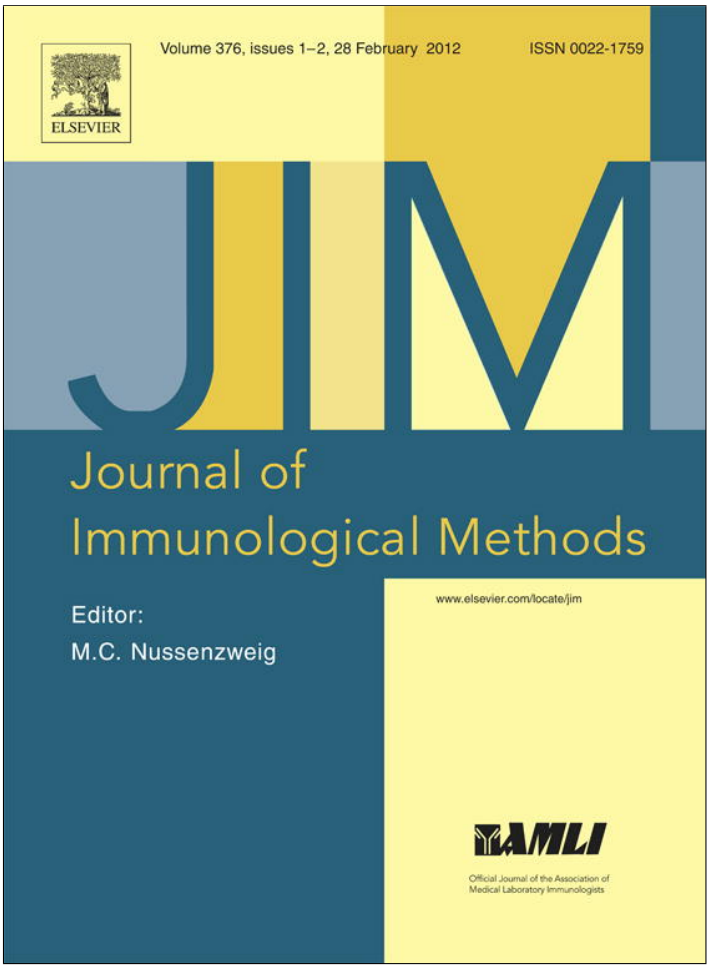

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Research paper

# Validation and long term performance characteristics of a quantitative enzyme linked immunosorbent assay (ELISA) for human anti-PA IgG ☆

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ABSTRACT

Accurate, reliable and standardized quantification of anti-protective antigen (PA) IgG antibody levels is essential for comparative analyses of anti-toxin immune responses in anthrax cases, recipients of PA-based anthrax vaccines and for evaluation of anti-PA based immunotherapies. We have previously reported the early performance characteristics and application of a quantitative anti-PA IgG enzyme linked immunosorbent assay. The principal application of this assay was in a Phase 4 human clinical trial of anthrax vaccine adsorbed (AVA, BioThrax), the central component of the CDC Anthrax Vaccine Research Program (AVRP) and in humans following bioterrorism associated *Bacillus anthracis* infection (Quinn et al., 2002; Quinn et al., 2004; Marano et al., 2008). The objective of the AVRP was to determine the feasibility of reducing the number of priming series and booster doses of the licensed Anthrax Vaccine Adsorbed (AVA) (BioThrax®; Emergent BioSolutions, Lansing, MI) and changing the route of administration from subcutaneous (SC) to intramuscular (IM) (Marano et al., 2008). In this paper we report the validation and long term performance characteristics of the assay during its six year application in the AVRP (2002–2008). The critical features are 1) extensive validation of the assay using two standard reference sera; 2) long term stability and 3) consistency of the data for quantitative analysis of human long term anti-PA IgG responses. The reportable value (RV) of the assay was expressed as anti-PA IgG concentration (μg/ml). Accuracy of the assay was high with a percent error (%ER) range of 1.6–11.4%. Overall intra-operator and intermediate precision were high with Coefficients of Variation (%CVs) of 2.5–15.4% and 6.3–13.2%, respectively. The assay demonstrated excellent dilutional linearity for human sera using $\log_{10}$ transformed data with the slope = 0.95 to 0.99, intercept = 0.02 to 0.06 and $r^2 = 0.980$–0.987. The assay was robust, tolerating changes in serum incubation temperatures from 35 to 39 °C, serum incubation times from 55 to 65 min and changes in key reagents. The long-term assay stability over 6 years using consecutive reference sera AVR414 and AVR801 demonstrated sustained high accuracy and precision for the assay, confirming its suitability for long term studies of PA protein-based anthrax vaccines.

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## 1. Introduction

Anthrax Vaccine Adsorbed (AVA, BioThrax) is currently the only licensed anthrax vaccine in the US (Grabenstein, 2003; Pittman et al., 2002; Joellenbeck et al., 2002). The principal immunogen of AVA is the anthrax toxin protective antigen (PA) component (Puziss and Wright, 1963). Anthrax vaccine immunogenicity is most frequently evaluated by quantitative enzyme-linked immunosorbent assay of anti-PA IgG (Turnbull

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et al., 1986; Welkos and Friedlander, 1988; Ivins et al., 1998; Pittman et al., 2000, 2002, 2005; Fellows et al., 2001; Fellows et al., 2002; Marano et al., 2008; Baillie et al., 2010). In 1999 the US Congress mandated the Centers for Disease Control and Prevention (CDC) to conduct a randomized, double-blind, placebo controlled Phase 4 clinical trial to assess safety and serological noninferiority of anti-PA IgG responses to reduced schedules and intramuscular (IM) administration of AVA. The CDC human clinical trial comprised 43 month participation for 1563 participants (Marano et al., 2008). Primary endpoint serological testing was implemented over a 6 year period from 2002 to 2008; from enrollment of the first participant until after completion of the last participant. In 2008, using data generated from an interim analysis of the first 1005 participants up to month 7 of their participation in the clinical trial, the AVA schedule was modified from subcutaneous (SC) to IM injections with the omission of the dose at week 2 (Marano et al., 2008; Food and Drug Administration, 2008). This modification was the first data driven change in use of the anthrax vaccine since it was licensed in 1970 (Joellenbeck et al., 2002; Wright et al., 2010).

Due to the long duration of the CDC study together with the public health impact of modifying the vaccine schedule it was necessary to develop a precise, accurate, specific, and sensitive serological assay with sustained performance, robustness and stability such that data generated early in the study were directly comparable to those generated in the later stages of enrollment. To address these needs, we developed, characterized and validated a quantitative enzyme-linked immunosorbent assay (ELISA) and a comprehensive set of serological reagents for assessment of anthrax toxin protective antigen (PA) specific immunoglobulin G (IgG) antibody levels in human serum (Quinn et al., 2002, 2004; Semenova et al., 2004; Gorse et al., 2006). We have previously reported the primary performance characteristics of the quantitative anti-PA IgG ELISA (Quinn et al., 2002). In this paper we report the validation and long term performance of the assay and demonstrate its consistency and robustness as applied to the pivotal CDC dose reduction studies of AVA in humans. The validated anti-PA IgG assay is the primary serological Laboratory Developed Test for confirmatory diagnosis of human anthrax (http://emergency.cdc.gov/agent/anthrax/faq/diagnosis.asp). The use of qualified reagents and analytical software together with the long term assay stability, precision and accuracy described in this report demonstrates the rigor required for use of the anti-PA IgG assay to provide data that may be used in consideration of anthrax vaccine licensure, evaluation of vaccine correlates of protection and in clinical diagnosis of human anthrax (Madore et al., 2010).

