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Development & validation of a quantitative anti-protective antigen IgG enzyme linked immunosorbent assay for serodiagnosis of cutaneous anthrax

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***Background & objectives*: Anthrax caused by *Bacillus anthracis* is primarily a disease of herbivorous animals, although several mammals are vulnerable to it. ELISA is the most widely accepted serodiagnostic assay for large scale surveillance of cutaneous anthrax. The aims of this study were to develop and evaluate a quantitative ELISA for determination of IgG antibodies against *B. anthracis* protective antigen (PA) in human cutaneous anthrax cases.**

***Methods*: Quantitative ELISA was developed using the recombinant PA for coating and standard reference serum AVR801 for quantification. A total of 116 human test and control serum samples were used in the study. The assay was evaluated for its precision, accuracy and linearity.**

***Results*: The minimum detection limit and lower limit of quantification of the assay for anti-PA IgG were 3.2 and 4 µg/ml, respectively. The serum samples collected from the anthrax infected patients were found to have anti-PA IgG concentrations of 5.2 to 166.3 µg/ml. The intra-assay precision per cent CV within an assay and within an operator ranged from 0.99 to 7.4 per cent and 1.7 to 3.9 per cent, respectively. The accuracy of the assay was high with a per cent error of 6.5 - 24.1 per cent. The described assay was found to be linear between the range of 4 to 80 ng/ml (R^2=0.9982; slope=0.9186; intercept = 0.1108).**

***Interpretation & conclusions*: The results suggested that the developed assay could be a useful tool for quantification of anti-PA IgG response in human after anthrax infection or vaccination.**

Key words Anthrax - *Bacillus anthracis* - protective antigen - quantitative ELISA - serodiagnosis

Anthrax, caused by *Bacillus anthracis* is a zoonotic disease, and primarily infects herbivorous livestock and wildlife species and then spreads to human through contact with infected animals or contaminated animal products[1]. Cutaneous anthrax is endemic and a disease of public health importance in several parts of developing countries.

Anthrax resulting from infection by *B. anthracis* spores has three clinical presentations depending upon the routes of infection: cutaneous, inhalation, and

gastrointestinal[2]. The pathogenicity and toxigenicity of bacterium is primarily due to a γ-linked poly-D-glutamic acid capsule and a tripartite toxin[3]. The tripartite toxin is comprised of three proteins, protective antigen (PA), oedema factor (EF) and lethal factor (LF), whose activities are well described[4,5].

Traditionally, the cutaneous anthrax is clinically diagnosed by conventional microbiological methods, such as culture isolation and Gram staining. Several other assays including PCR, latex agglutination, immunofluorescence and surface plasmon resonance are also used for detection of *B. anthracis* antigens or nucleic acids[6-9]. However, these methods often yield negative results when patients have received antibiotics. Hence, blood culture is recommended only if the patient has evidence of systemic anthrax and has not received the antibiotic therapy[10]. Therefore, an accurate diagnostic assay of cutaneous anthrax is required for timely treatment. Serodiagnostic assay for cutaneous anthrax is of utmost importance for surveillance and making strategy to prevent further spread of disease[11-13]. PA, being a common component of both the binary toxins is central to protection against *B. anthracis*[14].

We have previously reported the development and application of a qualitative anti-PA IgG enzyme linked immunosorbent assay (ELISA) for serodiagnosis of cutaneous anthrax[11]. Here, we report a quantitative ELISA for measurement of anti-PA IgG level in cutaneous anthrax cases.

Material & Methods

Protective antigen: The recombinant anthrax protective antigen (rPA) was obtained from Alpha Diagnostics International Company, USA. Lyophilized antigen was reconstituted and stored frozen at -80 °C in small aliquots (50 µl, 1mg/ml) in 5mM hydroxyethyl piperazineethane sulfonic (HEPES) acid buffer, *p*H 7.3.

