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Validation of high throughput screening of human sera for detection of anti-PA IgG by Enzyme-Linked Immunosorbent Assay (ELISA) as an emergency response to an anthrax incident

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ABSTRACT

To improve surge testing capability for a response to a release of *Bacillus anthracis*, the CDC anti-Protective Antigen (PA) IgG Enzyme-Linked Immunosorbent Assay (ELISA) was re-designed into a high throughput screening format.

The following assay performance parameters were evaluated: goodness of fit (measured as the mean reference standard r^2), accuracy (measured as percent error), precision (measured as coefficient of variance (CV)), lower limit of detection (LLOD), lower limit of quantification (LLOQ), dilutional linearity, diagnostic sensitivity (DSN) and diagnostic specificity (DSP). The paired sets of data for each sample were evaluated by Concordance Correlation Coefficient (CCC) analysis.

The goodness of fit was 0.999; percent error between the expected and observed concentration for each sample ranged from −4.6% to 14.4%. The coefficient of variance ranged from 9.0% to 21.2%. The assay LLOQ was 2.6 μg/mL. The regression analysis results for dilutional linearity data were $r^2 = 0.952$, slope = 1.02 and intercept = −0.03. CCC between assays was 0.974 for the median concentration of serum samples. The accuracy and precision components of CCC were 0.997 and 0.977, respectively.

This high throughput screening assay is precise, accurate, sensitive and specific. Anti-PA IgG concentrations determined using two different assays proved high levels of agreement. The method will improve surge testing capability 18-fold from 4 to 72 sera per assay plate.

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1. Introduction

The CDC validated ELISA for detection of *Bacillus anthracis* PA-specific IgG in human sera was originally developed as a laboratory assay for a Phase 4 human clinical trial on the safety and efficacy study of Anthrax Vaccine Adsorbed (AVA, BioThrax®) [1–3]. The assay has been shown to be highly accurate, precise, specific, sensitive and robust for quantification of anti-PA IgG in human serum. This assay was also used during the 2001 bioterrorism attack [4–7] and the CDC Anthrax Laboratory Surge Exercise in 2013 for quantification of anti-PA IgG in human sera. The surge exercise was performed at CDC in 2013 to test CDC's laboratory capacity for a response to a bioterrorism event involving the deliberate release of *B. anthracis*. These two events identified throughput as an opportunity for assay improvement for application to a large-scale response related to a release of *B. anthracis*. Emergency response requires rapid screening of a large number of human sera. The original assay allowed only 4 sera per plate with total 8 plates per operator per day, resulting in testing only 32 sera per operator per day. This issue raised the necessity to redesign the original validated anti-PA IgG ELISA to improve CDC surge testing capability for a large-scale response to a release of *B. anthracis*. In this paper we report the validation of the redesigned high throughput screening format.

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2. Materials and methods

2.1. Human test and control sera

The acquisition and use of human test serum in this study were approved by the CDC Human Subjects Institutional Review Board (IRB). Sera from the CDC Anthrax Vaccine Research Program (AVRP) clinical trial participants and clinical trial site IRB approvals were obtained as described previously [1]. The preparation of the standard AVR801, positive quality controls (QCs) (AVR1749, AVR1750 and AVR1751) and negative QC (AVR811) used in the study have been described previously [2,8].