## 2. Materials and methods

### 2.1. Human test and control sera

The acquisition and use of human serum in this study were approved by the Centers for Disease Control and Prevention (CDC) Human Subjects Institutional Review Board (IRB). Sera from clinical trial participants and clinical trial site IRB approvals were obtained as described by Marano et al. (2008).

The preparation and characterization of human standard reference sera AVR414 and AVR801 have been described previously. The assigned anti-PA IgG concentrations in AVR414 and AVR801 were 141.2 μg/ml and 109.4 μg/ml, respectively (Semenova et al., 2004). The standard reference sera were stored in 100 μl working aliquots at +4 °C for 14 days, at ≤−20 °C for short-term storage, or in 3 ml vials for long-term storage at ≤−70 °C. In addition, a panel of positive and negative quality control (QC) serum samples was prepared for validation studies and assay performance monitoring over the study duration. Positive QC sera were selected or constructed from both individual and pools of sera from healthy adult volunteers who received a minimum of four SC injections of AVA. Positive QC reagents AVR216, AVR284, AVR370 were individual sera from three independent human AVA-vaccinated donors. Positive QC sera AVR1749, AVR1750 and AVR1751 were prepared by pooling equal volumes of 9 positive sera for AVR1749, 13 sera for AVR1750, and 8 sera for AVR1751. Negative control sera AVR190 and AVR811 were from separate non-AVA vaccinated donors. Positive QC sera AVR216, AVR284, AVR370 and negative control AVR190 were used in experiments with standard reference serum AVR414. Positive QC sera AVR1749, AVR1750, AVR1751 and negative control AVR811 were used in experiments with the standard reference serum AVR801. The QC serum samples were stored in 100 μl working aliquots at +4 °C for 14 days, at ≤−20 °C for short-term storage, or in 3 ml vials for long-term storage at ≤−70 °C.

### 2.2. Protective antigen

Purified recombinant protective antigen (rPA) was obtained from three different sources; 1) Dr. Stephen H. Leppla, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; 2) List Biologicals, Campbell, CA or 3) BEI Resources (Manassas, VA). All lots of rPA were qualified in the ELISA before use in AVRP analyses to ensure consistent assay results. For qualification, all rPA lots were required to meet or exceed the acceptance criteria described in Section 2.4.

### 2.3. Quantitative anti-PA IgG enzyme-linked immunosorbent assay (ELISA)

Immulon® 2 HB microtiter plates (Thermo Labsystems, Franklin, MA) were coated with purified rPA (2 μg/ml) in 0.01 M phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Gaithersburg, MD) and incubated overnight (16–24 h) at +4 °C. Plates were washed 3 times with PBS containing 0.1% Tween-20, pH 7.4 (ELISA wash buffer). The standard reference serum was loaded into the first three wells of the plate. The test serum was loaded into the next four pairs of wells (each serum was tested in duplicates) and the last column on the plate was designated for positive and negative QC's. The first wells of each dilution series loaded with 100 μl of each test serum diluted in PBS containing 5% Skim Milk and 0.5% Tween-20, pH 7.4 (Serum Diluent) without a separate blocking step. Serum was mixed in the plate wells and serially transferred in 2-fold dilutions down the plate to make an 8-point dilution series. Plates were incubated for 60 min at 37 °C and washed 3 times with ELISA wash buffer. Horseradish peroxidase-conjugated (HRP) mouse monoclonal anti-human IgG Fc PAN clone HP6043 (Hybridoma Reagent Laboratory, Baldwin, MD) was added to all wells (1/16,000 dilution, 100 μl/well) and incubated at 37 °C for 60 min. Plates were washed 3 times and 100 μl of ABTS

Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to all wells. After 30 min incubation at 37 °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 with a MRX Revelation microtiter plate reader (Thermo Labsystems, Franklin, MA) at a wavelength of 405 nm with a 490 nm reference. Assay endpoints were reported as concentrations (μg/ml) of anti-PA IgG using the ELISA for Windows software Version 2.15 (http://www.cdc.gov/ncidod/dbmd/bimb/elisa.htm) (Plikaytis et al., 1996; Quinn et al., 2002).

### 2.4. Assay acceptance criteria

A set of 5 assay plate acceptance criteria and 2 test sample acceptance criteria was developed. These acceptance criteria were derived from empirical performance characteristics and the established anti-PA IgG concentrations for the reference standard serum and the positive QC sera. Specifically the 5 assay plate 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 R-squared correlation coefficient ($r^2$) 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%, else the concentration was censored; 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 serum anti-PA IgG concentrations were allowed to be >3SD from the expected value. In practice all 5 assay plate acceptance criteria were required to be met, otherwise all test samples on the plate were rejected and sample testing repeated. Based on the requirement for 2 of 3 control values to be within 2SD and one within 3SD, the calculated expected percentage of passing plates was 91% (Soroka et al., 2010).

Anti-PA IgG concentration was calculated for each sample dilution on the plate by interpolating to the reference standard curve. The concentration in the well was multiplied by the sample dilution factor to calculate the sample concentration. For each test sample the overall anti-PA IgG concentration reported was arithmetic mean of all calculable serial dilution pair concentrations. For test samples, the specific criteria to assess parallelism (Plikaytis et al., 1994) and intra-assay precision required that 1) at least three out of a potential 8 dilution pairs on a test sample curve had a calculable concentration and 2) the %CV of the overall dilution adjusted mean anti-PA IgG concentration of a test sample was ≤20%. Exceptions to these criteria included low reactivity samples which were unable to achieve calculable concentrations on three serial dilution pairs. These were accepted with 1 or 2 dilutions. All serum specimens were required to have at least two passing results generated by independent operators. Sample concentrations from ≥2 passing results (inter-operator precision) were required to have %CV of ≤30%.

### 2.5. Assay validation samples

Accuracy and precision of the assay were determined using both AVR801 and AVR414 reference standards. Precision was evaluated within assay, between assays within operator, and between operators (intermediate precision). These parameters were evaluated by the repeated analysis of 3 human sera with predetermined anti-PA IgG concentrations and representing the range of the standard curve; one at the lower region of the standard (AVR1490, 10.9 μg/ml); one at the mid-range of the standard (AVR1497, 72.9 μg/ml); and one at the upper region of the standard (AVR802, 109.4 μg/ml).

