approximately one year. At each sampling 20 ml of whole blood was collected into syringes pre-loaded with sodium citrate. Each sample was centrifuged at 5000 x g for 30 minutes. The plasma was then aspirated from the red cells and divided into 2 ml aliquots and stored at -80 °C until ready to use.

Four plasma samples were randomly chosen from the samples collected after 56 days (representing fully primed individuals) and pooled to make a reference plasma sample (REF) that would be used to develop a titration curve and would then serve as the positive standard for each subsequent assay.

Plasma used for negative standards were obtained from two horses presumed to have had no previous exposure to *B. anthracis* or the vaccine (Normal horse plasma, NHP). Samples were collected and processed as described above.

Quantification of anti-PA IgG antibodies

Wells of an Immulon 2 HB, 96-well round bottom microtiter plate (Southern Biological, Birmingham, AL) were coated with 100 µl of a coating solution (rPA diluted at 1 µg/ml in a 0.01 M PBS solution, pH 7.4). Plates were sealed with parafilm and incubated overnight (16 +/- 2 hours) refrigerated at 4 ºC. Following antigen coating, the plates were washed three times using 300 µl/well of washing buffer (0.01 M PBS and 0.1% Tween 20) each wash. Unknown, REF, and NHP samples were thawed at 4 ºC overnight and kept on ice until ready to use. Unknown plasma samples were pre-diluted 1:500 in dilution buffer and 200 µL were added to wells of the first row. REF plasma samples were also pre-diluted 1:500 and 200 µL were added to two wells of the first row. NHP samples were diluted 1:50 and 200 µL were added to the remaining two wells in the first row. Two-fold serial dilutions of these preparations (unknown, REF, and NHP) were then made down the plate by mixing and transferring 100 µl of diluted plasma between adjacent wells containing 100 µl of dilution buffer (0.01 M PBS, 5% goat serum, and 0.5% Tween 20). For the final row, 100 µl were discarded. The plate was sealed and incubated at 37 oC for 1 hour. After the plate was washed three times as described above, 100 µl of 1:1000 dilution of goat anti-horse antibody conjugated to HRP (Jackson Immunoresearch Lab, INC, West Grove, PA) was added to each well and the plate was sealed and incubated again at 37 ºC for 1 hour.

Following three additional post-incubation washes, 100 µl of the substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt, Thermo Scientific, Rockford, IL) was added to each well. The plate was sealed and incubated at room temperature protected from light for 20 min. Absorbance was read at dual wavelength (405 and 550 nm) in a Bio-Tek Elx800 plate reader (Bio-Tek Instruments, Inc. Winooski, VT).

Titration Curve

Eight rPA-coated microtiter plates were prepared. Following overnight incubation, approximately 200 µl of REF plasma that had been diluted to 1:500 was added to the first well of three rows on each plate then two-fold serial dilutions carried out. In this way, each plate contained REF plasma samples in triplicate. A titration curve was drawn using individual absorbance values at each dilution point from the 24 assays relating the log¬10 experimental units/mL vs. log10 OD. Once established, REF plasma was used as a reference standard in each assay thereafter.

Specificity

Two experiments were carried out in order to demonstrate the specificity of this ELISA. In the first, a 750 µl volume of a 1:500 dilution of REF plasma was mixed with an equal volume of rPA at 100 µg/ml and incubated for 1 hour at 37 ºC. After incubation, 100 µl of the mixture were deposited in three wells in each of two rPA-coated microtiter plates. Then, 100 µl of NHP, diluted 1:50 was added to an additional four wells on each of the plates. Finally, un-treated REF plasma samples diluted 1:500, were added to four wells on each microtiter plate that had been left uncoated. Following these procedures, the ELISA was carried out as described.

In the second experiment, two rPA co ated microtiter plates were prepared, except that ovalbumin (100 µl diluted at 1 µg/ml in a 0.01 M PBS solution), a heterologous protein to *B. anthracis* PA, was also used to coat six wells in each plate. Following overnight incubation, REF plasma, pre-diluted 1:500 and serially diluted twofold into 8 additional wells. In addition, 100 µl of NHP, diluted 1:50 was added to an additional four wells on each of the plates. Finally, 100 µl of pre-diluted 1:500 REF plasma was added to each of the six wells coated with ovalbumin on each plate. Both plates were subjected to the anti-PA IgG ELISA as described previously.

