HHS Public Access

Author manuscript

Biologicals. Author manuscript; available in PMC 2018 January 01.

Published in final edited form as:

Biologicals. 2017 January ; 45: 61–68. doi:10.1016/j.biologicals.2016.09.012.

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.

Keywords

Emergency response; Anthrax; Screening; Human sera; Validation

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Required disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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 bio-terrorism 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.

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). Briefiy, 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 concentrations within 2 standard deviations (SD) of their expected values and 5) no positive control sera anti-PA IgG concentrations were allowed to be >3SD from the expected value. All five acceptance criteria were required to be met, otherwise all test samples on the plate were rejected and sample testing repeated. During normal testing, all serum specimens are required to have at least two passing results generated by two independent operators and expressed as a mean of those two passing results. Inter-operator precision is required to have a %CV of ≤30%. Samples with >30% CV between operators would be repeated up to a total of 4 times, and the median result would be reported. Note that during validation, samples were tested up to 12 times for accuracy, precision, lower limits of detection and quantification and dilutional linearity of the assay. Concordance with the historical ELISA, diagnostic sensitivity (DSN) and diagnostic specificity (DSP) were tested according to the normal 2 operator test method described here.

2.4. Validation parameters

Validation was done in accordance with the Food and Drug Administration guidance [10]. Acceptance criteria were derived from the assay development data (Table 1), and are consistent with the criteria used for the original assay. The following validation parameters were tested during validation experiments: accuracy, precision, goodness of fit, Lower Limit of Detection (LLOD), Lower Limit of Quantification (LLOQ), dilutional linearity, and range.

2.4.1. Accuracy—Accuracy, a measure of the exactness of the assay, was determined by the repeated analysis of nine sera sample with predetermined (expected) anti-PA IgG concentrations ranging from 10.9 μg/mL to 72.9 μg/mL: AVR1489, AVR1490, AVR1491, AVR1492, AVR1493, AVR1494, AVR1495, AVR1496, and AVR1497. Preparation and determination of the expected values for samples AVR1489, AVR1490, AVR1491, AVR1492, and AVR1497 has been described previously [2]. The samples AVR1493, AVR1494, AVR1495, and AVR1496 were prepared by spiking positive serum AVR802 into four negative human serum pools (AVR1412, AVR1413, AVR1408, and AVR1436, respectively) in different ratios. The assay-based mean observed concentrations were calculated and compared to the expected concentration of the sample to determine the percent error (%E) of the assay. The %E was calculated as [(observed–expected)/expected] × 100%. A %E of ≤25% was adopted as an acceptable level of accuracy based on the data from the development experiments.

2.4.2. Precision—Inter-assay precision, a measure of the degree of repeatability of the assay between operators under normal operating conditions, was determined by repeated analysis of the same nine sera samples used for determination of accuracy (AVR1489, AVR1490, AVR1491, AVR1492, AVR1493, AVR1494, AVR1495, AVR1496, and AVR1497). Precision was expressed as the coefficient of variation (% CV = standard deviation/mean) of the reported anti-PA IgG concentrations between operators. The acceptable level of inter-assay precision was ≤25%.

2.4.3. Goodness of fit—Goodness of fit was expressed as the estimated non-linear coefficient of determination (r^2) of the standards data. An r^2 value that approaches 1.0 is indicative of a precise fit for the data to the standard curve. The standard's ODs were fitted to a 4-PL model by SAS program ELISA HT 3.sas. The goodness of fit was determined by averaging the r^2 values of 12 independent standard reference curves for AVR801 from the validation experiments.