The empirical Lower Limit of Quantification (LLOQ) was also determined separately for each of the reference standards by testing a range of serum samples with known anti-PA IgG concentrations approaching and spanning the theoretical LOQ of 3.0 μg/ml as described in Section 2.6.4 using the assay Minimum Detectable Concentration (MDC) (O'Connell et al., 1993; Quinn et al., 2002). To determine the empirical LLOQ two positive serum samples (AVR1490 and AVR802) were spiked into three negative human serum pools (AVR1410, AVR1411 and AVR1413) in different ratios to create 9 validation samples for AVR414 (AVR2252, AVR2253, AVR2254, AVR2255, AVR2256, AVR2257, AVR2258, AVR2259 and AVR1490) with the range of concentrations from 2.1 to 10.9 μg/ml and 8 validation serum samples for AVR801 (AVR1853, AVR1854, AVR1855, AVR1856, AVR1857, AVR1858, AVR1859 and AVR1860) with a range of concentrations from 2.1to 7.4 μg/ml anti-PA IgG.

For evaluation of dilutional linearity 7 additional samples for AVR414 (AVR802, AVR1489, AVR1490, AVR1491, AVR1492, AVR1497 and AVR1490/2.5) with a concentration range from 2.7 to 109.4 μg/ml and 7 different samples for AVR801 (AVR1854, AVR802, AVR1490, AVR1491, AVR1492, AVR1489, and AVR1497) with a concentration range from 2.6 to 109.4 μg/ml were prepared by spiking 7 negative serum pools (AVR1408, AVR1410, AVR1411, AVR1412, AVR1413, AVR1435 and AVR1436) with positive serum AVR802 in different ratios and used in experiments with standards AVR414 and AVR801, respectively.

The robustness of the assay was determined using the AVR414 reference standard. The robustness parameters were evaluated using 11 serum samples with predetermined anti-PA IgG concentrations (AVR351, AVR353, AVR370, AVR376, AVR369, AVR361, AVR390, AVR351, AVR368, AVR396, AVR324) with a range of concentrations from 56.0 to 547.9 μg/ml anti-PA IgG. The three different QC samples also were used as test samples (AVR216, AVR284, and AVR286) with the range of concentrations from 59.8 to 102.0 μg/ml anti-PA IgG. Each parameter was evaluated from a minimum of three experiments performed by 3 independent operators over at least three non-consecutive days.

### 2.6. Validation parameters

Validation was done in accordance with the Food and Drug Administration guidance (2001). Acceptance criteria were derived from extensive assay development data (Table 1).

#### 2.6.1. Accuracy

Accuracy, a measure of the exactness of the assay, was determined by the repeated analysis of 3 sera (AVR1490, AVR1497 and AVR802) with predetermined anti-PA IgG concentrations and representing the range of the standard curve. Accuracy was determined from a minimum of 3 assay runs per operator per day, performed by 3 or more independent operators over 3 non-consecutive days. The assay-based mean

**Table 1**
Summary of the anti-PA IgG ELISA performance and validation characteristics.

| Validation parameter | Acceptance criteria | Observed results | |
|---|---|---|---|
| | | Standard AVR414 | Standard AVR801 |
| Accuracy | ≤25% error between the expected and observed concentration for each validation sample | 1.6%–11.4% | 6.2%–6.4% |
| Intra-assay precision | CV≤10% for each validation sample on a assay plate | 1.6%–6.2% | 3.1%–10.0% |
| Inter-assay precision | CV≤20% for each validation sample | | |
| — Intermediate precision | | 7.5%–10.7% | 6.3%–13.2% |
| — Intra-operator precision | | 2.5%–15.4% | 4.2%–11.0% |
| The theoretical lower limits of detection (LLD) and the theoretical lower limit of quantification (LLQ) | N/A | 2.1 μg/ml and 3.1 μg/ml | 3.4 μg/ml and 5.2 μg/ml |
| Lower Limits of Detection (LLOD) | No established accuracy and precision limits. | 1.1 μg/ml | 1.7 μg/ml |
| Lower Limit of Quantification (LLOQ) | ≤50% error | 2.6 μg/ml | 3.7 μg/ml |
| | CV≤20% for each validation sample | 3.1% error, CV = 11.2. % | 7.5% error, CV = 10.7% |
| Dilutional Linearity: | | | |
| — $r^2$ | ≥0.850 | $r^2 = 0.980$ | $r^2 = 0.987$ |
| — Slope | 0.8 to 1.2 | Slope = 0.95 | Slope = 0.99 |
| — Intercept | −4.0 to 4.0 | Intercept = 0.06 | Intercept = 0.02 |
| — Range | ≥LLOQ | 0.03–1.41 μg/ml anti-PA IgG 'in the well' | 0.07–2.19 μg/ml anti-PA IgG 'in the well' |

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.

concentrations were calculated and compared to the predetermined concentration of the sample to determine the percent error of the ELISA. Accuracy of ELISA was expressed as the percent error (%E) between the assay-determined value and the predetermined value for the serum samples. The percent error was calculated as the absolute value of [(observed − expected)/expected] × 100. A %E of ≤25% was adopted as an acceptable level of accuracy based on the data from development experiments. Due to the high analytic sensitivity of the assay, an acceptable %E at the lower limit of quantification (empirical LLOQ; 3.7 μg/ml) was ≤50% error with %CV of <20%. FDA CBER recommends that the mean value should be within 15% of the actual value (30%E) except at LLOQ, where it should not deviate by more than 20% (40%E). Accuracy criteria were not applied to intra-assay results, since these represented the data from a single assay rather than a mean of multiple assays.

*2.6.2. Precision*

Precision, a measure of the degree of repeatability of the assay under normal operating conditions, was determined by repeated analysis of the same three validation serum samples used for accuracy. It was determined from a minimum of 3 assay runs per operator per day, performed by 3 or more independent operators over 3 non-consecutive days. Precision for each sample was calculated both within each operator and over all operators (intermediate precision). Precision was expressed as the coefficient of variation (%CV) of the reported anti-PA IgG concentrations under the specified test conditions. The acceptable level of inter-assay precision was ≤20%. FDA CBER recommends the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV (Food and Drug Administration, 2001).

Intra-assay precision for each of 3 samples (AVR802, AVR1490, and AVR1497) was analyzed by loading each validation serum sample on the whole plate in all four test positions on the assay plate. This was performed by three or more different operators in a single day, generating a total of 4 replicates per sample per operator. The acceptance criterion for intra-assay precision for the ELISA was ≤10% derived from assay development data (Table 1).

*2.6.3. Goodness of fit*

Goodness of fit was expressed as the estimated non-linear squared correlation coefficient ($r^2$) 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 standards data were fitted to a 4-PL model by the ELISA for Windows analytical software (ELISA for Windows, Version 2.15) using the 'Robust' fit algorithm. Long term goodness of fit was determined by averaging the $r^2$ values of 5435 independent standard reference curves for AVR414 and 7862 curves for AVR801. These standards data were from all independent ELISA experiments generating reportable values for the AVRP human clinical trial.