An additional experiment was conducted to determine what effect treatment with milk based blocking solution demonstrated on absorbance values. Plates were prepared with rPA and following the overnight incubation, one of the plates was treated with a blocking solution containing 0.5% skim milk and 0.05% Tween 20 in PBS for 1.5 hours at room temperature (Plate I), while the other remained untreated (Plate II). REF plasma samples diluted 1:500 were added in triplicate to each plate and serially diluted. Both plates were then subjected to the anti-PA IgG ELISA procedure.

Limit of Detection

Two samples of NHP were diluted 1:50 and subjected to the anti-PA IgG ELISA in triplicate in two microtiter plates, on each of the five days within two weeks. Fresh dilutions and rPA-coated plates were individually prepared and used daily. Limit of detection was estimated by interpolating the mean of 60 absorbance values, plus three standard deviations, in a titration curve of REF plasma relating log10 -assigned units/ml per well vs. log10 absorbance. The antilog of the interpolated value was subsequently corrected by the dilution of the sample (1:50).

Linearity

Seven plasma samples of varying antibody concentrations, S1 through S7, were used for the assessment of assay linearity. S1 was randomly selected from the collection of hyper-immune plasma samples. Samples S2 through S7 consisted of individually prepared dilutions of S1 in two-fold increments, from 1:2 through 1:64. All samples were diluted 1:500 into NHP and subjected to the anti-

PA IgG ELISA. All seven samples were randomly distributed in each of three coated microtiter plates. Log10 estimates of antibody concentration per sample (three reads, one per plate) were plotted vs. corresponding log10 reciprocal dilution, and the correlation coefficient, the y-intercept, the slope of the regression line and also the p-value for the lack of fit were calculated.

Accuracy

Units per ml of each of four freshly made samples (S1, S3, S5 and S6 equivalents to the linearity assay) were estimated by anti-PA IgG ELISA. Each sample was assayed in triplicate in each of three plates. Accuracy is expressed as percent recovery and calculated as: mean estimated units per ml/assigned units per ml x 100. The assigned concentration of each sample represents the geometric mean estimate of S1 units (n=16), corrected for each sample's dilution.

Precision

Three samples (S1, S3 and S5) were individually prepared and diluted 1:500. A triplicate of each sample was serially diluted two-fold in each of three microtiter plates and subjected to the anti-PA IgG ELISA on three consecutive days by two analysts. Results are expressed as %CV (SD/mean x 100).

Limit of Quantification

Three samples showing relatively low antibody titers (equivalent to samples S5, S6, and S7) were freshly prepared. Each sample was diluted 1:500 in triplicate and serially diluted in each of three microtiter plates. Anti-PA IgG ELISA was performed on three consecutive days. Results are expressed as % CV (standard deviation/mean x 100).

Statistical Analysis

The titration and linearity curves were drawn in Microsoft Excel® 2010 and were analyzed by linear regression analysis. Student's T tests were used compare mean absorbance values for the specificity experiment. All statistical analyses were conducted using IBM SPSS Statistics version 21.

Results

Specificity

Plasma from naïve horses (NHP) demonstrated no significant reactivity with rPA in the ELISA comparable to REF plasma when applied to uncoated wells (p = 0.092), Table 1. The pre-incubation of free rPA with REF plasma reduced the binding activity to background levels which was not significantly different from the absorbance values observed for NHP (p = 0.213). The results of Table 2 indicate that a separate blocking step is not required in this system, as the blocking solution tested only resulted in a significant effect for the highest REF dilution (1:500). In addition, the REF plasma demonstrated no significant difference in binding activity compared to NHP when ovalbumin was used to coat to the wells of the microtiter plate (p=0.482), Table 3.

Sample ID	**Sample Pre-treatment**	**Dilution in well**	**Absorbance values**	**p value**
REF	Pre-incubated with rPA	1:500	0.076	0.21*
NHP	None	1:50	0.064	0.092**
REF	Uncoated Wells	1:500	0.056	

* p value of significance between REF pre-incubated with rPA and NHP

** p value of significance between control wells and NHP

Table 1: Specificity: Free rPA successfully binds with anti-PA antibodies and reduces the interaction of the antibodies with bound rPA; REF plasma was pre-incubated with free rPA for 1 hour and then subjected to the anti-PA ELISA alongside naïve plasma (NHP) and un-treated REF plasma in uncoated wells. Reported absorbance values represent the mean for triplicate assays