Human test and control serum: The cutaneous anthrax serum samples were collected between 2010-2012 from the Paderu and Munchingput Mandals, the anthrax endemic areas of Visakhapatnam district, Andhra Pradesh, India. The patients were contacted with the help of District Medical Health Officer (DMHO), Visakhapatnam. The patients were diagnosed on the basis of cutaneous anthrax-like symptoms. The control serum samples from healthy persons having no anthrax-like symptoms were collected from the same area. Both, the patient and control samples were collected randomly. The ethical clearance for the study was obtained from the Institutional Ethical Committee of Andhra Medical College, Vishakhapatnam. The serum samples were subdivided into the following three groups.

(i) Group I (*n*= 68): Control serum samples. The selection of individuals was made on the basis of no early exposures to anthrax or related infections or vaccinations. These samples were negative for anti-PA IgG[11].

(ii) Group II: Human standard reference serum (AVR801). The anti-AVA standard human reference serum, AVR801 was obtained from Centers for Disease Control and Prevention (CDC), Atlanta, USA. The assigned value of total anti-PA IgG concentration of serum pool was 109µg/ml.

(iii) Group III (*n* = 47). Samples from patients with clinically defined cutaneous anthrax. The patients were clinically and epidemiologically well-defined. The samples were confirmed for the presence of anti-PA IgG in the serum by Western blot analysis using rPA[11]. These samples were positive for anti-PA IgG[11].

Quantitative anti-PA ELISA: For measurement of anti-PA IgG antibodies, Maxisorp flat bottom microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 µl/well of rPA (1 µg/ml) in 0.01 M phosphate buffered saline (PBS), *p*H 7.4 and incubated overnight for 16 h at 4 °C. After coating, plates were washed three times with wash buffer (phosphate-buffered saline, *p*H 7.4, supplemented with 0.1% Tween-20). Non-specific binding sites were saturated by adding 300 µl of blocking buffer (3% skimmed milk in PBS, *p*H 7.4) in each well for 1 h at 37 °C. The plates were again washed with wash buffer. Serial two-fold dilutions of reference, controls and test samples (initial dilution 1:200 v/v) in dilution buffer (PBS containing 1% skimmed milk) were added (100 µl/well) and incubated for 1 h at 37 °C. Each serum was tested in duplicate. After incubation, the plate was washed three times with wash buffer and 100 µl of goat anti-human IgG (F_c specific) conjugated to horseradish peroxidase (Sigma, USA) diluted to 1:60,000 in dilution buffer was added to each well and incubated at 37 °C for 60 min. Plates were washed three times with wash buffer and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB), containing hydrogen peroxide (Sigma) was added to all the wells as substrate. After incubation for 15 min at room temperature, 100 µl of 1N H_2SO_4 was added to

each well to arrest the enzymatic reaction. The plates were read within 30 min at a 450 nm using an ELx808 Microplate Reader (BioTek Instruments Inc, USA). The optical density (OD) values after subtraction of the control OD values (background) were taken for final calculations.

Limit of detection for quantitative ELISA: A standard curve was generated using the serial two-fold dilutions of reference serum AVR801 (anti-PA IgG ranging from 109 to 0.21 µg/ml) and was fitted using the 4-PL regression function using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, *www.graphpad.com*). These data exhibited a sigmoidal shape when plotted on an OD-$\log_{10}$ anti-PA IgG concentration in serum. The quality of curve fit was assessed by back calculation of the concentration in standards after the regression has been completed using the formula: (Observed concentration/Expected concentration) x 100[15].

Detection limit (LOD) was calculated from the frequency distribution of OD values from a panel of control human serum samples (Group I, *n*=68). The LOD was estimated by interpolating the mean of all 68 OD values, plus three standard deviations in the standard curve of $\log_{10}$ anti-PA IgG concentration vs. OD values. The antilog of interpolated value was subsequently corrected by the dilution of the sample (1:200).

The theoretical lower limit of quantification (LLOQ) was calculated by interpolating the mean of all 68 OD values, plus ten standard deviations in the standard curve of $\log_{10}$ anti-PA IgG concentration vs. OD values. The empirical lower limit of quantification (LLOQ) was determined experimentally by testing three different sets of serum samples with anti-PA IgG concentrations spanning the theoretical LOD. To determine the lower limit of quantification (LLOQ), positive serum (AVR801) was spiked into a negative human serum pool in different ratios to create six validation samples with the range of concentrations from 1 to 10 µg/ml (1, 2, 4, 6, 8 & 10 µg/ml). Anti-PA IgG ELISA was performed in duplicate by three operators over three non-consecutive days to generate a total of 36 values for each sample. LLOQ was calculated as the lowest anti-PA IgG concentration of the test serum that was measured with a per cent CV of ≤ 20 per cent and an error of ≤ 50 per cent.