2.4.4. Lower limit of detection and lower limit of quantification—LLOD of the assay is the lowest concentration of anti-PA IgG that can be empirically detected in a diluted serum sample independent of criteria for assay accuracy and precision. Because concentrations are not extrapolated outside the range of the reference standard, this is the concentration of the most diluted reference standard (109.4/6400) in a well divided by the dilution of the test serum (1/50). LLOQ of the assay is the lowest concentration of anti-PA IgG that can be measured in a diluted serum sample with a fixed degree of precision and accuracy. The degree of precision and accuracy at LLOQ for this assay was selected as a

coefficient of variation (%CV) of ≤25% for the calibrated antibody concentration and ≤50% error (unpublished development data). LLOQ was determined experimentally by testing 14 different serum samples with anti-PA IgG concentrations ranging from 0.3 μg/mL to 7.4 μg/mL (AVR2252, AVR2253, AVR2254, AVR2255, AVR2256, AVR2257, AVR2258, AVR2259, MCR0147, MCR0148, MCR0150, MCR0152, MCR0153, and MCR0155). Preparation of samples AVR2252, AVR2253, AVR2254, AVR2255, AVR2256, AVR2257, AVR2258 and AVR2259 has been described previously [2]. The validation serum samples MCR0147, MCR0148, MCR0150, MCR0152, MCR0153, and MCR0155 were prepared by spiking a positive serum AVR1490 into a negative human serum pool AVR411 in different ratios with concentrations from 0.4 μg/mL to 1.9 μg/mL.

2.4.5. Dilutional linearity and range—The dilutional linearity of the assay is its ability to generate results that have a linear concentration response in a particular diluent that is directly, or by a well-defined mathematical transformation, proportional to the concentration of anti-PA IgG in the sample. Note that dilutional linearity is measuring the linearity of concentrations interpolated from the reference standard, and is measure of the reference standard's ability to accurately determine concentrations across the full range of the assay. Expected and observed anti-PA IgG concentrations in serum samples were $\log_{10}$-transformed for analysis. Twenty-three serum samples with the range of concentrations from 0.3 μg/mL to 72.9 μg/mL were used for determination of dilutional linearity (AVR1489, AVR1490, AVR1491, AVR1492, AVR1493, AVR1494, AVR1495, AVR1496, AVR1497, AVR2252, AVR2253, AVR2254, AVR2255, AVR2256, AVR2257, AVR2258, AVR2259, MCR0147, MCR0148, MCR0150, MCR0152, MCR0153, and MCR0155). Dilutional linearity was determined from regression analysis of empirically observed anti-PA IgG concentrations for the validation serum samples versus the expected concentrations for those samples. The fit of the data to the regression line required a mean $r^2 \geq 0.850$ with a slope between 0.9 and 1.1 and an intercept between −0.1 and 0.1 in linear scale (unpublished development data).

The assay range was calculated as the interval of anti-PA IgG concentrations that can be interpolated from the standard curve with acceptable accuracy, precision and linearity. The lower limit of the range was established at LLOQ and the upper limit was established at the maximum detectable concentration. The starting dilution of 1/50 of the reference standard serum AVR801 allowed for a detection of a maximum concentration of 109.4 μg/mL.

2.5. Reactivity threshold, diagnostic sensitivity and specificity

Serum samples from AVA vaccinated volunteers and control samples (n = 320) were used for calculation of DSP and DSN on sample and on patient levels. At least two experiments were performed by two different operators. A panel of sera from human adult volunteers from the CDC Anthrax Vaccine Research Program (AVRP) clinical trial were chosen to simulate positive and negative samples and patients. Samples from week 0 (pre-vaccination) were chosen to simulate an acute sample, and samples from week 8 were chosen to simulate convalescent samples. There were 80 participants from the control (unvaccinated) group to simulate true negative patients (expected to have no anti-PA IgG at either week 0 or week 8), and 80 participants from the treatment (vaccinated) group to simulate true positive patients

(expected to have no anti-PA IgG at week 0, but detectable anti-PA IgG at week 8 after vaccination). At the sample level, all samples from the control group and the week 0 samples from treatment group were defined as negative, and the week 8 samples from the treatment group were defined as positive. At the patient level, the control group were defined as negative and treatment group were defined as positive.

A sample is considered positive if its concentration is greater than or equal to the reactivity threshold (RT). A range of reactivity threshold values were evaluated from 1.7 μg/mL to 13.4 μg/mL (the highest concentration of anti-PA IgG in a false positive sample). The historical RT was 3.7 μg/mL [2]. Diagnostic Specificity (DSP) measures the ability of the assay to identify a true negative result for a serum sample DSP = TN/(TN + FP) × 100% and Diagnostic Sensitivity (DSN) measures the ability of the assay to identify a true positive result for a serum sample DSN = TP/(TP + FN) × 100%. TN = true negative, negative result on a negative sample. FP = false positive, positive result on a negative sample. TP = true positive, positive result on a positive sample. FN = false negative, negative result on a positive sample.