Sample ID	**Plate coating**	**Dilution in well**	**Absorbance values**	**p values**
REF	Ovalbumin	1:500	0.041	0.482
REF	rPA	1:500	1.292	
NHP	Ovalbumin	1:50	0.038	

Table 2: Specificity: REF plasma specifically binds to rPA coated wells but does not bind ovalbumin coated wells; A microtiter plate was coated with both rPA and ovalbumin. REF plasma and NHP were assayed in each coated wells and subjected to the anti-PA ELISA

			Absorbance values (mean)		
Sample ID	**Sample Pre-treatment**	**Dilution in well**	**Plate I**	**Plate II**	**p values***
REF	None	1:500	1.367	1.217	0.021
		1:1000	0.918	1.045	0.166
		1:2000	0.755	0.063	0.345
		1:4000	0.601	0.823	0.366
		1:8000	0.490	0.735	0.343
		1:16000	0.364	0.569	0.295
		1:32000	0.221	0.391	0.381
		1:64000	0.152	0.266	0.380
		1:128000	0.106	0.161	0.413
		1:256000	0.087	0.120	0.374
		1:512000	0.078	0.100	0.444
		1:1024000	0.061	0.186	0.207

*p value of significance between absorbance values obtained from each plate

Table 3: Specificity: Blocking a microtiter plate with a milk based blocking agent does not significantly alter the absorbance values obtained; Two microtiter plates were coated with rPA. Following overnight incubation Plate I was treated with a blocking agent, while Plate II was left untreated. A sample of REF plasma was serially diluted in duplicate and subjected to the anti-PA ELISA with each plate to determine if a blocking step induced significant changes in absorbance values

Limit of Detection

Table 4 summarizes the absorbance values obtained from repeated testing in the PA-ELISA of the two NHP samples. The mean absorbance value for NHP and standard deviation was calculated (0.045 and 0.019, respectively) and interpolated in the titration curve, Table 5 and Figure 1. The anti-PA IgG ELISA is capable of detecting antibody levels above 2.25 EU/ml.

Day		**Sample**			**Absorbance Values**	
		NHP		Plate I		Plate II
1		1		0.032		0.025
				0.029		0.028
				0.031		0.026
		2		0.033		0.028
				0.031		0.022
				0.023		0.015
2		1		0.040		0.036
				0.040		0.029
				0.035		0.018
		2		0.079		0.056
				0.063		0.062
				0.055		0.076
3		1		0.085		0.092
				0.090		0.056
				0.086		0.045
		2		0.048		0.062
				0.046		0.063
				0.043		0.061
4		1		0.045		0.072
				0.045		0.07
				0.041		0.069
		2		0.034		0.044
				0.032		0.042

Day		Sample			Absorbance Values	
		NHP		Plate I		Plate II
				0.025		0.043
5		1		0.035		0.057
				0.032		0.052
				0.035		0.056
		2		0.041		0.028
				0.028		0.036
				0.021		0.042
Overall Mean				0.045		
St. Dev				0.019		

Mean NHP absorbance value = 0.045 ± 0.019
LOD = 0.045 + (3 X 0.019) = 0.102

Table 4: Limit of Detection; Two NHP samples were subjected to repeated anti-PA ELISA to establish the lowest limit of detection for this assay

Mean NHP + 3 STD Absorbance Value	0.102
Log_{10} (0.102)	-0.9914
Interpolated EU/ml	0.045 EU/ml
Corrected for dilution	2.25 EU/ml

Table 5: Interpolated Limit of Detection; The interpolated limit of detection for the anti-PA ELISA was derived from the addition of the mean absorbance value for naïve plasma and three standard deviations and the interpolation of this value from the titration curve. This measure represents the lowest effective assessment of anti-PA antibody titers (EU/ml) in this assay

