Anti-Protective Antigen IgG Enzyme-Linked Immunosorbent Assay for Diagnosis of Cutaneous Anthrax in India

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Anthrax caused by *Bacillus anthracis* is a public health problem in several developing countries whose main source of income is farming. Anthrax is a disease of herbivorous animals, and humans can be infected by handling infected animals or contaminated animal products. Specific diagnostic tests are unavailable in India for the detection and confirmation of cutaneous anthrax in humans. Here, we describe the development of an enzyme-linked immunosorbent assay (ELISA) for detection of serum antibodies against *Bacillus anthracis* protective antigen in the Indian population. A total of 405 serum samples collected from different groups were tested by the developed ELISA. The assay provided a specificity of 99.41% (95% confidence interval [CI], 97.89 to 99.93) and a sensitivity of 100% (CI, 94.4 to 100) using a cutoff value of 0.29 ELISA unit (EU). The positive predictive value (PPV) and negative predictive value (NPV) of the assay were 97% and 100%, respectively. The efficiency and J index for the reliability of the assay were 99.5% and 0.994, respectively. The assay can be a very useful tool for surveillance as well as for diagnosis of cutaneous anthrax cases in India.

Anthrax is an acute bacterial infection caused by *Bacillus anthracis*, a large, Gram-positive, spore-forming, nonmotile bacterium. Anthrax is primarily a zoonotic disease, but humans can acquire this disease through contact with infected animals and their products under natural conditions (30). Depending upon the route of exposure to *B. anthracis*, anthrax can be of three types; cutaneous, gastrointestinal, or inhalational, and case fatality rates in human have been estimated at 1 to 20%, 25 to 60%, and 86 to 89%, respectively, in untreated anthrax cases globally (http://www.bt.cdc.gov/agent/anthrax/faq/signs.asp) (29). The cutaneous route accounts for 95% and the inhalation route for 5% of total reported anthrax cases, while gastrointestinal anthrax is quite rare, mainly due to underreporting of the disease (4, 26). Anthrax is known to occur globally; however, unreliable reporting makes it difficult to estimate the true incidences of human anthrax. Cutaneous anthrax is the most common, with an estimated 2,000 cases reported worldwide annually (4, 9).

The virulence of *B. anthracis* is attributed to two major factors, i.e., a tripartite toxin and the poly-γ-D-glutamic acid capsule (5). Virulent *B. anthracis* vegetative cells form capsules of poly-D-glutamic acid which impede the host immune system and inhibit macrophages from engulfing and destroying the bacteria (7). The anthrax toxins are secreted as three distinct proteins, namely, protective antigen (PA), lethal factor (LF), and edema factor (EF), and their activities have been well described (13, 27). The exotoxins are binary, composed of a B (binding protein) and an A (enzymatically active) protein. PA acts as a B component and combines with EF and LF to form the binary toxins edema toxin (ETx) and lethal toxin (LTx), respectively (25). Thus, PA is the pivotal protein of the anthrax toxin complex. Studies in animal models have confirmed that the immune response to PA is central to protection against *B. anthracis* (17). Therefore, the presence of anti-PA IgG in human serum can be an accurate indicator of anthrax exposure. Simultaneously, it can confirm the efficacy of vaccines in humans as well animals. Earlier reports also showed that anti-PA IgG enzyme-linked immunosorbent assay (ELISA) is a valuable tool for confirmation of cutaneous and inhalational anthrax cases (19). In a recent study, anti-PA IgG and anthrax lethal toxin neutralization activity levels were found to be very useful for detection and confirmation of natural cutaneous anthrax cases in Bangladesh (1).