Since reactive samples can be caused by vaccination as well as infection, patients are diagnosed as positive if there is a 4-fold or greater increase in concentration between the acute and convalescent samples [11]. Thus, DSP and DSN at the patient level measure the ability of the assay to identify a true negative result and a true positive result for a patient using the fold change over time. Concentrations below the LLOQ (2.6 μg/mL) were replaced by ½ LLOQ (1.3 μg/mL) for purposes of calculating fold increase. The starting dilution of 1/50 of the reference standard serum AVR801 allows detection of a maximum concentration of 109.4 μg/mL. Therefore the values above 109.4 μg/mL were replaced with 110.0 μg/mL as the next whole number above 109.4. In cases where quantification of concentration above 110 μg/mL would be required to determine if the fold increase is >4 (i.e. for patients who had >27.4 μg/mL in their acute sample), high concentration samples can be retested either at higher starting dilution in the HT format or in dilution series in the original assay format. No such samples were observed in this sample set.

2.6. Concordance correlation analysis

Comparative analysis of anti-PA IgG concentrations (μg/mL) obtained by the High Throughput anti-PA IgG by Enzyme-Linked Immunosorbent Assay (ELISA) for Screening of Human Sera as an Emergency Response to an Anthrax Incident and the validated CDC ELISA for Detection of *B. anthracis* PA-Specific IgG in Human Sera [2] was performed using Deming regression [12]. Selected samples consisted of a panel of 95 human sera from AVA vaccinated volunteers. The samples encompassed a range of anti-PA IgG concentrations from the non-vaccinated volunteers (n = 19, week 0) and vaccinated volunteers (n = 76, week 8). Samples with concentration of anti-PA IgG < LLOD and >109.4 μg/mL were omitted from the analyses. The paired sets of data for each sample were evaluated by determining the Concordance Correlation Coefficient (CCC) and goodness of fit r^2 value was calculated from the Deming regression of the $\log_{10}$ transformed median anti-PA IgG concentrations of AVR801. A CCC value of ≥0.95 was considered an acceptable level of agreement between the data sets. Median concentration of anti-PA IgG

concentration obtained by the original CDC validated ELISA was plotted on the X axis and median anti-PA IgG concentration by the High Throughput anti-PA IgG ELISA (HTE) is plotted on the Y-axis. The accuracy component of the CCC is calculated from the distances of the Deming regression best-fit line from the line of unity, where slope = 1 and intercept = 0. Concentrations below LLOD (0.85 μg/mL) could neither be measured nor replaced by 0.0 μg/mL because a non-zero value was required to calculate the ratio of fold-increase. Therefore, all such values were replaced with 0.425 μg/mL (1/2 of LLOD) rounded up to 0.43. The values above 109.4 μg/mL were replaced with 110.0 μg/mL as the next whole number above 109.4. Samples with results < LLOD and >109.4 μg/mL in both sets were omitted from the analyses. Graphs of the Deming regression and the CCC calculations were done with SAS® (SAS Institute Inc., Cary, NC). At least two experiments were performed by two different operators over nonconsecutive days.

2.7. Data analysis

The raw data were imported into and analyzed by an ELISA analysis program ELISA for SAS (ELISA HT 3.sas) generated in the laboratory for calculation of the antibody concentration for standard, positive and negative quality control sera, and the validation test samples. Mean anti-PA IgG concentration, standard deviation and coefficient of variation (% CV) s were used to calculate the validation parameters except CCC analysis where the median anti-PA IgG concentration was used. All calculations for regression analyses, DSN/DSP analysis and CCC analysis were performed with $\log_{10}$ transformed data using SAS® (SAS Institute Inc., Cary, NC) version 9.3. The calculation procedure for each validation parameter is described in its respective designated section.