Total anti-PA IgG concentration in clinical serum samples: The total anti-PA IgG concentration in clinical serum samples (Group III, n=47) was calculated using the standard curve. The concentration was determined at three different dilution from 1:200 to 1:800 in all the samples. The frequency distribution of total anti-PA IgG in this group was calculated using a bin width of 10.

Assay characteristics: The quantitative anti-PA ELISA was characterized using the validation parameters suggested by Food and Drug Administration (FDA), USA as guidance for industry[16]. Acceptance parameters for the assay are given in Table I.

Table I. Acceptance criteria and performance characteristics of human anti-PA IgG ELISA

Validation parameter	Acceptance criteria	Observed results
Accuracy	≤ 25% error between the expected and observed concentration for each validation sample	6.55 - 24.1%
Intra-assay precision	CV ≤ 10% for each validation sample on a assay plate	0.99 - 7.4%
Inter-assay precision	CV ≤ 20% for each validation sample	3.3 - 14.1%
Lower limit of detection (LOD)	NA	3.2 µg/ml
Lower limit of quantification (LLOQ)	NA	4.0 µg/ml
Dilutional linearity		
- R^2	≥ 0.850	0.9982
- slope	0.8 to 1.2	0.9186
- intercept	4.0 to 4.0	0.1108
- range	≥ LLOQ	4.0-80.0 ng/ml

LLOQ, lower limit of quantification

Accuracy of the assay: The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte[17]. In the present study, accuracy to measure a known, true value of anti-PA IgG was determined by the repeated analysis of three clinical serum samples with predetermined anti-PA IgG concentrations (high, mid and low concentrations). Accuracy was determined from three assay runs per operator per day, performed by three independent operators over three non-consecutive days (a total of 27 assays per sample). The per cent error of ELISA was determined by comparing the anti-PA IgG mean concentration of assay with the predetermined concentration and calculated using the formula [(observed concentration-expected concentration)/expected concentration] X 100. An error of ≤ 25 per cent was considered as an acceptable level of accuracy.

Precision: Precision was determined by repeated analysis of the same three validation serum samples used for accuracy. Precision for each sample was calculated both within each operator and overall operator (intermediate precision) and expressed as the per cent coefficient of variation (% CV) of the reported anti-PA IgG concentrations. For the determination of repeatability (intra-assay precision), each sample was tested in quadruplicate on the same assay plate, independently by three operators in a single day. For the determination of intermediate precision, the samples were measured by three different operators on three different days, equivalent to a total of 27 measurements per sample per operator. The per cent CV was calculated by the sample standard deviation divided by the sample mean and multiplied by 100. The acceptable levels of intra-assay precision and inter-assay precision for ELISA were considered ≤ 10 per cent and ≤ 20 per cent, respectively.

Dilutional linearity and recovery: The dilutional linearity of the assay provides information about the precision of assay results for samples tested at different levels of dilution in the chosen sample diluents. A total of seven serum samples of varying anti-PA IgG concentrations, S1 through S7, were used for the assessment of assay linearity. Samples S1 through S7 consisted of 2 to 80 µg/ml anti-PA IgG and were individually made by adding AVR801 in a pool of control serum samples. All samples were pre-diluted 1:200 in the dilution buffer and subjected to anti-PA ELISA. A total of 16 replicates were generated for each sample by two different operators over four non-consecutive days. The dilutional linearity was calculated from the regression analysis of the empirically observed $\log_{10}$ anti-PA IgG concentrations in the samples versus the expected $\log_{10}$ antibody concentration per sample. The correlation coefficient, the y-intercept and slope of the regression line were calculated.

To perform a spike and recovery experiment, the same seven samples with different anti-PA IgG concentrations were used. The anti-PA IgG ELISA was performed to estimate the amount of IgG in spiked samples. The final observed concentration was corrected using the dilution factor of the sample. The per cent recovery was calculated using the formula (expected concentration/observed concentration) x 100.