India has the largest livestock population in the world. In many regions in India, anthrax is still enzootic; however, more sporadic cases of human anthrax are reported from southern states than from northern states (20). In some states, like Orissa and Andhra Pradesh, anthrax is endemic and a public health problem in many areas (11, 15). The clinical diagnosis of cutaneous anthrax is traditionally established by conventional microbiological methods, such as culture and Gram staining. 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 antibiotic therapy (22). Molecular tests, including PCR and real-time PCR, also do not work well for detection of cutaneous anthrax cases after antibiotic therapy, and wounds are sometimes superinfected by several other pathogens, most often with staphylococcus or streptococcus (6). It has been found that anti-PA IgG can be detected just 11 days after the onset of symptoms or 15 days after likely exposure of anthrax (18). Hence, detection of anti-PA IgG can be a very good diagnostic tool for anthrax infection. In addition, the anti-PA IgG response remains detectable 8 to 16 months after the onset of symptoms (18). Therefore, determination of the immune response to PA can be a very good tool for surveillance as well.

In this study, we report the development and evaluation of an enzyme-linked immunosorbent assay for detection of anti-protective antigen (PA) IgG in human serum samples from an Indian population.
MATERIALS AND METHODS

Antigen preparation. Recombinant anthrax toxin protective antigen (rPA) was obtained from Alpha Diagnostics International Company, San Antonio, TX. Lyophilized antigen was reconstituted in ultrapure water and was stored at −80°C in small aliquots (50 μL, 1 mg/ml) in 5 mM HEPES, pH 7.3.

Serum samples. A total of 405 serum samples were employed in this study. The sera were subdivided into five distinct groups (groups I to V).

(i) Group I (n = 253): samples from healthy persons. Group I samples were obtained from healthy blood donors from north and middle parts of India and are representative of the Indian population in areas where anthrax is not endemic. The selection of individuals was made on the basis of no early exposures to anthrax or related infections or vaccinations. The donors included volunteers of various age groups.

(ii) Group II (n = 71): samples from healthy persons from an area where anthrax is endemic. Groups II samples were obtained from healthy blood donors from areas (south India) where anthrax is endemic. The selection of individuals was made on the basis of no early exposures to anthrax or related infections or vaccinations.

(iii) Group III (n = 16): samples from patients with clinically confirmed nonanthrax infections. Group III samples were obtained from patients with infections clinically proved not to be anthrax, from the area where anthrax is endemic. The selection of individuals was made on the basis of ailments other than anthrax.

(iv) Group IV (n = 5): samples from persons vaccinated with anthrax vaccine adsorbed (AVA). Group IV sera were obtained from persons vaccinated with AVA. These sera served as positive controls for the presence of anti-PA IgG.

(v) Group V (n = 60): samples from patients with clinically and immunologically defined cutaneous anthrax. Group V samples were obtained from patients with clinically proved anthrax from the area where anthrax is endemic. The patients lived in southern India, and their clinical and epidemiological parameters were well defined. The presence of anti-PA IgG in the sera was confirmed by Western blot analysis.

ELISA procedure. Anti-PA IgG was measured by ELISA as described earlier, with some modifications (1, 19). Maxisorp flat-bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 μl per well of phosphate-buffered saline (PBS), pH 7.4, containing 1 mg/ml of rPA and incubated overnight at 4°C. The antigen-coated plates were washed three times with wash buffer (PBS containing 0.1% Tween 20) using ELX 508 μl microplate washer (Biotek Instruments Inc.). The wells were blocked with 300 μl of blocking buffer (3% skim milk in PBS) for 1 h at 37°C. After washing, the plate was blotted dry on a paper towel. A series of positive, negative, and test sera were diluted 1:250 in PBS containing 1% skim milk, pH 7.4. The final volume in each well was 100 μl, and all wells were incubated for 60 min at 37°C. The plate was then washed three times with wash buffer and blotted dry on a paper towel. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma–Aldrich) diluted 1:3,000 in PBS containing 1% skim milk (100 μl/well) was added and incubated for 60 min to detect the bound anti-PA IgG. Again, the plate was washed three times with wash buffer, and anti-PA IgG was detected colorimetrically by using 100 μl per well of TMB (3,3′,5,5′-tetramethylbenzidine) in sodium acetate-citrate buffer, pH 6.0, containing hydrogen peroxide as the substrate (Sigma–Aldrich). The color development was read after 15 min of incubation at room temperature at 630 nm using an ELISA plate reader (Biotek Instruments Inc.). The optical densities (ODs) were expressed in ELISA units (EU). All the serum samples were tested in duplicate in two different assays.