3. Results

The assay's results for detection of anti-PA IgG *B. anthracis* PA-specific IgG in validation test human sera, validation parameters, and acceptance criteria are summarized in Table 2. All validation parameters were met or exceeded.

3.1. Goodness of fit, accuracy and precision

The mean r^2 of the standard's ODs were fitted to a 4-PL model by SAS program ELISA HT 3.sas. The standard's data were obtained from all validation experiments performed by four operators. The mean r^2 was 0.999 with the range from 0.995 to 1.00. The assay demonstrated high accuracy and precision for this assay. The %E ranged from −4.6% to 14.4% and inter-assay (intermediate) precision expressed as %CV of the reportable value ranged from 9.0% to 21.2%. Each validation sample had a ≤25% E and ≤25% CV. These data are indicative of a high level of accuracy and reproducibility of this assay between four independent operators and met the predetermined assay acceptance criteria (Tables 1 and 2).

3.2. Lower limit of detection and lower limit of quantification

LLOD of the standard AVR801 has been determined as 1.7 μg/mL [2] at the 1/1600 dilution. The current format of the plate allowed us to use an additional 1/6400 dilution of the standard, so concentrations 0.85 μg/mL was accepted as LLOD of the High Throughput Screening assay. Similarly, the values above the concentration of the standard 109.4 μg/mL

could not be measured and were replaced with 110.0 μg/mL (110 is the next whole number after 109.4, used as a marker to indicate >109.4). If quantitation is necessary for high samples they can be repeated at higher starting dilution or run in the multi-dilution original assay format.

LLOQ was determined experimentally by testing 14 serum samples. The sample AVR2253 had the lowest expected concentration 2.6 μg/mL that met the validation acceptance criteria and therefore is LLOQ for this assay (≤50% error and CV ≤ 25% for each validation sample) (Tables 1 and 2).

3.3. Dilutional linearity and range

The $\log_{10}$ transformed concentrations of anti-PA IgG (μg/mL) for 23 validation serum samples with the range of anti-PA IgG concentrations 0.3–72.9 μg/mL were used for determination of dilutional linearity. The $\log_{10}$ transformed observed concentrations were plotted on the Y-axis and the $\log_{10}$ transformed expected mean value of those serum samples on the X-axis. A line of best fitness was generated to represent the trend of the data points and the slope and intercept of this line was calculated. Observations of 0 were masked as ½ LLOD (0.43 μg/mL). Regression analysis showed the fit of the data with $r^2 \geq 0.952$, slope = 1.029 and intercept = −0.03 that met the acceptance criteria ($r^2 \geq 0.850$ with a slope between 0.9 and 1.1 and an intercept between −0.1 and 0.1) (Table 1, Fig. 2).

The assay range was calculated as the interval of anti-PA IgG concentrations that can be interpolated from the standard curve with acceptable accuracy, precision and linearity. The lower limit of the range was established at LLOQ = 2.6 μg and the upper limit was established at the maximum detectable concentration 109.4 μg/mL.

3.4. Diagnostic sensitivity and specificity

DSP and DSN on a sample level measures the ability of the assay to identify a true negative and a true positive result for a serum sample, respectively. A sample is considered positive if its concentration is greater than or equal to the reactivity threshold of 3.7 μg/mL [2]. DSP and DSN was calculated for a range of values from 1.7 μl/mL (the LLOD for the Standard Reference serum AVR801) to 13.4 μg/mL (the highest concentration of anti-PA IgG in a false positive sample).

Analysis of serum anti-PA IgG responses from unvaccinated volunteers at week 0 indicated 236 non-reactive samples (True Negative) and four reactive sera (False Positive). Based on these data the assay DSP on a sample level was 98.3%. While increasing the RT above the historical levels could increase the DSP in this set, the historical level of 3.7 μg/mL was retained to maintain high sensitivity. Analysis of serum anti-PA IgG responses of vaccinated volunteers indicated 100% DSN (80 of 80 sera > LLOQ) (Fig. 3).

DSP and DSN at the patient level measures the ability of the assay to identify a true negative and a true positive result for a patient, respectively. A patient is diagnosed as positive if there is a 4-fold or greater increase in concentration between the simulated acute (non-vaccinated volunteers, time point 0) and simulated convalescent samples (vaccinated volunteers) [11]. All 80sera pairs from vaccinated volunteers indicated a 4-fold or greater increase in

concentration between simulated acute and simulated convalescent samples (100% DSN). There were also no False Positive patients (100% DSP).