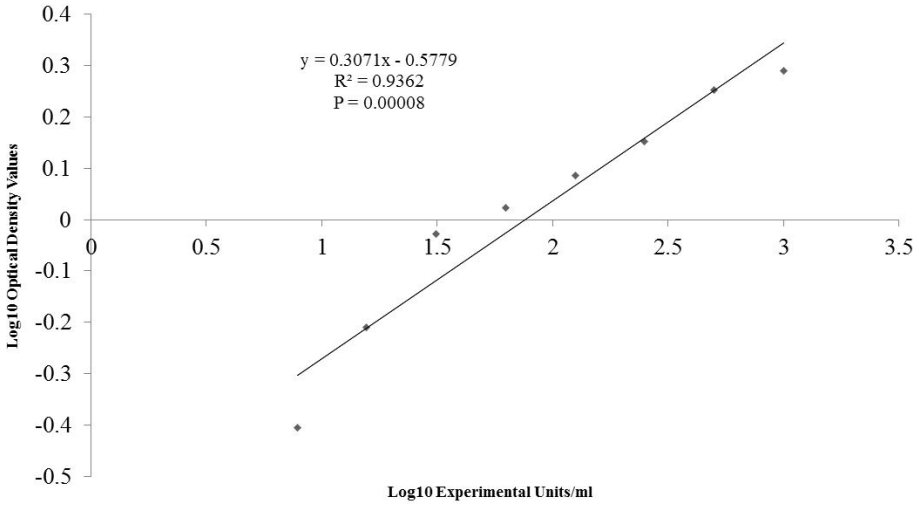

Figure 1: Titration Curve; The titration curve was drawn using individual absorbance values from 24 assays (8 assays in triplicate)

Linearity

In the accuracy experiment the mean estimated EU/ml for S1 was approximately 1500 EU/ml. Table 6 displays the predicted EU/ml for each sample (S1 – S7) following two-fold dilution. Subjecting each sample in triplicate to the anti-PA IgG ELISA demonstrated the assay to be linear between 25 and 1,662 EU/ml. The relationship between the EU/ml estimated in each sample and its dilution is significantly linear (R^2 = 0.9988, P value = 0.000018), Figure 2, indicating that the assay accurately predicted the estimated EU/ml at each dilution point, Table 8.

Mean S1 Absorbance Value	2.494
Log_{10} Absorbance	0.396
Interpolated Log10 (EU/ml)	3.174
Assigned EU/ml for S1	1494

Table 6: Accuracy; The mean absorbance value for S1 after 9 assays was 2.494 (Log_{10} = 0.396). The mean absorbance value was interpolated to represent an estimated 1494 EU/ml

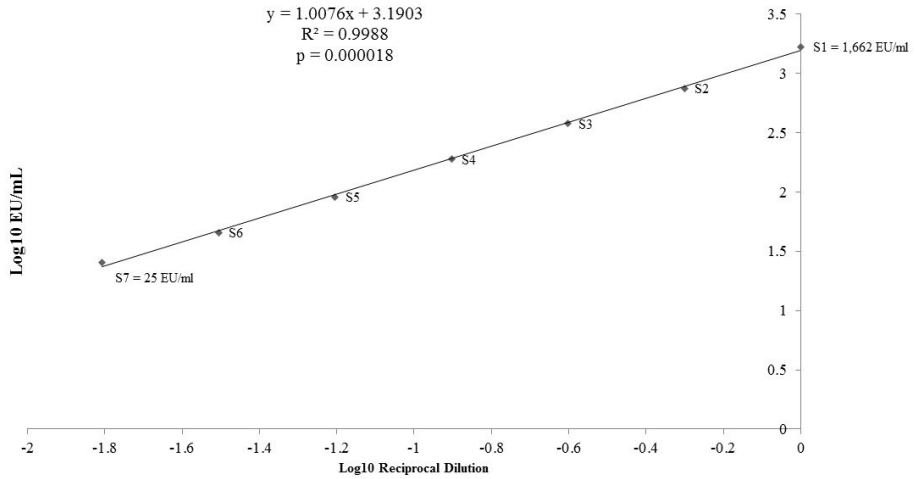

Figure 2: Linearity; An unknown plasma sample (S1) was serially diluted twofold (S2 – S7) and subjected to the anti-PA ELISA in triplicate. The observed absorbance values were used to interpolate the estimated EU/ml for each sample from the titration curve. The $\log_{10}$ EU/ml was then plotted against the log10 of the reciprocal dilution to determine if a linear relationship existed between the estimated EU/ml and the dilution factor

Sample ID	Predicted EU/ml	Estimated EU/ml
S1	1500	1662
S2	750	742
S3	375	376
S4	188	189
S5	94	91
S6	47	45
S7	23	25

Table 8: Linearity; Comparison of the predicted EU/ml and the assay estimated EU/ml of twofold serially diluted samples