*2.6.4. Theoretical and empirical limits of detection and quantification of the assay*

The theoretical lower limits of detection and qualification are lowest concentration of analyte that can be measured in a diluted serum sample with a specific degree of probability. These limits were derived from a 4-PL model based on the modification of the method described by O'Connell et al. (1993) and calculated from the 95% confidence intervals (95%CI) curves of standard reference sera AVR414 and AVR801. The approach uses the upper 95% confidence limit of the lowest calculated point on the graph rather than the asymptote as the limit, and chose the first predicted point on the graph above that limit for the minimum detectable concentration (MDC), and the first lower 95% confidence limit point on the graph above that limit as the reliable detection limit (RDL). The MDC and RDL of the assay were calculated from 5435 and 7862 independent standard curves of standards AVR414 and AVR801, respectively. The theoretical lower limits of detection (LLD) and the theoretical lower limit of quantification (LLQ) were calculated as the derivates of MDC and RDL, respectively, multiplied by the reciprocal of the lowest serum starting dilution (1/50).

The empirical lower limit of detection (LLOD) is the lowest concentration of analyte that can be empirically detected in a diluted serum sample independent of criteria for assay accuracy and precision. Since we do not extrapolate outside the range of the reference standard, this is simply the concentration of the most dilute reference standard well multiplied by initial dilution of the test serum. The empirical lower limit of quantification (LLOQ) is the lowest concentration of analyte 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 ≤20% for the calibrated antibody concentration and ≤50% error (unpublished development data).

LLOQ was determined experimentally by testing two different sets of serum samples with anti-PA IgG concentrations spanning the theoretical LLQ. One sample set was used for each of the two standards. The serum sets were created by spiking two positive serum samples into 3 negative serum pools in different ratios. The first set consisted of 9 serum samples with anti-PA IgG concentrations ranging from 2.1 μg/ml to 10.9 μg/ml for AVR414 and 8 serum samples from 2.1 μg/ml to 7.4 μg/ml for AVR801. Three experiments were performed by 3 or more different operators over at least 3 non-consecutive days. LLOQ was calculated as the lowest anti-PA IgG concentration of the test sera that was measured with %CV of ≤20% and ≤50% error.

*2.6.5. Dilutional linearity and range*

The dilutional linearity of the assay was its ability to elicit results that were directly, or by a well-defined mathematical transformation, proportional to the concentration of anti-PA IgG in the sample. Two sets of seven samples with a range of concentrations from 2.7 to 109.4 μg/ml and seven samples with a range concentration from 2.6 to 109.4 μg/ml were used in experiments with each of the standards AVR414 and AVR801, respectively. 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 0.8 and 1.2 and an intercept between −4 and 4 in linear scale (unpublished development data). Three experiments were performed by 3 or more different operators over at least 3 non-consecutive days to generate at least 27 replicates for each sample.

The assay range was calculated as the interval of anti-PA IgG concentrations "in the well" that can be interpolated from the standard curve with acceptable linearity, accuracy and precision as described above and including those concentrations used to determine the lower limits of quantification. The upper bound of the range was determined by the highest concentration of analyte that may be interpolated to the standard curve with acceptable accuracy and precision.

*2.6.6. Robustness of the assay*

The robustness of a procedure is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters. Robustness provides an indication of an assay's reliability in normal usage. All experiments were performed using reference standard AVR414 and 11 serum samples as described in Section 2.5. Variations in assay procedures were assessed for their effect on the assay reportable value. Robustness parameters evaluated in this study were: serum incubation temperature and time, conjugate binding time, enzyme-substrate incubation time and stop solution incubation time and test matrix interference.

Test matrix interference was evaluated in normal negative human serum (AVR190), normal human plasma (Biomeda Corp., Foster City, CA) normal rabbit serum (AVR818) and by addition of 0.15%, 1.25% or 5% of human hemoglobin (ICN Biomedicals, Inc., Costa Mesa, CA) to the ELISA diluent buffer. Three plates per operator per day were analyzed from three separate days for each of the conditions tested. Mean anti-PA IgG concentrations, standard deviations and %CVs for positive serum controls and test samples were analyzed and compared to the standard assay procedure.

*2.7. Diagnostic sensitivity and specificity*

The Diagnostic Sensitivity (DSN) measures the ability of the assay to identify a true positive result. The Diagnostic Specificity (DSP) measures the ability of the assay to identify a true negative result. High levels of DSN and DSP are important in ensuring that data generated by the assay were accurate and reliable representations of the immune response to vaccination. Using unblinded AVRP study group assignments, sera were first classified as Positive (0–2–4 week sub-cutaneous AVA vaccinated donors at week 8, n = 235) or Negative (unvaccinated and placebo controls at week 0, n = 1563). The DSN of the assay was calculated as $[TP/(TP+FN)]\times 100$ where TP = true positive and FN = false negative. The DSP of the assay was calculated as $[TN/(TN+FP)]\times 100$ where TN = true negative and FP = false positive. DSN was also compared between SC and IM for 0–2–4 week AVA vaccinated donors at the 8 week study time point.

*2.8. Comparative analysis of anti-PA IgG concentrations determined using two different standard reference sera*

Due to the size, complexity and duration of the human clinical trial two different standard sera AVR414 and AVR801 were used over the course of the study. To demonstrate that data generated using both standards were compatible, anti-PA IgG concentrations (μg/ml) for a panel of 125 human sera obtained by ELISA using standard AVR414 were, after a minimum 4 year interval, retested against AVR801. The test set of samples was selected from the first 1005 participants of the AVRP up to month 7 of their 43 month study involvement. The range of anti-PA IgG concentrations in test sera was 3.05–119.5 μg/ml. The paired sets of data for each sample were evaluated by Concordance Correlation Coefficient (CCC) calculated from Deming regression analysis of the $\log_{10}$ transformed median anti-PA IgG concentrations of AVR801 and AVR414 (Martin, 2000). The acceptable concordance between the two reference standards required a CCC ≥ 0.95.

*2.9. Assay performance monitoring*

A two-stage, multilevel quality control system was established for evaluation of the assay performance and long-term monitoring over the course of the study (Soroka et al., 2010). In brief, the first stage consisted in evaluating of QC acceptance criteria of the assay. The second stage consisted in monitoring multiple assays and multiple variables via quality

control monitoring program (QC-Mon) using SAS®. This approach allowed rejecting an unacceptable assay and controlling stability of the assay performance over the duration of the study. Consistency of the standards' performance was assessed by evaluating the standards' parameters over a 75-month testing period fitted to a 4-PL model. The fit parameters were Lower Asymptote (A), Upper Asymptote (B), midpoint dilution (C) and Slope Factor (D) in the equation

$$OD = B + \frac{A-B}{1 + \left({}^{Dil}/_{C}\right)^{D}}.$$

### 2.10. Statistical analyses

For all comparative analyses between conditions for robustness (Section 2.6.6), mean anti-PA IgG concentrations, standard deviations, and CVs were collected for positive serum controls and test samples, and were analyzed and compared to the standard assay procedure using Analysis of Variance (ANOVA). A p-value $\leq 0.05$ was used to determine statistical difference. Test conditions were considered equivalent unless they were both statistically significantly different ($p \leq 0.05$) and had $\geq 20\%$CV. Analysis was performed using PROC MIXED in SAS® (SAS Institute Inc., Cary, NC).