3.5. Concordance correlation analysis

Comparative analysis of anti-PA IgG concentrations (μg/mL) for 95 serum samples obtained by two assays showed that CCC = 0.974. A CCC value of ≥0.95 was considered an acceptable level of agreement between the data sets [12]. The measure of accuracy was 0.997 and measure of precision was 0.977 (Fig. 4).

4. Discussion

Serological testing is a necessary part of anthrax diagnostics [13]. Assessment of acute and convalescent serum specimens for evaluation of patient's immune status to anthrax requires a validated serological assay. The newly established and validated assay for high throughput screening of human sera was developed in our laboratory as an adaptation of the CDC validated ELISA for detection of *B. anthracis* PA-Specific IgG in human sera (Semenova, 2012). Together these two assays can be used as a complement to each other and tool during an emergency response to the deliberate release of *B. anthracis*. During anthrax attacks in 2001, over 70,000 samples were tested in laboratories across the country and many states' public health laboratories were overwhelmed by the volume of testing [14]. CDC also processed thousands of samples during the anthrax outbreak 2001 investigation. It was mentioned that "this was a relatively small-scale situation. Had it been a massive release, we would have been stretched beyond capacity…. Laboratory capacity must be sufficient to support a large-scale event" [15]. To address one of these challenges for laboratory capacity, we developed an assay for a screening of a large number of human sera for detection of anti-PA IgG. The high throughput format allows testing of 72 serum samples using one dilution instead of 4 serially diluted serum samples per plate thus providing capacity to handle a large incident. The results obtained from a single 1/50 serum dilution instead of serially diluted showed that it does not compromise the assay's accuracy, precision, sensitivity and specificity. Moreover, the comparative analysis of anti-PA IgG concentrations (μg/mL) for serum samples obtained by two assays showed that this assay generated an adequate level of agreement between the results obtained using a single and serially diluted serum sample with the CCC = 0.974. The high level of patient sensitivity and specificity of the assay (100%) makes this assay a reliable instrument to measure the ability of the assay to identify a true negative result and a true positive result for a patient.

The starting dilution of 1/50 of the reference standard serum AVR801 allows detecting a maximum concentration of 109.4 μg/mL in a serum sample, thus limiting testing of highly concentrated serum samples by the high throughput screening assay. Initial quantifiable anti-PA IgG concentration for four of six patients with bioterrorism-related inhalation anthrax identified during the bio-terrorist attacks of October to November 2001 had concentration lower than 100 μg/mL and only one serum sample out of 10 for patients with cutaneous anthrax had IgG concentration over 100 μg/mL. However, peak detected anti-PA IgG levels in inhalation anthrax patients ranged from 168.5 to 1449.5 μg/mL [5]. In this situation the CDC anti-PA IgG ELISA can be used to test highly concentrated serum samples using

serially diluted samples and supplement the high throughput screening format of the assay at later stages of an emergency response. Note that for diagnostic purposes, the 109.4 μg/mL upper limit is sufficient to determine 4-fold rise in patients whose acute sample was below 27.4 μg/mL, which should include anyone who has not been previously exposed or vaccinated against anthrax.

The high throughput assay will be incorporated into a diagnostic algorithm as follows: Initial testing of samples by high throughput ELISA. Acute samples that are >109.4 μg/mL will be tested by standard ELISA in multiple dilutions to determine concentration. Convalescent samples that are >109.4 μg/mL will be compared to the acute sample from the same patient, if the acute sample was <27.4 μg/mL the patient has >4-fold response and is positive with no further testing required. If the acute sample is ≥ 27.4 μg/mL then the sample will be tested by the standard ELISA in multiple dilutions to determine concentration.