Statistical analysis of data for calculation of performance characteristics of the assay. EU values (OD at 630 nm [OD [630]]) from different samples were plotted using computer graphics software (GraphPad Prism, version 5.0; GraphPad, San Diego, CA) to determine the background for different control groups in the assay as well as distribution of OD [630] data for different sera tested (14). Cutoff ELISA values were calculated as the mean OD [630] of sera from different groups plus three standard deviations (SD). Cutoff values were also established using a receiver-operating characteristic (ROC) curve, and the performance of the test was evaluated based on area under the ROC curve (AUC). The variables measured for the calculation of performance indices of the assay were the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). The sensitivity and specificity were calculated as [TP/(TP + FN)] × 100 and [TN/(TN + FP)] × 100, respectively. The positive predictive value (PPV) and the negative predictive value (NPV) were calculated as TP/(TP + FP) and TN/(TN + FN), respectively. Sensitivity and specificity were used to calculate the false-positive rate (FPR) as 1 specificity and the false-negative rate (FNR) as 1 sensitivity. The efficiency or test accuracy was calculated as (TP + TN)/(TP + TN + FP + FN). Reliability was expressed as Youden’s (J) index and calculated as sensitivity + (specificity − 1) (16). To test the significance of the differences in mean anti-PA IgG titers in different groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey’s multiple comparison tests to analyze pairwise comparisons, by using GraphPad Prism, version 5.0.

RESULTS

Determination of reactivity of anthrax protective antigen with sera from various groups. The mean EU values of IgG against PA in different groups are given in Table 1. The mean values (overall 95% confidence interval) in sera from groups I, II, III, IV, and V varied between 0.09 and 0.1, 0.17 and 0.2, 0.13 and 0.19, 1.44 and 2.58, and 1.23 and 1.61 EU, respectively. The overall mean value (overall 95% confidence interval) of sera from all control groups (groups I, II, and III; n = 340) was 0.11 to 0.12 EU. The frequency distribution of anti-PA IgG EU in different groups is given in Fig. 3. The minimum cutoff value was measured in EU in different control populations as the mean plus 3 SD (Table 1). The calculated cutoff values were 0.21 EU in the healthy control group (group I), 0.34 EU in the control population from the area of endemicity (group II), and 0.33 EU in the nonanthrax infection group (group III). The cumulative cutoff value of all the control (groups I, II, and III) was 0.29 EU.

The presence of high levels of anti-PA IgG was detected in sera from individuals who had received the AVA vaccination (group IV) and in most of the sera from those with clinical anthrax (group V), in contrast to control groups (Fig. 2). There was very little overlap in the mean titers of control sera and clinical anthrax sera (Table 1; Fig. 2). Only 33 (9.7%) and 2 (0.58%) of all the control sera showed EU values higher than 0.21 and 0.29, respectively, while 100% of the vaccinated and anthrax-infected subjects (group IV and group V) were above such thresholds (Fig. 2). The mean anti-PA EU values for the AVA vaccination (group IV) and clinical anthrax (group V) sera were significantly higher than the mean EU values for the other three groups (P < 0.001, Tukey’s

| Group (no. of samples) | Mean (SD) anti-PA IgG (EU) | No. of positive tests |
|------------------------|----------------------------|----------------------|
| Group I (253)          | 0.097 (0.038)              | 0                    |
| Group II (71)          | 0.191 (0.051)              | 33                   |
| Group III (16)         | 0.168 (0.054)              | 0                    |
| Group IV (5)           | 2.015 (0.456)              | 5                    |
| Group V (60)           | 1.426 (0.738)              | 60                   |
| All control sera (340)| 0.120 (0.057)              | 33                   |

Table 1 Comparison of titers of anti-PA IgG in sera from different groups

a For the definitions of the groups, see the text.
No significant difference was observed between the mean EU of control sera from individuals in areas of endemicity (group II) and sera from individuals with nonanthrax infections (group III). Likewise, difference between the mean EU of healthy control sera (group I) and nonanthrax infection sera (group III) was also not significant. However, a significant difference was observed between the mean EU values of healthy control sera (group I) and control sera from areas of endemicity (group II) \((P < 0.05, \text{Tukey's test})\). All the false-positive sera from the area of endemicity were re-examined by Western blot analysis and competitive inhibition assay \((19)\) and were found to be negative for anti-PA IgG (data not shown).