All regression analyses were performed on $\log_{10}$ transformed data. For the comparison between reference standards, the Deming regression (Martin, 2000) and Concordance Correlation Coefficient (CCC) were used. The Deming regression assumes equal variance on both the X and Y axes; as opposed to linear regression which assumes 0 variance on the X axis. The Concordance Correlation Coefficient takes both accuracy and precision into account. The CCC is calculated from the Deming regression of the AVR801 values (median concentrations) vs. the AVR414 values (median concentrations) for the same samples on the $\log_{10}$ scale. The $r^2$ statistic (Pearson correlation coefficient) of the Deming regression is the precision component of the CCC. 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. The CCC is the product of the precision and accuracy components. A perfect concordance would produce a CCC value of 1. A CCC $\geq 0.95$ was considered acceptable for this assay. Graphs of the Deming regression were created with JMP® (SAS Institute Inc., Cary, NC), and the CCC was calculated in SAS® (SAS Institute Inc., Cary, NC).

## 3. Results

### 3.1. Validation of the assay

The assay's validation parameters, acceptance criteria and observed results for detection of anti-PA IgG *Bacillus anthracis* PA-specific IgG in human sera are summarized in Table 1. All validation parameters were met or exceeded.

#### 3.1.1. Accuracy and precision

Accuracy of the assay using both standards AVR414 and AVR801 was determined by multiple spike-recovery analyses of 3 different sera with predetermined anti-PA IgG concentrations and representing the range of the standard curve; one at the lower region of the standard (AVR1490, 10.9 μg/ml), one at the mid-range of the standard (AVR1497, 72.9 μg/ml) and one at the upper region of the standard (AVR802, 109.4 μg/ml). The assay demonstrated high accuracy (low %E) using both standards. The %E ranged from 1.6% to 11.4% for AVR414 and from 6.2% to 6.4% for AVR801. Each validation serum sample had a %E $\leq 25\%$ and therefore met the pre-determined criteria for validation (Table 2).

The intra-assay precision %CV using these sera ranged from 1.6% to 6.2% and from 3.1% to 10.0% for reference standard AVR414 and AVR801, respectively (Table 2). Inter-assay (intermediate) precision expressed as %CV of the reportable value ranged from 7.5% to 10.7% and 6.3% to 13.2% for reference standards AVR414 and AVR801, respectively (Table 2). These data are indicative of a high level of assay reproducibility using both standards AVR414 and AVR801.

#### 3.1.2. Theoretical and empirical limits of detection and quantification

The MDC and RDL values for the anti-PA IgG ELISA using AVR414 as the reference standard were 0.042 μg/ml and 0.068 μg/ml, respectively, as calculated from the 95%CI of 5435 independent standard curves. The MDC and RDL values for the anti-PA IgG ELISA using AVR801 as the reference standard were 0.062 μg/ml and 0.10 μg/ml, respectively (n = 7862). The theoretical LLD and LLQ adjusted for undiluted serum samples were determined as 2.1 μg/ml and 3.1 μg/ml for AVR414 and 3.4 μg/ml and 5.2 μg/ml for AVR801, respectively. The lowest concentration that could be detected empirically using standard AVR414 (LLOD) is 1.1 μg/ml, the LLOD using AVR801 is 1.7 μg/ml (Table 1).

The empirical LLOQ for the different standards was calculated from two different sets of serum samples with a similar range of concentrations. The serum sample AVR2253 had the lowest expected concentration (2.6 μg/ml, 3.1%E, 11.2%CV) that met the validation criteria and was therefore the LLOQ for this assay using reference standard AVR414. For standard AVR801, serum sample AVR1856 had the lowest expected concentration (3.7 μg/ml, 7.5%E, 10.7% CV) which met the validation criteria and established LLOQ for this assay as 3.7 μg/ml using reference standard AVR801. These data indicate that LLOQ for both standards are in a good agreement (Table 1). In practice and for consistency of data analyses interpretation the more conservative empirical value of 3.7 μg/ml was adopted as the overall assay LLOQ.

#### 3.1.3. Dilutional linearity and range

Dilutional linearity of the assay is its ability to obtain results that are directly, or by a well defined mathematical transformation, proportional to the concentration of anti-PA IgG in the sample. Seven sera of varying concentrations of anti-PA IgG were included in the analyses, including samples with reactivity below the empirical LLOQ. Regression analyses of $\log_{10}$ transformed dilutional linearity data using AVR414 as a standard were $r^2 = 0.980$, slope = 0.95 and intercept = 0.06; using AVR801 as a standard, $r^2 = 0.987$, slope = 0.99 and intercept = 0.02 (Table 1, Fig. 1A, 1B). These data demonstrated a high level of dilutional linearity for the quantification of anti-PA IgG human sera using both standards.

The range of the assay is the interval of the “in the well” anti-PA IgG concentrations that can be interpolated from