Goodness of fit: The "goodness of fit" of the assay is, for comparative purpose, an indication of how closely the data points of the reference serum standard fit the 4-PL model. Goodness of fit is expressed as the regression coefficient (R^2) of the standard curve. An R^2 value that approaches unity is indicative of a good fit for the data to the curve[16].

Results

A qualitative ELISA for detection of IgG antibodies against PA of *B. anthracis* in human serum has been described earlier[11]. In this assay, the total amount of anti-PA IgG antibodies were calculated by a quantitative ELISA using the AVR801 as a reference serum (109 µg/ml anti-PA IgG). The different parameters of the present quantitative ELISA were assessed using the reference as well as clinical serum samples. All validation parameters were met or exceeded when serum samples were tested in a minimum dilution of 1:200.

Reference standard 4-parameter logistic curve: The absorbance values at 450 nm and the known concentration of anti-PA IgG (log values) were plotted using GraphPad Prism version 6.00 software by a sigmoid curve defined by a 4-parameter logistic equation, Y=Bottom +(Top-Bottom)/(1+10^((LogIC50-X)^HillSlope). The reference standard curve (Fig. 1) consisted of 10, two-fold serial dilutions of reference serum AVR109 from 545 ng/ml (1:200 dilution) to 1.06 ng/ml (1:1,02,400 dilution). The per cent recovery of all the standards was calculated from the standard curve and found to be between 80-120 per cent for all the standards except the lowest one, *i.e.* 1.06 ng/ml. The goodness of fit (mean R^2) for the AVR801 standard curve calculated over multiple run was 0.989.

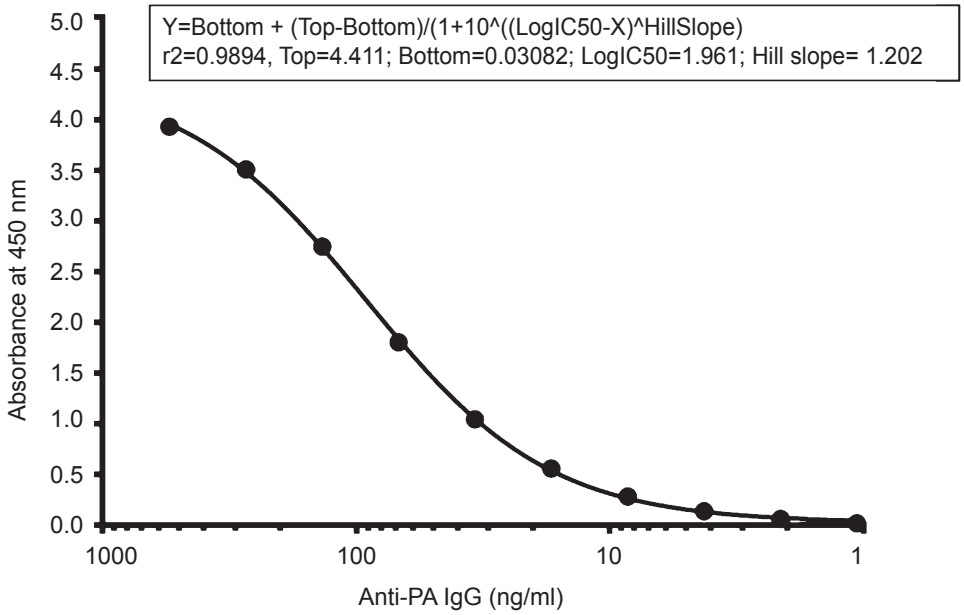

Fig. 1. Four-parameter logistic curve of the anti-protective antigen (PA) IgG.