The method of quantitative anti-PA IgG ELISA of the high throughput screening assay has the same procedures, reagents, and similar calculation of the assay endpoint as the CDC anti-PA IgG ELISA used as the primary serological laboratory test for confirmatory diagnosis of human anthrax during the bioterrorist attacks of October to November 2001 [2,5]. The quality control system for this assay is also similar; it is well established and effectively used for evaluation of humoral immune responses for the CDC Anthrax Vaccine Research Program Human Clinical Trial [9]. All these features make the high throughput screening format of the assay as a valuable laboratory tool and can be adapted by other laboratories and applied broadly during an emergency response to an anthrax incident.

Acknowledgments

This research was performed in the Microbial Pathogenesis and Immune Response Laboratory at the Centers for Disease Control and Prevention within the Meningitis and Vaccine Preventable Diseases Branch, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases. It was funded by CDC's Office of Public Health Preparedness and Emergency Response.

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	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

Figure 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).

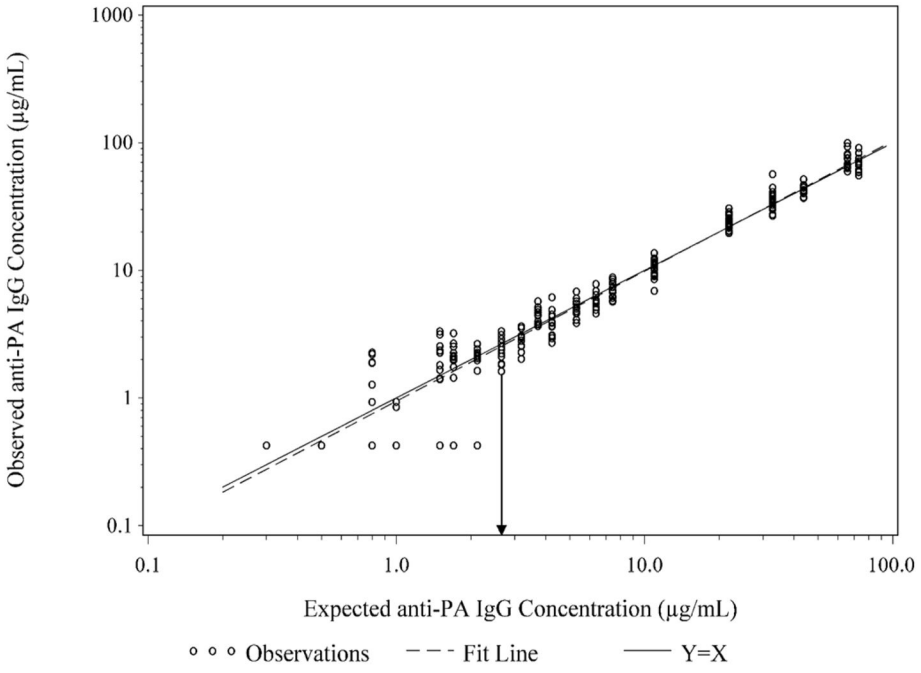

Figure 2.

Dilutional linearity of high throughput screening of human sera for detection of anti-PA IgG by ELISA.

Twenty three serum samples with a range of concentrations from 0.3 to 72.9 μg/mL (LLOQ = 2.6 μg/mL; downward arrow) were used in experiments for determination of dilutional linearity. Dilutional linearity was determined from regression analysis of empirically observed anti-PA IgG concentrations for the serum samples versus the expected concentrations for those samples. Regression analysis showed the fit of the data with $r2 \geq 0.952$, slope = 1.029 and intercept = −0.03 that met the acceptance criteria ($r2 \geq 0.850$ with a slope between 0.9 and 1.1 and an intercept between −0.1 and 0.1). Three experiments were performed by 4 different operators over at least 3 non-consecutive days.