Accuracy

Results of the accuracy assessment are presented in Table 7. The mean absorbance value for S1 after 9 assays was 2.494 (Log10 = 0.396) which represents an interpolated estimate of 1494 EU/ml (approximately 1500 EU/ml). Based on this observation, the anticipated antibody concentration for S3, S5 and S6 was 375 EU/ml, 93.75 EU/ml, and 46.87 EU/ml, respectively. The percent

	Replicates	Target sample (EU/ml)			
		S1 (1500)	S3 (375)	S5 (93.75)	S6 (46.87)
		% Recovery	% Recovery	% Recovery	% Recovery
Plate 1	1	98.9	98.1	95.0	98.1
	2	106.9	99.1	94.1	98.5
	3	107.4	98.1	95.3	95.2
Plate 2	4	113.3	98.1	94.4	94.1
	5	107.2	99.9	95.6	95.9
	6	102.2	99.5	94.1	98.5
Plate 3	7	113.0	99.1	95.9	97.7
	8	102.5	98.1	95.6	100.0
	9	97.1	99.7	93.8	93.7
Mean		105.4	98.9	94.8	96.8

Table 7: Accuracy; Units per ml of each of four freshly made samples (S1, S3, S5 and S6 equivalents to the linearity assay) were estimated by anti-PA ELISA. Each sample was assayed in triplicate in each of three plates. Accuracy is expressed as percent (%) recovery: mean estimated units per ml/assigned units per ml x 100

recovery for S1 ranged from 1456.5 - 1699.5 EU/ml (97.1% - 113.3%). The percent recovery for S3 ranged from 367.87 – 374.62 EU/ml (98.1% - 99.9%). The percent recovery for S5 ranged from 88.2 – 89.62 EU/ml (94.1% - 95.6 %). The percent recovery for S6 ranged from 43.92 – 46.87 (93.7% - 100%). On the basis of the criterion established by the ICH outlined in Table 11, the assay is accurate between 46 and 1500 EU/ml (94.8 - 105.4 % recovery within this range).

Assay Characteristics	Acceptance Criteria	Results
Precision-Repeatability	% CV < 20	
Intra-plate		< 17.6
Inter-plate		< 16.4
Intermediate Precision		
Days	% CV < 20	< 15.7
Analyst	% CV < 20	< 13.1
Accuracy	80 – 120 %	94.8 – 105.4 %
Limit of Detection	—	2.25 EU/ml
Limit of Quantification	—	25 EU/ml
Linearity	—	Working Range: 25 and 1,662 EU/ml $R^2 = 0.9988$ P value = 0.000018

Table 11: Acceptance Criteria and Characteristics of the anti-PA Equine ELISA; Summary of the performance characteristics of the anti-PA ELISA and the acceptance criteria set forth by the ICH

Precision

Table 9 summarizes assay variability expressed as % CV at two levels of repeatability: intra-plate (triplicate estimates per sample per plate) and intra-day (three assays performed each day) and two levels of intermediate precision: inter-day (three days), and inter-analyst (two analysts on the same day). The assay displayed acceptable repeatability and intermediate precision on the basis of the validity criteria in the 73-1581 EU/ml range establishing this as the reliable working range of anti-PA IgG antibodies for this assay.

Sample	Mean (EU/ml)	%CV						Inter-analyst
		Analyst 1			Analyst 2			
		Intra-plate	Intra-day	Inter-day	Intra-plate	Intra-day	Inter-day	
S1	1581	3.3	7.0	9.0	12.6	14.0	15.7	13.1
		4.5			1.3			
		3.9			9.8			
		11.1	9.4		1.3	10.1		
		9.3			7.7			
		4.0			1.7			
		0.9	7.7		6.1	16.4		
		11.1			15.3			
		5.3			16.6			
S3	323	15.9	12.3	10.4	9.2	11.8	10.4	10.5
		7.2			1.3			
		1.9			9.9			
		11.0	9.4		11.2	7.8		
		0.3			6.7			
		5.4			2.8			
		1.7	8.3		6.3	10.5		
		12.0			16.8			
		0.9			7.5			
S5	73	17.6	13.8	10.9	3.7	8.2	10.7	10.8
		9.3			2.5			
		4.9			12.0			

Sample	Mean (EU/ml)	%CV						Inter-analyst
		Analyst 1			Analyst 2			
		Intra-plate	Intra-day	Inter-day	Intra-plate	Intra-day	Inter-day	
		4.0	9.7		3.0	5.9		
		6.1			3.1			
		10.3			9.3			
		1.2	4.4		1.4	12.8		
		5.8			15.1			
		2.5			7.7			

Table 9: Precision; Results of repeated analyses of three samples were compared across multiple plates performed on multiple dates by two analysts to determine the repeatability and robustness of the assay. Results are expressed as %CV (SD/mean x 100)

Limit of Quantitation

To define the limit of quantitation, precision was estimated in the range 25-71 EU/ml. The calculated % CV for this range fell well below that of the acceptable % CV (< 20%) for repeatability establishing 25 EU/ml as the lowest limit anti-PA IgG antibodies can be reliably quantified, Table 10.