**Table 2**
Assessment of accuracy, inter- and intra-assay precision.

| Validation parameter | Sample | n | Expected mean IgG (μg/ml) | Standard AVR414 | | | | Standard AVR801 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Observed mean IgG (μg/ml) | St. dev (%) | CV (%) | % Error | Observed mean IgG (μg/ml) | St. dev (%) | CV (%) | % Error |
| Accuracy and intermediate precision | AVR1490 | 27 | 10.9 | 9.7 | 1.0 | 10.7 | 11.4 | 11.6 | 1.5 | 13.2 | 6.2 |
| | AVR1497 | 27 | 72.9 | 69.4 | 5.2 | 7.5 | 4.8 | 77.6 | 6.3 | 8.1 | 6.4 |
| | AVR802 | 27 | 109.4 | 111.1 | 8.7 | 7.8 | 1.6 | 116.2 | 7.3 | 6.3 | 6.2 |
| Intra-operator precision: | | | | | | | | | | | |
| Operator A | AVR1490 | 9 | 10.9 | 9.7 | 0.4 | 4.1 | 11.0 | 11.2 | 0.6 | 5.7 | 2.8 |
| | AVR1497 | 9 | 72.9 | 71.9 | 1.8 | 2.5 | 1.4 | 79.4 | 4.9 | 6.2 | 8.9 |
| | AVR802 | 9 | 109.4 | 116.8 | 2.9 | 2.5 | 6.8 | 112.2 | 5.7 | 5.1 | 2.6 |
| Operator B | AVR1490 | 9 | 10.9 | 10.1 | 1.6 | 15.4 | 7.3 | 13.2 | 1.5 | 11.0 | 21.1 |
| | AVR1497 | 9 | 72.9 | 68.3 | 7.3 | 10.7 | 6.3 | 81.8 | 5.6 | 6.8 | 12.2 |
| | AVR802 | 9 | 109.4 | 109.4 | 11.4 | 10.4 | 0.0 | 122.9 | 5.2 | 4.2 | 12.3 |
| Operator C | AVR1490 | 9 | 10.9 | 9.3 | 0.8 | 8.1 | 14.7 | 10.4 | 0.7 | 6.4 | 4.6 |
| | AVR1497 | 9 | 72.9 | 68.0 | 4.7 | 6.8 | 6.7 | 71.7 | 3.5 | 4.9 | 1.6 |
| | AVR802 | 9 | 109.4 | 107.3 | 7.1 | 6.6 | 1.9 | 113.4 | 6.1 | 5.4 | 3.7 |
| Intra-assay precision: | | | | | | | | | | | |
| Operator A | AVR1490 | 4 | 10.9 | 10.7 | 0.7 | 6.2 | 2.2 | 11.3 | 0.6 | 4.9 | 3.5 |
| | AVR1497 | 4 | 72.9 | 70.4 | 2.0 | 2.8 | 4.6 | 75.5 | 7.3 | 9.6 | 3.5 |
| | AVR802 | 4 | 109.4 | 94.7 | 4.9 | 5.2 | 13.4 | 113.2 | 3.5 | 3.1 | 3.6 |
| Operator B | AVR1490 | 4 | 10.9 | 10.0 | 0.3 | 3.4 | 8.6 | 11.0 | 0.9 | 7.9 | 0.5 |
| | AVR1497 | 4 | 72.9 | 64.8 | 1.8 | 2.8 | 10.2 | 71.0 | 3.0 | 4.2 | 2.0 |
| | AVR802 | 4 | 109.4 | 81.6 | 2.8 | 3.4 | 25.7* | 110.2 | 5.0 | 4.5 | 0.2 |
| Operator C | AVR1490 | 4 | 10.9 | 9.7 | 0.3 | 3.5 | 11.3 | 10.6 | 1.1 | 10.0 | 3.3 |
| | AVR1497 | 4 | 72.9 | 62.2 | 1.0 | 1.6 | 13.8 | 71.2 | 5.9 | 8.2 | 2.6 |
| | AVR802 | 4 | 109.4 | 107.4 | 3.9 | 3.7 | 1.7 | 113.4 | 3.9 | 3.4 | 3.2 |

* Note that accuracy criteria are not applied to inter- and intra-assay results, since these represent the results from a single assay rather than a mean of multiple assays.

the standard curve inclusive of the lower limits of quantification and the highest concentration of the standard with acceptable accuracy, precision, and linearity. The LLOQ for the assay with AVR414 and AVR801 standard was 2.6 μg/ml and 3.7 μg/ml, respectively, which equates to an "in the well" anti-PA IgG concentration of 0.03 μg/ml for AVR414 and 0.074 μg/ml for AVR801. Since the first dilution on a plate is 1/100 for AVR414 and 1/50 for AVR801, the highest concentration "in the well" for the standards are 1.41 μg/ml and 2.19 μg/ml, respectively. Thus, the 'in the well' range of this assay is 0.03–1.41 μg/ml 'in the well' with the standard AVR414 and. 0.07–2.19 μg/ml with the standard AVR801 (Table 1).

*3.1.4. Robustness of the assay*

Incubation times for each of the four key steps of the assay's procedure were tested (serum, conjugate, substrate, and stop solution incubation times). There was no statistical difference between the standard serum incubation time of 60 min and the varying conditions for each of the four key steps (55 min or 65 min). The CVs between conditions ranged from 0.0 to 3.4%, 0.2 to 12.4%, 0.1 to 6.1% and 0.1 to 1.8% for serum, conjugate, substrate and stop solution incubation time, respectively.

Change in incubation temperature from 37 °C to 35 °C or to 41 °C resulted in %CVs from 0.5 to 3.3%, 0.1 to 5.0% and 0.3 to 5.6% for serum, conjugate binding, and substrate incubation, respectively. No discernible difference was found among temperatures for all reagents; CVs ranged from 0.0 to 6.4%.

The effect of matrix interference on the reportable values was evaluated by 'spike and recovery' analyses of AVR414 in human negative serum, human plasma, normal rabbit serum and addition of different amounts of purified human hemoglobin to ELISA diluents. The results showed that there was no interference by human and rabbit negative serum on the assay endpoints. Recovery ranged from 89.3 to 104.1% and from 97.3 to 130.8%, respectively. There was no discernable effect on assay endpoints at any of the tested levels of hemoglobin; recovery ranged from 89.3 to 100%. There was, however, interference by human plasma, with recovery rates ranging from 114.3 to 170.6%. The possible explanation of human plasma effect is fibrinogen in the plasma, which can be adsorbed on polystyrene surface (Slack and Horbett, 1988, 1992), thus causing nonspecific modifications on the plate surface and nonspecific binding.

*3.2. Diagnostic sensitivity and specificity*

Analysis of serum anti-PA IgG responses at week 0 (unvaccinated) indicated 1538 non-reactive samples (<LLOQ; 3.7 μg/ml anti-PA IgG, True Negatives) and 25 reactive sera (False Positives; concentrations ranged from 3.9 to 17.8 μg/ml anti-PA IgG). Based on these data the assay DSP was 98.4%. DSN in vaccinees receiving the 0–2–4 wk schedule by the subcutaneous route and tested at week 8 (4-SQ) was 100% (235 out of 235 samples > LLOQ). DSN of the combined group was 98.6% (1151 out of 1167).

*3.3. Comparative analysis of anti-PA IgG concentrations determined using two different standard reference sera*

Orthogonal regression analyses demonstrated the concordance correlation coefficient (CCC) between reference standards AVR414 and AVR801 was 0.979 for the median anti-PA IgG concentration, which is greater than minimum requirement of 0.95. The measure of precision was 0.985 and the measure of accuracy was 0.9940 (Fig. 2). These data indicate acceptable concordance between the two reference standard sera for determination of anti-IgG concentrations.