2.2. Anti-PA IgG Enzyme-Linked Immunosorbent Assay (ELISA) for high throughput screening of human sera

The method of quantitative anti-PA IgG ELISA has been described previously [2]. The main difference in the current assay is in the plate format: the reference standard is run in duplicate instead of in triplicate and test samples are run in a single well at one dilution instead of in duplicate and at 8 serial dilutions. The reagent control was removed to allow an additional dilution of the reference standard (see Fig. 1). Briefly, the procedure consists is as follows: Immulon® 2 HB microtiter plates (Thermo Labsystems, Franklin, MA) were coated with purified recombinant PA (2 μg/mL) (BEI Resources, Manassas, VA) in 0.01 M phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Gaithersburg, MD) and incubated overnight (16–24 h) at 2–8 °C. Plates were washed three times with PBS containing 0.1% Tween-20, pH 7.4 (ELISA wash buffer). Master Plate Diluent (PBS containing 5% skim milk and 0.5% Tween-20, pH 7.4) was added to the first two columns of the plate (100 μl per well). The standard reference serum AVR801 was diluted 1:25 in Master Plate Diluent and 100 μl was loaded into the first two wells of the plate and serially transferred in 2-fold dilutions down the plate to make an 8-point dilution series. The test sera were diluted 1/50 in Master Plate Diluent and 100 μl of each test serum was loaded into 72 wells of the plate (Fig. 1). The last column on the plate was designated for positive and negative QC samples. Plates were incubated for 60 ± 5 min at 37°±2 °C and washed three times with ELISA wash buffer. Horseradish peroxidase conjugated mouse monoclonal anti-human IgG Fc PAN clone HP6043 (lot #061010 at 1:27,000 dilution, Hybridoma Reagent Laboratory, Baldwin, MD) was added to all wells (100 μl/well) and incubated at 37°±2 °C for 60 ± 5 min. Plates were washed three times and 100 μl of ABTS Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to all wells. After 30 ± 5 min incubation at 37°±2 °C 100 μl of ABTS Peroxidase Stop Solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and plates were read within 30 min using the BioTek spectrophotometer at a wavelength of 405 nm with a 490 nm reference utilizing the Gen5 software (Bio-Tek Instruments, Inc., Winooski, VT). Assay endpoints were reported as concentrations (μg/mL) of anti-PA IgG for quality control sera and test samples using the ELISA for SAS program (ELISA HT 3.sas) version 9.3. The reference standard is fit to a 4-parameter logistic (4-PL) curve, and sample concentrations are calculated by interpolating to the reference standard, which has a pre-established concentration of 109.4 μg/mL. Samples with ODs below the lowest standard OD are reported as 0, and samples with ODs above the highest reference standard OD are reported as >109.4. High samples will be retested in serial dilution in the original assay format if quantification of high results is required. All values for validation parameters were obtained from at least three experiments performed by four operators on non-consecutive days.

2.3. Assay acceptance criteria

A set of five acceptance criteria for assay performance in the high throughput screening format were similar to the acceptance criteria for the CDC validated anti-PA IgG ELISA [2,9]. These five assay acceptance criteria were: 1) the mean Optical Density (OD) value of the negative control was required to be less than 0.200 OD units; 2) the standard reference serum was required to have a weighted coefficient of determination (r^2) value of ≥0.990 to the 4-Parameter Logistic (4-PL) model; 3) the mean anti-PA IgG concentration for each of three positive quality control sera were required to have coefficients of variation (CV) <20%; 4) at least 2 of 3 positive control sera were required to have anti-PA IgG

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	T#1	T#2	T#3	T#4	T#5	T#6	T#7	T#8	T#9	QC1
B	S2	S2	T#10	T#11	T#12	T#13	T#14	T#15	T#16	T#17	T#18	QC1
C	S3	S3	T#19	T#20	T#21	T#22	T#23	T#24	T#25	T#26	T#27	QC2
D	S4	S4	T#28	T#29	T#30	T#31	T#32	T#33	T#34	T#35	T#36	QC2
E	S5	S5	T#37	T#38	T#39	T#40	T#41	T#42	T#42	T#44	T#45	QC3
F	S6	S6	T#46	T#47	T#48	T#49	T#50	T#51	T#52	T#53	T#54	QC3
G	S7	S7	T#55	T#56	T#57	T#58	T#59	T#60	T#61	T#62	T#63	N
H	S8	S8	T#64	T#65	T#66	T#67	T#68	T#69	T#70	T#71	T#72	N

Fig. 1. Plate layout for performance of validation experiments for high throughput screening of human sera for detection of anti-PA IgG as an emergency response to an anthrax incident. The Standard Reference serum AVR801 (S) was loaded in duplicated in the first two columns (1–2). The test serum was diluted 1/50 in Master Plate Diluent (PBS containing 5% Skim Milk and 0.5% Tween-20, pH 7.4) and loaded into the 72 wells of the plate in (columns 3–11). The last column on the plate was designated for three positive (QC1, QC2 and QC3) and one negative QC (N1).