Sample	Mean	% CV		
	(EU/ml)	Intra-plate	Intra-day	Inter-day
S5	**71**	**8.1**	**10.5**	**10.2**
		9.7		
		12.7		
		3.7	10.1	
		10.9		
		10.4		
		4.8	9.1	
		6.2		
		7.5		
S6	44	7.3	5.6	6.6
		3.8		
		4.0		
		5.7	6.5	
		3.8		
		7.4		
		5.2	6.3	
		6.2		
		6.6		
S7	25	1.7	5.5	7.0
		10.4		
		3.0		
		7.0	7.6	
		5.8		
		9.7		
		4.8	7.3	
		5.0		
		6.2		

Table 10: Limit of Quantitation; Three samples showing relatively low antibody titers were analyzed across multiple plates performed on multiple days to determine the lowest antibody concentration that demonstrated adequate repeatability. Results are expressed as %CV (SD/mean x 100)

Discussion

Protective antigen is a critical component of the protective immune response to *B. anthracis* infection [21,22]. The pursuit of new vaccines as well as monoclonal and polyclonal immunotherapies requires an efficient and effective assessment of antibody response

during the initial stages of vaccine candidate development. We have developed an anti-PA IgG ELISA designed to measure the immune response against PA in horses. This assay was developed using the validation parameters suggested by the ICH and CBER and is consistent with other literature where serologic assays have been used to verify immunologic responses and vaccine analyses [23-25]. Acceptance criteria for the validation of this assay were based on the performance of similar ELISAs [15,26]. The intended use of this assay is to measure antibody titers against PA in the plasma of horses vaccinated with anthrax vaccines. The vaccine used in this study was the *B. anthracis* Sterne strain spore vaccine which produces all three components of the lethal and edema toxins [5,6]. In addition, other epitopes such as exterior spore antigens and bacterial cell surface antigens are present and have been shown to induce an immune response in other species [27,28]. Despite the presence of this diverse immune response, it was demonstrated that the ELISA validated here can consistently quantify anti-PA IgG antibodies in the plasma of horses. Saturating a plasma mixture with free PA reduced the level of binding to that equivalent of naïve horse plasma. When ovalbumin was used as a heterologous antigen in the solid phase of the ELISA, plasma from horses immunized against anthrax demonstrated negligible reactivity indicating that the response seen to PA in this assay was not the result of non-specific binding.

The results of the accuracy and precision assessment fell well below the guidelines established by the ICH indicating that this ELISA is both highly repeatable and robust for the range of samples assayed. Some sources recommend a lower standard of 10% CV necessary to define suitable precision. The samples evaluated in the precision assessment represented approximately 100, 80, 50 and 20% of maximal activity. Because both the highest and lowest concentrations of antibody sampled in the present study were demonstrated to be suitably repeatable the true working range of EU/ml was not fully established. Often, extremes in antibody concentration show less precise measurements due to non-uniform error in the assay. Therefore, further evaluation will be necessary to determine what these levels are for this assay.

The limit of quantitation can be defined as the assay's measure of sensitivity or capacity to measure the smallest amount of target analyte. In the present study the lowest derived antibody concentration was estimated to be 25 EU/ml, which displayed acceptable repeatability. The limit of detection is a means of establishing background levels of reactivity in the plasma of horses expected to be naïve to *B. anthracis*. Based on the results of several repeated assays of two normal horse plasma samples, the lowest detectable anti-PA IgG antibody concentration that was statistically significant from background levels was 2.25 EU/ml. The incorporation of three standard deviations above the mean absorbance value for NHP yields a 99% confidence level in the difference observed.

Conclusion

In conclusion, the results of this study validate this immunoassay for the accurate assessment of specific anti-PA IgG titers in horses. The application for this assay is in defining the immunogenicity of future vaccine strategies in horses ensuring its usefulness in accurately and reliably assessing the antibody response to PA in the plasma of horses.

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