Detection limits of the assay: The minimum detectable limit of the assay was calculated from the frequency distribution of OD values of healthy control population (Fig. 2). The mean OD value (overall 95% confidence interval) of all 68 control serum samples varied between 0.21 and 0.25. The LOD as calculated by mean plus 3 SD was 0.489. This corresponded to an anti-PA IgG concentration of 3.2 µg/ml after correcting with dilution factor. Therefore, the assay could detect a minimum of 3.2 µg/ml of anti-PA IgG in human serum. The theoretical LLOQ (Mean + 10SD) of the assay was 1.07 OD, corresponding to an anti-PA IgG concentration of 6.9 µg/ml after multiplication with dilution factor. The empirical LLOQ was estimated from a set of six serum samples having anti-PA IgG concentration of 1 to 10 µg/ml. The serum sample with 4 µg/ml had the lowest expected concentration (17% CV, 3.5% error) and thus met the criteria of 20 per cent CV and 50 per cent error. The serum samples with 2 and 1 µg/ml anti-PA IgG could not meet these criteria as these were having CV and error of more than 20 per cent and 50 per cent, respectively. The LLOQ of 4.0 µg/ml equates to an "in the well" anti-PA IgG concentration of 20 ng/ml and hence was within the range of the assay.

Human serum samples with clinically defined anthrax cases: The total anti-PA IgG concentration varied from 5.2-166.3 µg/ml in different anthrax infected clinical serum samples from Group III (Fig 3). All the cutaneous anthrax serum samples (n=47) could be divided into 10 bins according to the circulating antibody levels (Fig. 3). Most of the samples (n=25) could be grouped in the bin of 5-15 µg/ml anti-PA IgG followed by eight samples falling into the bin of 15-25 µg/ml (bin width of 10). The study also showed that only three dilutions of test samples (with high as well as low analyte) were sufficient to determine the anti-PA IgG concentration.

Accuracy and precision: A total of three human serum samples of pre-known anti-PA IgG concentrations were used to establish accuracy of the ELISA. The per cent error between the assigned value and the assay-determined value was determined from the independent analysis of three positive samples. The three pre-determined samples with anti-PA IgG of high (S1, 80 µg/ml), mid (S2, 67.5 µg/ml) and low concentrations (S3, 31 µg/ml) were used in the study. The assay demonstrated high accuracy using AVR801 standard. The per cent error ranged between 6.5-24.1 per cent. Each validation serum sample should have a per cent error ≤ 25 and, therefore, met the predetermined criteria for validation (Table II).

The intra-assay precision per cent CV within an assay and within an operator ranged from 0.99 -7.4 per cent and 1.7-3.9 per cent (except in S2 in which % CV was 12.1 per cent for operator 1 and 14.6 per

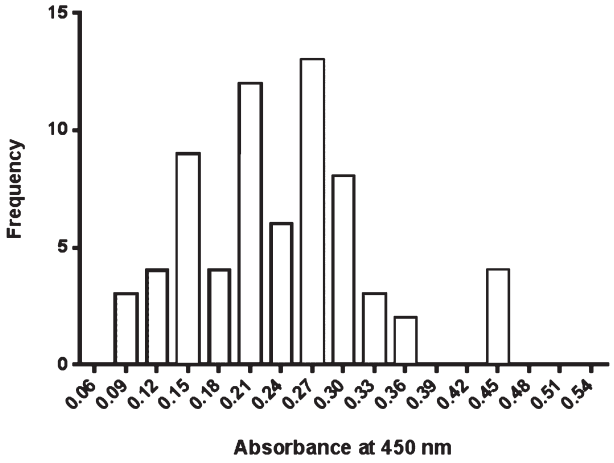

Fig. 2. Frequency distribution of anti-PA IgG absorbance values at 450 nm in serum samples from healthy control population.

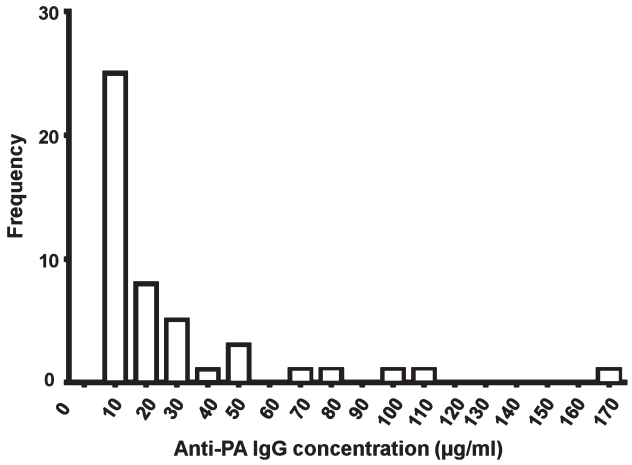

Fig. 3. Frequency distribution of anti-PA IgG concentration (µg/ml) in clinical serum samples from anthrax infected cases.