**Characteristic features of anthrax protective antigen IgG detection ELISA.** The sensitivity and specificity of the assay were determined at various cutoff values. At a cutoff value of \(\geq 0.21\) (calculated from group I), the sensitivity and specificity were 100% \((95\% \text{ CI, 94.4 to 100})\) and 90.29\% \((95\% \text{ CI, 86.64 to 93.23})\), respectively (Table 2). The best combination of sensitivity and specificity was observed at a cutoff value of 0.29 EU (calculated test). No significant difference was observed between the mean EU of control sera from individuals in areas of endemicity (group II) and sera from individuals with nonanthrax infections (group III). Likewise, difference between the mean EU of healthy control sera (group I) and nonanthrax infection sera (group III) was also not significant. However, a significant difference was observed between the mean EU values of healthy control sera (group I) and control sera from areas of endemicity (group II) \((P < 0.05, \text{Tukey's test})\). All the false-positive sera from the area of endemicity were re-examined by Western blot analysis and competitive inhibition assay \((19)\) and were found to be negative for anti-PA IgG (data not shown).

**FIG 1** Frequency distribution of the anti-PA IgG titers (EU) in different population groups. (A) Sera from healthy controls (group I, \(n = 253\)); (B) sera from controls in an area where anthrax is endemic (group II, \(n = 71\)); (C) sera from individuals with nonanthrax infections (group III, \(n = 16\)); (D) all control sera (groups I, II, and III, \(n = 340\)); (E) sera from AVA-vaccinated individuals (group IV, \(n = 5\)); (F) sera from individuals with clinically confirmed anthrax (group V, \(n = 60\)). Vertical dotted bars indicate an IgG antibody titer of 0.29 (cutoff value) against PA.

**FIG 2** Anti-PA IgG titer in serum (diluted 1:250) obtained from different groups of human subjects. (A) Sera from healthy controls (group I, \(n = 253\)); (B) sera from controls in an area where anthrax is endemic (group II, \(n = 71\)); (C) sera from individuals with nonanthrax infections (group III, \(n = 71\)); (D) all control sera (groups I, II, and III, \(n = 340\)); (E) sera from AVA-vaccinated individuals (group IV, \(n = 5\)); (F) sera from individuals with clinically confirmed anthrax (group V, \(n = 60\)). Each data point represents one person. Bars show the means with SD for each group. The horizontal dotted bar indicates an IgG antibody titer of 0.29 (cutoff value) against PA.
from cumulative control population, n = 340). At this cutoff, ELISA could diagnose 100% of infections with a specificity of 99.41% (95% CI, 97.89 to 99.93). The AUC index of the IgG test with a cutoff value of 0.29 EU by ROC curve analysis was 1.0 (95% CI, 1.000 to 1.000; P < 0.0001). The positive predictive value (PPV) of the assay was 97%, and the negative predictive value (NPV) was 100% (Table 2), using a cutoff point of 0.29 EU. The efficiency or test accuracy in terms of all the tests that gave correct results was 99.5%. The J index for the reliability of the assay was 0.994. The corresponding false-positive rate (FPR) and false-negative rate (FNR) of the assay were 0.59% and 0%, respectively.