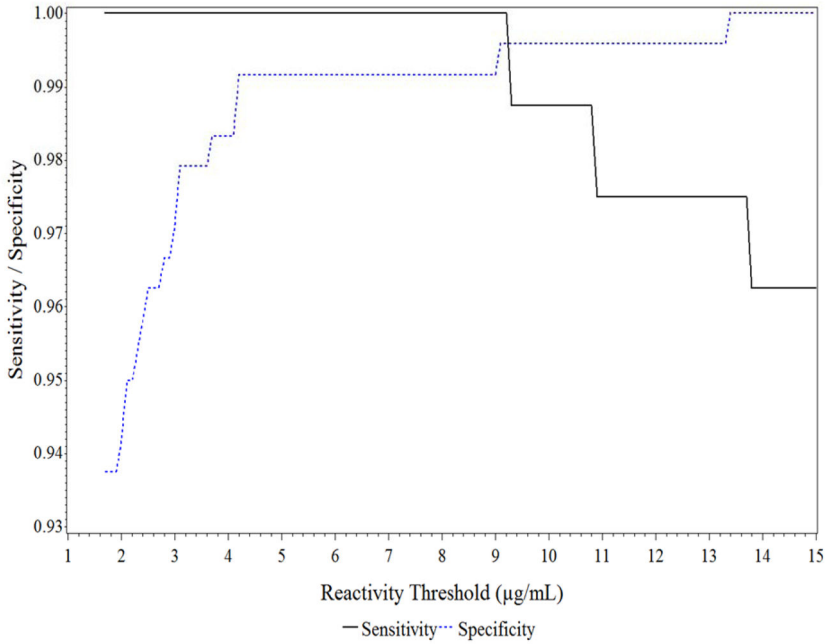

Figure 3.

Analysis of Sample Diagnostic Specificity (DSP) and Diagnostic Sensitivity (DSN)

Serum samples from AVA vaccinated volunteers and control samples (n=320) were used to calculate DSP and DSN for a range of RT values from 1.7 µg/mL (the empirical Lower Limit of Detection (LLOD) for the Standard Reference serum AVR801) to 13.4 µg/mL (the highest concentration of anti- PA IgG in a negative sample, beyond which DSP is 100% and cannot increase further). DSP and DSN were plotted up to RT = 15 µg/mL to demonstrate the continuity in their trend).

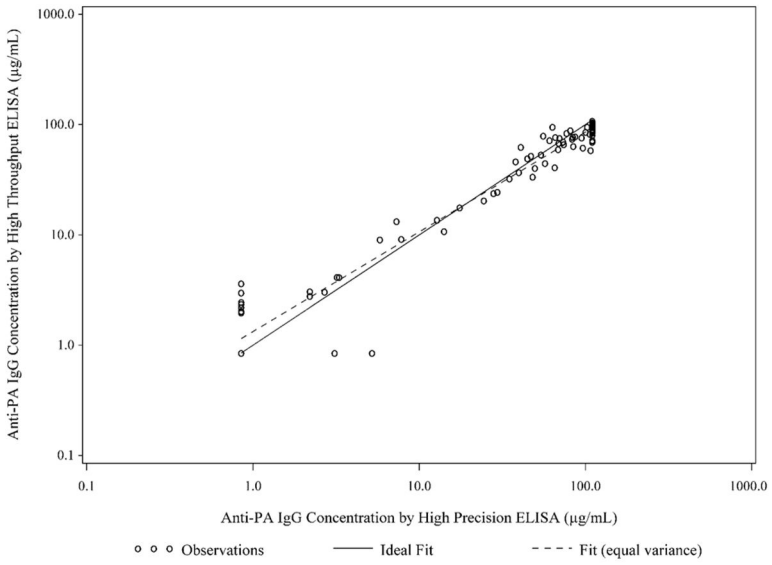

Figure 4.

Comparative analysis of anti-PA IgG concentrations (μg/mL) obtained by two assays: the high throughput anti-PA IgG by ELISA for screening of human sera and the validated CDC ELISA for detection of Bacillus anthracis PA-Specific IgG in human sera

Selected Selected samples consisted of a panel of 95 human sera from AVA vaccinated volunteers. The samples encompassed a range of anti-PA IgG concentrations from acute and convalescent sera. The paired sets of data for each sample were evaluated by Concordance Correlation Coefficient (CCC = 0.9741; accuracy = 0.997 and precision = 0.977).

Table 1

Summary of assay performance characteristics as determined during validation process.