Table II. Precision assessment of the quantitative ELISA

Validation parameter	Sample	Sample number (n)	Expected IgG (µg/ml)*	Standard AVR801			
				Observed mean IgG (µg/ml)	Standard deviation	% CV	% Error***
Accuracy and intermediate precision	S1	27	80	74.76	2.5	3.3	6.55
	S2	27	67.5	58.5	3.01	5.1	13.3
	S3	27	31	24.7	3.5	14.1	24.1
Intra-operator precision							
Operator 1	S1	9	80	76.2	2.7	3.5	4.75
	S2	9	67.5	61.6	7.5	12.1**	8.7
	S3	9	31	24.4	0.7	2.8	21.2
Operator 2	S1	9	80	76.2	2.7	3.5	4.75
	S2	9	67.5	59.0	2.05	3.4	12.5
	S3	9	31	25.1	0.98	3.9	19.0
Operator 3	S1	9	80	77.2	2.2	2.0	3.5
	S2	9	67.5	62.6	1.6	14.6**	7.2
	S3	9	31	26.8	9.2	1.7	13.5
Intra-assay precision							
Operator 1	S1	4	80	73.2	3.5	4.7	8.5
	S2	4	67.5	54.1	4.02	7.4	19.8
	S3	4	31	21.1	0.73	3.4	21.1
Operator 2	S1	4	80	75.8	1.03	0.99	5.25
	S2	4	67.5	61.2	1.6	2.6	9.3
	S3	4	31	25.8	0.86	3.3	16.7
Operator 3	S1	4	80	74.2	2.6	3.5	7.25
	S2	4	67.5	59.7	2.7	4.5	11.5
	S3	4	31	25.8	0.86	3.3	16.7

CV, coefficient of variation
*The concentration of the samples obtained after diluting the known reference standard
**Values outside the range
*** % Error = (Estimated value-Actual value)/Actual value

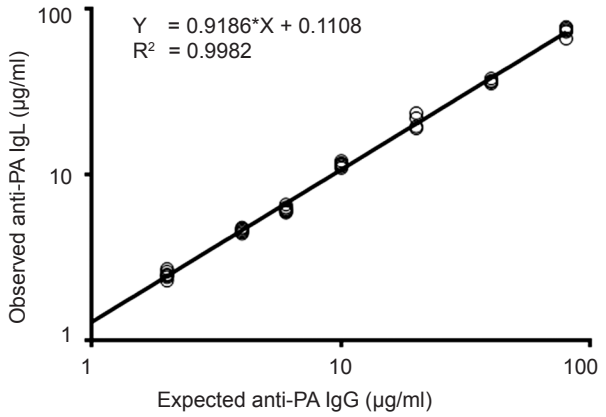

Fig. 4. Dilutional linearity of the quantitative ELISA for human anti-PA IgG.

cent for operator 3), respectively (Table II). These data indicated the reproducibility of the assay.

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[18]. The described assay was shown to be linear between the range of 2 to 80 ng/ml. The best-fit slope was 0.9186, the intercept was 0.1108 and the R^2 of the curve was 0.9982 (Fig 4). The per cent recovery of all the samples ranged between 80-120 per cent, except for sample S7 having 2 µg/ml anti-PA IgG concentration (Table III). Thus, the range of the assay for quantification 'in the well' anti-PA IgG was 20 to 400 ng (4-80 µg/ ml in the serum). The range of the assay is the interval of the "in the well" anti-PA IgG concentrations that can be interpolated from the standard curve inclusive of the lower limits of quantification and the highest concentration of the standard with acceptable accuracy, precision and linearity[19].