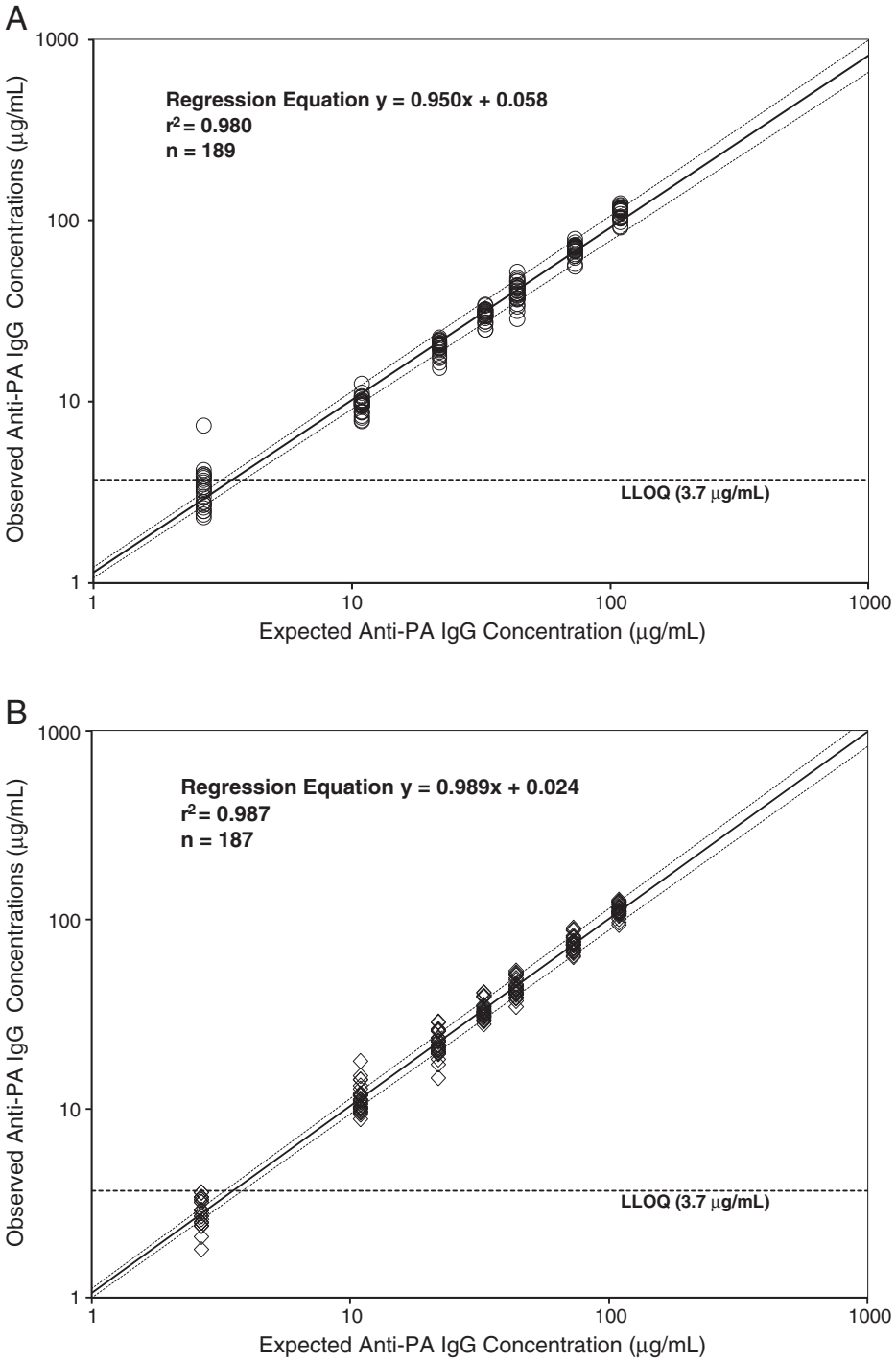


**Fig. 1.** Dilutional linearity of the Quantitative Enzyme Linked Immunosorbent Assay (ELISA) for Human Anti-PA IgG using human reference standard AVR414 (A) and AVR801 (B).Dilutional linearity of the assay is its ability to obtain results that are directly, or by a well defined mathematical proportional to the concentration of anti-PA IgG in the sample. Seven samples with a range of concentrations from 2.7 to 109.4 μg/ml and seven samples with a range concentration from 2.6 to 109.4 μg/ml were used in experiments with both standards AVR414 and AVR801, respectively. 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. The acceptance criteria required a mean $r^2 \geq 0.850$ with a slope between 0.8 and 1.2 and an intercept between −4 and 4 in linear scale. Three experiments were performed by 3 or more different operators over at least 3 non-consecutive days to generate at least 27 replicates for each sample.Note: Concentration results of 0.0 μg/ml were removed from the log scale regression analysis thus resulting in total n = 187 in Fig. 1B. _____ Linear Regression Fit, ..... 95% Confidence limits on Linear Regression Fit, - - - - - - - - Lower Limit of Quantification (3.7 μg/ml).

### 3.4. Assay performance monitoring

The long-term performance of the standards, determined from 5410 assays using standard AVR414 and 7925 assays using standard AVR801 indicated good stability of the standards' parameters over the time of testing (Table 3). The goodness of fit (mean $r^2$) for these data was $r^2 = 0.9961$ and $r^2 = 0.9960$ for standard AVR414 and 801, respectively (assays with $r^2 < 0.99$ failed QC and were repeated). No corresponding change was evident in the standards' first dilution

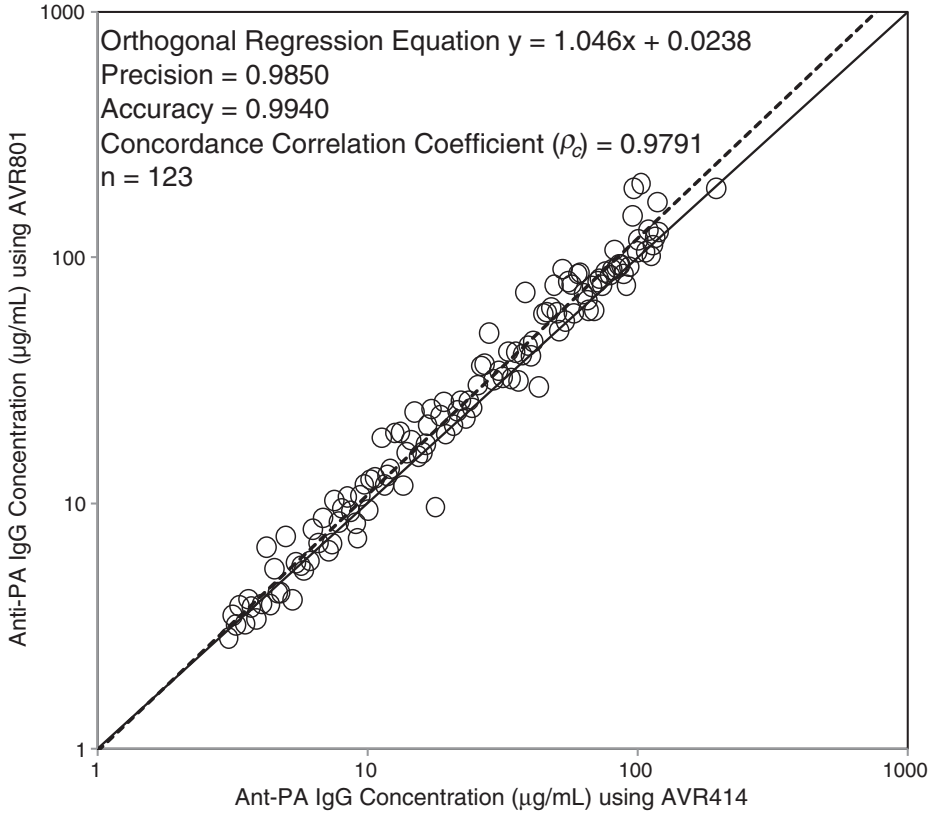


**Fig. 2.** Comparative analysis of anti-PA IgG concentrations determined using two different standards reference sera AVR414 and AVR801.Anti-PA IgG concentrations (µg/ml) for a panel of 125 human sera that were obtained by ELISA using standard AVR414 were retested against AVR801. The test set of samples was selected from the first 1005 participants of the AVRP up to month 7 of their 43 month study involvement. The samples encompassed a range of anti-PA IgG concentrations from below the LLOQ up to the upper range of the standard (3.05–119.5 µg/ml). The paired sets of data for each sample were evaluated by Concordance Correlation Coefficient (CCC) calculated from the Deming regression of the $\log_{10}$ transformed median anti-PA IgG concentrations of AVR801 and AVR414. A CCC value of ≥0.95 was considered an acceptable level of agreement between data sets. Median concentration by AVR414 is plotted on the X axis and median concentration by AVR801 is plotted on the Y-axis.Note: Concentration results of 0.0 µg/ml were removed from the log scale Deming regression analysis of the log10 transformed median anti-PA IgG concentrations thus resulting in total n = 123 in Fig. 2. _____ Ideal fit (Y = X), - - - - - - - - Orthogonal Regression Fit, ..... 95% Confidence limits on Orthogonal Regression Fit.