Parameter	Acceptance Criteria	Performance
Goodness of Fit	Mean standard	
	r^2 = 0.999 (0.999–1.000)	r^2 = 0.999 (0.995–1.000)
Accuracy	Percent Error ≤ 25% between the observed and expected concentration for each validation sample	−4.6%–14.4%
Precision	CV ≤25% for each validation sample	CV = 9.0%–21.2%
The lower limit of detection (LLOD)	The lowest dilution of the reference standard curve, which is the lowest concentration possible to interpolate. No established accuracy and precision limits	0.85 μg/mL
Lower limit of quantification (LLOQ)		2.6 μg/mL
•Accuracy	≤ 50% Error	−9.6% Error
•Precision	CV ≤ 25% for each validation sample	CV = 23.5%
Dilutional linearity		
- r^2	≥0.850	r^2 = 0.952
- Slope	0.9 ≤ slope ≤ 1.1	Slope = 1.02
- Intercept	−0.1 ≤ Intercept ≤ 0.1	Intercept = −0.03
Range	≥LLOQ-≤109.4	2.4–109.4 μg/mL

Assay validation parameters, acceptance criteria and observed results for detection of anti-PA IgG *Bacillus anthracis* PA-specific IgG in human sera are described in detail in Results Section 3.1. All validation parameters were met or exceeded, including recommendations provided as Guidance for Industry by FDA CBER. CV = coefficient of variation.

Table 2

Assessment of accuracy, precision, LLOQ and accuracy and precision at LLOQ.

Parameter	Sample	n	Expected Concentration (μg/mL)	Observed Concentration (μg/mL)	Observed Concentration Range (μg/mL)	Standard Deviation (μg/mL)	CV (%)	% Error*
Accuracy and Precision	AVR1497	12	72.9	69.5	56.2–92.0	10.4	15.0	− 4.6
	AVR1496	12	65.6	75.1	60.4–100.5	12.6	16.7	14.4
	AVR1489	12	43.7	43.3	37.4–52.4	4.1	9.5	−1.0
	AVR1492	12	32.8	33.6	27.1–39.3	3.3	10.0	2.4
	AVR1494	12	32.8	37.5	27.7–57.4	7.9	21.2	14.3
	AVR1491	12	21.8	23.2	20.0–27.3	2.1	9.0	6.0
	AVR1493	12	21.8	24.6	19.8–31.0	3.3	13.4	12.4
	AVR1490	12	10.9	10.9	8.6–12.6	1.3	12.1	−0.2
	AVR1495	12	10.9	10.4	7.0–13.8	1.7	16.0	−4.5
LLOQ	AVR2259	12	7.4	7.2	5.7–8.9	1.1	14.9	−2.6
	AVR2258	12	6.3	5.9	4.6–7.9	0.9	15.2	−6.7
	AVR2257	12	5.3	5.1	3.9–6.9	0.9	17.3	−4.6
	AVR2256	12	4.2	3.9	2.7–6.2	1.0	25.0	−7.0
	AVR2255	12	3.7	4.4	3.7–5.8	0.7	14.9	18.6
	AVR2254	12	3.1	2.8	2.0–3.7	0.5	17.5	−10.7
	AVR2253	12	2.6	2.4	1.6–3.4	0.6	23.5	−9.6
	AVR2252	12	2.1	1.9	0.4–2.7	0.7	38.4	−10.1
	MCR0148	12	1.7	2.1	0.4–3.2	0.7	33.3	22.1
	MCR0147	12	1.5	1.9	0.4–3.4	0.9	48.0	29.6
	MCR0155	12	1.0	0.5	0.4–0.9	0.2	36.2	−49.2
	MCR0150	12	0.8	1.1	0.4–2.3	0.8	71.6	37.1
	MCR0153	12	0.5	0.4**	0.4–0.4	0	0	14.0
	MCR0152	12	0.3	0.4**	0.4–0.4	0	0	43.3

AVR2253 is the LLOQ (2.6 μg/mL).

*The reported percent error was calculated based on unrounded values of observed concentration.

**Note that the lowest concentration of the Standard Reference Serum AVR801 on the plate is 0.85 μg/mL. Results below that read as 0 and were masked at one ½ of 0.85 μg/mL (i.e. 0.43 μg/mL). MCR0152 and MCR0152 were below the LLOD, all results read as 0.