Discussion

Cutaneous anthrax is a public health problem in several countries including India where agriculture is the prime resource. Serological testing is the most important diagnostic tool for confirmation of anthrax where time- or treatment-sensitive methods like polymerase chain reaction are not applicable. Serological assays for anthrax have primarily been applied to the evaluation of immune responses to anthrax vaccines, in epidemiologic investigations of the disease in animals, and in confirmatory diagnosis of the various manifestations of anthrax in humans[20,21]. ELISA continues to be a fundamental analytical tool for serodiagnosis or detection of antibody responses to anthrax infection and vaccination. PA is the major antigen that contributes to a protective immune response to anthrax[22]. Besides serological diagnosis of anthrax, antibody to PA is also used as the protective correlate for the licensed and research anthrax vaccines[23]. Antibodies against PA appear as early as 11 days after the onset of disease or 15 days after likely exposure to *B. anthracis*[24]. The antibody response against the protective antigen of *B. anthracis* persists for a long time, even after 1-2 years of infections, and hence detection of anti PA IgG is a good method for the surveillance of anthrax infection[24]. We developed and validated a quantitative ELISA for determination of anti-PA IgG in cutaneous anthrax cases. The anti-PA IgG concentration in the patients' serum samples varied from 5.2-166.3 µg/ml. In a previous study, immune response to protective antigen in human was evaluated and found that peak concentrations of anti-PA IgG was between 168.5 to 1449.5 µg/ml after 18 days of onset of symptoms and varied between 12.6- 107.8 µg/ml after 8-16 months of onset of symptoms[25]. The anti-PA IgG concentration in the serum samples used in this study was less because samples were collected at the later stages of infection. The developed assay could detect 3 µg/ml antibodies against the PA in human serum with a quantitative limit of 4 µg/ml. Hence, the assay was sensitive enough to detect and quantify anti-PA IgG levels even after long time post-exposure to infection, when the antibody level has dropped down to residual level. The LOD and LLOQ of a quantitative ELISA developed by Semenova *et al*[19] were derived from a 4-PL model of the standard curve and found to be 3 and 3.7 µg/ml, respectively. However, we calculated the detection limit on the basis of frequency distribution of OD values in the control serum. The theoretical and empirical LLOQ was 6.9 and 4 µg/ml, respectively. This limit of detection indicates a highly-sensitive assay. All the validation parameters used in the study were met or exceeded. An earlier developed assay reported the LOD of 0.55 µg/ml with a positive cut-off of 9.4 µg/ml. The developed assay yielded 3.41 and 16.0 per cent intra and inter-assay coefficients of variation, respectively[25]. The developed assay can be a useful method for serodiagnosis of cutaneous anthrax and for exact measurement of anti-PA antibodies.

Table III. Per cent recovery of anti-PA IgG in spiked from samples by quantitative ELISA

Sample	Dilution factor (DF)	Observed (µg/ml) X DF	Expected (µg/ml) neat value	Per cent recovery (%)
S1	1:200	Out of range	80	-
	1:400	70.7	80	88.3
	1:800	72.7	80	90.8
	1:1600	77.2	80	96.5
	1:3200	76.7	80	95.8
	1:6400	77.1	80	96.3
	1:12800	74.9	80	93.6
S2	1:200	36.9	40	92.2
	1:400	37.55	40	93.8
	1:800	36.4	40	91
	1:1600	36.3	40	90.7
	1:3200	36.6	40	91.5
	1:6400	34.95	40	87.3
S3	1:200	19.8	20	99
	1:400	19.3	20	96.5
	1:800	19.45	20	97.2
	1:1600	23.55	20	117.7
	1:3200	22.9	20	114.5
S4	1:200	11.5	10	115
	1:400	11.55	10	115.5
	1:800	11.89	10	119
	1:1600	12.05	10	120.5
	1:3200	11.14	10	111
S5	1:200	6.6	6	110
	1:400	6.26	6	104.3
	1:800	6.08	6	101.3
	1:1600	6.02	6	100.3
S6	1:200	4.6	4	115
	1:400	4.51	4	112.7
	1:800	4.64	4	116
	1:1600	4.77	4	119.2
S7	1:200	2.71	2	135.5
	1:400	2.7	2	135
	1:800	2.65	2	132.5
	1:1600	2.47	2	123.5

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***Conflict of interest*:** None

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