Anthrax, caused by *Bacillus anthracis*, is primarily a zoonotic disease. Being a public health problem also in several developing countries, its early diagnosis is very important in human cases. In this study, we describe the use of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of anti-lethal factor (anti-LF) IgG in human serum samples. A panel of 203 human serum samples consisting of 50 samples from patients with confirmed cutaneous anthrax, 93 samples from healthy controls from areas of India where anthrax is nonendemic, 44 samples from controls from an area of India where anthrax is endemic, and 16 patients with a disease confirmed not to be anthrax were evaluated with an anti-LF ELISA. The combined mean anti-LF ELISA titer for the three control groups was 0.136 ELISA unit (EU), with a 95% confidence interval (CI) of 0.120 to 0.151 EU. The observed sensitivity and specificity of the ELISA were 100% (95% CI, 92.89 to 100%) and 97.39% (95% CI, 93.44 to 99.28%), respectively, at a cutoff value of 0.375 EU, as decided by receiver operating characteristic (ROC) curve analysis. The likelihood ratio was found to be 49.98. The positive predictive value (PPV), negative predictive value (NPV), efficiency, and Youden’s index (J) for reliability of the assay were 92.5%, 100%, 98.02%, and 0.97, respectively. The false-positive predictive rate and false-negative predictive rate of the assay were 2.61% and 0%. The assay could be a very useful tool for early diagnosis of cutaneous anthrax cases, as antibodies against LF appear much earlier than those against other anthrax toxins in human serum samples.

Anthrax, a potentially deadly disease caused by a spore-forming, Gram-positive bacterium, *Bacillus anthracis*, has been known since antiquity. Herbivores, including cattle, goats, horses, and sheep, are susceptible to infection with *B. anthracis* spores. Anthrax is acquired by humans through contact with contaminated meat, hair, hides, and wool containing *B. anthracis* spores from infected animals. Anthrax infection in humans occurs by any of the three major routes the skin, the respiratory tract, or the gastrointestinal tract, generating three different primary forms of the disease—cutaneous, inhalational, and gastrointestinal anthrax, respectively (1). The case-fatality rates of untreated cutaneous, gastrointestinal, and inhalational anthrax in humans vary from 1 to 20%, 25 to 60%, and 86 to 89%, respectively (2; http://www.bt.cdc.gov/agent/anthrax/faq/signs.asp). The majority of reported anthrax cases are cutaneous (95%), with inhalational anthrax accounting for about 5% of reported anthrax cases, while gastrointestinal anthrax is rarely reported (3). Notably, anthrax is known to occur globally; however, most of the cases remain unreported (4).

*B. anthracis* harbors two major virulence factors, a tripartite toxin and a poly-γ-d-glutamic acid capsule, carried by two distinct plasmids, pX01 and pXO2, respectively. The capsule interferes with phagocytosis of the organism. The anthrax exotoxin is formed by combinations of lethal factor (LF) and/or edema factor (EF) with protective antigen (PA) to form lethal toxin (LTx) or edema toxin (ETx), respectively (5). PA binds to cellular receptors and mediates the entry into the cytosol of both LF, a zinc metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK), and EF, an adenylate cyclase that converts ATP to cyclic AMP (cAMP) and promotes lethal tissue edema (6, 7). A consequence of the combined activities of these toxins is to inhibit innate and acquired immune responses, allowing the bacteria to replicate unchecked in the host (8, 9).

Protection against anthrax is mediated by antitoxin antibody responses either actively induced or passively administered. Studies in animal models have confirmed that the immune response to PA is central to protection against *B. anthracis* (10). Therefore, the presence of anti-PA IgG in human serum has been targeted as a major indicator of anthrax, especially for the cutaneous form (11–13). However, animal vaccine studies have shown that LF elicits higher IgG antibody titers than PA (14, 15). In natural cutaneous anthrax cases, the majority of the toxin-specific antibody responses are directed against LF, with IgG detected as early as 4 days after the onset of symptoms, in contrast to the delayed and lower EF- and PA-specific IgG responses (16). Therefore, the development of an anti-LF ELISA can be a good retrospective diagnostic or epidemiologic tool for investigation of cutaneous anthrax cases. Moreover, an anti-LF ELISA should differentiate between the vaccinated and naturally anthrax-infected persons as PA is the major component of anthrax vaccine.