**DISCUSSION**

The main objective of the study was to develop a serological test for diagnosis of cutaneous anthrax on the basis of anti-PA IgG antibodies in human sera from Indian population. Being a zoonotic disease, anthrax infection occurs when the bacterium enters a cut or abrasion on the skin, such as when an individual handles contaminated wool, hides, leather, or hair products of infected animals. Skin infection begins as a raised itchy bump that resembles an insect bite but within 1 to 2 days develops into a vesicle and then a painless ulcer. Cutaneous anthrax is characterized by the formation of a black eschar surrounded by prominent edema and vesicles, which may resemble many other skin lesions, such as ulceroglandular tularemia (21), plague (12), ecthyma gangrenosum (8), various spotted fever group rickettsial infections (10), and scrub typhus (3). The clinical diagnosis of cutaneous anthrax is traditionally established by microbiological methods like demonstrating Gram-positive, capsulated bacilli on the smear of the lesion or isolating *B. anthracis* in culture (2, 24). However, Gram staining and culture for *B. anthracis* can be unrevealing for patients who have received antibiotic therapy before the collection of samples (23). Moreover, cutaneous anthrax lesions are very difficult to discriminate from many other skin infections. An immune response to PA is detectable after 11 days of onset of symptoms and remains detectable after 8 to 16 months of exposure (18). Therefore, for surveillance or during outbreaks, when time- or treatment-sensitive diagnostic methods (e.g., culture, PCR, and biopsy) may not be applicable, serological testing may be the only confirmatory diagnostic tool that can be used.

India is a predominantly agricultural country. Anthrax is endemic in certain areas, but many cases remain undocumented due to lack of a proper diagnostic system. Hence, in this study an anti-PA IgG ELISA was optimized to detect the level of immunoglobulin G antibodies in the infected serum of individuals with cutaneous anthrax. The mean IgG titer in healthy human sera from an area of anthrax endemicity was much higher than that in control sera from healthy individuals in areas where anthrax is not endemic (Table 1). When a cutoff value was selected on the basis of these individuals (group I), 33 of 71 control sera (46.4%) from the area of anthrax endemicity (group II) were found to have mean values higher than 0.21 EU. This confirmed that human sera from different populations have different reactivity with PA. Statistically, a significant difference (P < 0.05) was observed in the mean anti-PA EU of healthy control sera from groups I and II. Turnbull et al. also reported the detection of anti-PA antibodies in humans and animals in the Etosha National Park and concluded that there is a residual antibody level in these populations in an area where disease is endemic (28). In this study, although all the sera from AVA-vaccinated and anthrax-infected individuals were positive at a cutoff value of 0.21 EU, specificity was only 90.29%. Therefore, a cutoff value should be selected on the basis of prevalence of disease in a particular population. The best combination of sensitivity and specificity was observed at an antibody titer cutoff of 0.29 EU, which was selected by taking mean plus 3 SD for all control sera (groups I, II, and III). Sensitivity and specificity of 100% and 99.41% were observed at this cutoff value. The results were significant, and the 95% confidence intervals for both sensitivity and specificity were narrow (Table 2). The sensitivity of the assay measures the proportion of actual positives which are correctly identified, while the specificity measures the proportion of negatives which are correctly identified. The area under a ROC curve quantifies the overall ability of the test to discriminate between individuals with the disease and those without the disease. There were only two false-positive serum samples from the area where anthrax is endemic. These were truly negative for anti-PA IgG EU values and were detected by ELISA due to a high background. Previously, Quinn et al. described a quantitative ELISA for human IgG antibodies to PA (19). The diagnostic sensitivity and specificity of the assay were 97.8% and 97.6%, respectively. They also suggested that when a serological assay is being evaluated, the prevalence of disease in the group should be considered. Hence, the ELISA developed in the present study can be used in areas where anthrax is endemic. Moreover, during a surveillance program, the same ELISA can also be used for detection of anti-PA antibodies in animals by using the suitable enzyme-conjugated antibody. To our knowledge, this is the first study from India which describes the sensitivity and specificity of an anti-PA IgG ELISA. The study demonstrated that for the Indian population, the optimized parameters of this ELISA serve as the best serological test that can be used for epidemiological surveillance, screening of blood plasma and serum, and diagnosis and confirmation of cutaneous anthrax.

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