(maximum OD) with %CV 9.38% and 13.61%, for AVR414 and AVR801, respectively. The high %CV of the lower asymptote A parameter (42.7% and 48.31% for AVR414 and AVR801, respectively) was due to its low mean value for both standards (0.061 and 0.046, respectively). The combined data for both standards indicated high level of agreement in the standards parameters over the time of testing and a high degree of fit to the model.

**Table 3**
Assessment of standards' performance over time of testing. The standard curves were fitted to a 4-PL model. The fit parameters are A (Lower Asymptote), B (Upper Asymptote), C (midpoint dilution) and D (Slope Factor) in the equation OD = B + (A − B) / (1 + C/Dil) D. The Top OD is the median OD of the first dilution of the reference standard. The $r^2$ is the unweighted correlation coefficient between the curve fit and the observed data points. All tests were performed using each reference standard, sorted by date, and then divided into 3 groups containing approximately equal numbers of tests.

| | AVR414 mean performance by time period (months) | | | Combined AVR414 data and precision (N = 5410) | | | AVR801 mean performance by time period (months) | | | Combined AVR801 data and precision (N = 7925) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | 1–13 N = 1803 | 14–16 N = 1803 | 17–30 N = 1804 | Mean | Standard error | CV% | 31–52 N = 2642 | 53–62 N = 2642 | 62-75 N = 2641 | Mean | Standard error | CV% |
| A | 0.062 | 0.066 | 0.056 | 0.061 | 0.0004 | 42.70% | 0.050 | 0.037 | 0.050 | 0.046 | 0.0002 | 48.31% |
| B | 3.344 | 3.189 | 3.106 | 3.213 | 0.0055 | 12.57% | 2.721 | 2.671 | 2.814 | 2.735 | 0.0044 | 14.32% |
| C | 281 | 301 | 263 | 282 | 1.26 | 32.80% | 140 | 109 | 131 | 127 | 0.52 | 36.40% |
| D | −1.294 | −1.373 | −1.351 | −1.339 | 0.0020 | 10.72% | −1.317 | −1.270 | −1.347 | −1.311 | 0.0014 | 9.41% |
| Top OD | 2.560 | 2.530 | 2.351 | 2.480 | 0.0032 | 9.38% | 2.086 | 1.884 | 2.135 | 2.035 | 0.0031 | 13.61% |
| $r^2$ | 0.9956 | 0.9960 | 0.9967 | 0.9961 | 0.0001 | 1.06% | 0.9960 | 0.9966 | 0.9955 | 0.9960 | 0.0001 | 1.24% |

a — The upper asymptote of the standard curve.
b — The lower asymptote.
c — Parameter, related to the dilution at the midpoint of the assay.
d — Parameter, related to the slope of the curve.

## 4. Discussion and conclusions

Immunoassays are pivotal tools for the diagnosis of a wide variety of bacterial and viral infectious diseases and for providing key patient management data for disease prevention and treatment. They may also provide pivotal data for quantitative determination of immune correlates of protection. Enzyme linked immunosorbent assay (ELISA) technology continues to be a fundamental analytical tool for quantification of antibody responses to vaccination and infection. The assay technology developed in our laboratory followed the FDA guidance for bioanalytical method validation (Food and Drug Administration, 2001) and is aligned with literature guidance on the use of serologic assays to support vaccine efficacy analyses (Madore et al., 2010). In this context the study provides one example of a model for immunoassay development and validation that has broad applicability to standardization of quantitative immunoassays.

Various ELISAs for anti-PA IgG have been used widely to confirm anthrax in humans and animals (Berthold et al., 2005; Little et al., 2006; Grunow et al., 2007; Shakya et al., 2007; Turnbull et al., 2008; Good et al., 2008). A standardized, validated anti-PA ELISA platform with qualified reagents and analytical software that can be made available to the research community is therefore a valuable asset to the study of anthrax disease and for development of medical countermeasures (Joellenbeck et al., 2002; Committee to Review the CDC Anthrax Vaccine Safety and Efficacy Research Program, 2003; Hughes et al., 2009).

Previously we have reported the development of human reference standard materials for anti-PA quantification, the development and performance characteristics of a quantitative ELISA for evaluation of anti-PA IgG responses in human anthrax vaccinees, and their application in evaluating natural and bioterrorism associated *B. anthracis* infection (Quinn et al., 2002, 2004; Hsu et al., 2002; Dewan et al., 2002; Semenova et al., 2004; Walsh et al., 2007). The data presented here are derived from rigorous validation of the assay and its continuous extended use over multiple years. These data demonstrate the assay to be highly accurate, precise, specific, sensitive and robust for quantification of anti-PA IgG in human serum. The standardization of assay procedures, reagents, the calculation of the assay endpoint and the quality control system allowed us to successfully apply this tool for evaluation of humoral immune responses for the CDC Anthrax Vaccine Research Program human clinical trial. Consistency of the assay's performance over the extended duration of the testing for the CDC AVRP was a critical component in ensuring the accuracy and reliability of the serological data for that study (Soroka et al., 2010). Due to the size, complexity and duration of the trial two reference standard sera were used, AVR414 and AVR801. The data presented here demonstrate that serum anti-PA mass value assignments generated using both standards have a very high level of agreement.

The standardized, validated assay reported here has been used extensively in a variety of laboratories, thus facilitating direct quantitative comparison of human anti-PA IgG responses for different applications (Purvis et al., 2004; Gorse et al., 2006; Semenova et al., 2007; Marano et al., 2008; Pittman et al., 2005; Lininger et al., 2007; Bienek et al., 2007, 2008; Gubbins et al., 2007; Walsh et al., 2007; Singer et al., 2008). The anti-PA ELISA and the standardized validated anthrax lethal toxin neutralization (TNA) assay developed in our laboratory (Li et al., 2008) together provide a valuable point of reference for standardized quantification and functional analyses of anti-PA antibody responses to *B. anthracis* infection and anthrax vaccination. This model can be adapted and applied broadly when developing relevant, high stringency methods for quantitative measurement of biomarkers as clinical trial endpoints or as an immune correlates of protection.

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