The incidence of natural anthrax in industrialized countries remains quite low, and the disease is not a major public health issue in developed countries. However, cutaneous anthrax remains a public health problem in several developing countries, where the main source of income is farming or where communities live in interface with wildlife (17, 18). India has the largest livestock population in the world, and there are several areas of the country where cutaneous anthrax is endemic (19, 20). There is a scarcity of specific diagnostic tests for cutaneous anthrax in India.
Hence, accurate diagnostic assay of cutaneous anthrax is of utmost importance for timely treatment and for the development of strategies to prevent further spread of disease.

In this study, we report the development and evaluation of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of anti-lethal factor (anti-LF) IgG in human serum samples from several Indian populations.

MATERIALS AND METHODS

Antigen preparation. Recombinant *B. anthracis* lethal factor (rLF) was obtained from the Alpha Diagnostics International Company, San Antonio, TX. LF was reconstituted in ultrapure water, and 50-μl aliquots were stored at −80°C.

Serum samples. To determine the background level of anti-LF ELISA reactivity in the Indian population, a total of 203 serum samples were collected from different population groups. A total of 93 samples (group I) were collected from healthy blood donors from the north and middle parts of India, representing an area of anthrax nonendemicity. Another set of 44 sera (group II) was obtained from the healthy blood donors from south India, where anthrax is endemic. The selection of individuals was made on the basis of their having no prior exposure to anthrax or related infections or vaccinations. The donors included volunteers of various age groups. A total of 16 sera (group III) were collected from the patients with clinically confirmed non-anthrax infections from the area of anthrax endemicity. The selection of individuals in this group was made on the basis of ailments other than anthrax. Group IV consisted of a total of 50 serum samples from patients with clinically diagnosed anthrax from the area where anthrax is endemic. The cutaneous anthrax cases were confirmed by clinical symptoms, case history, or PCR of swabs from cutaneous lesions of patients.

**ELISA procedure.** Maxisorp flat-bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 μg per well of 2 μg/ml rLF suspended in coating buffer (0.06 M Na₂CO₃ and 0.14 M NaHCO₃ [pH 9.5]) and incubated overnight at 4°C. The antigen-coated plates were washed three times with wash buffer (phosphate-buffered saline [PBS] containing 0.1% Tween 20) using the ELx50 microplate washer (BioTek Instruments, Inc.). The wells were blocked with 300 μl of blocking buffer (3% skim milk in PBS [pH 7.4]) for 1 h at 37°C. The blocking buffer was decanted, and the plates were blotted dry on a paper towel without washing. Positive and negative test sera were diluted to 1:200 in PBS containing 1% skim milk (pH 7.4). One hundred microliters of each well of the plates, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma-Aldrich) was added to produce a color change. The plates were washed three times with wash buffer and blotted dry on a paper towel. In each well of the plates, 100 μl of substrate (Sigma-Aldrich) was added to 1:25,000 in PBS containing 1% skim milk was added to detect the bound anti-LF-IgG. The plates were then again incubated at 37°C for 60 min. The plates were then washed three times with wash buffer, and to each well 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMDB) in sodium acetate/citrate buffer (pH 6.0) containing hydrogen peroxide as the substrate (Sigma-Aldrich) was added to produce a color change. The plates were read at 630 nm using an ELx808 microplate reader with Gen5 data analysis software (BioTek Instruments, Inc.) after incubation for 10 min at room temperature. All sera were tested twice in duplicate without heat inactivation. The average absorbance (optical density at 630 nm [OD₆₃₀]) values are expressed in ELISA units (EU).

**Selection of cutoff values.** EU values (OD₆₃₀) from different groups of the population were plotted using computer graphics software (GraphPad Prism, version 6.00 for Windows; GraphPad, La Jolla, CA) to determine the background levels from different control groups in the assay as well as the distribution of anti-LF EU in different tested sera (21). Cutoff values in the ELISA were calculated as the mean OD₆₃₀ of sera from different groups + 3 standard deviations (SD). In addition, cutoff values at a 95% accuracy level were determined using a receiver operating characteristic (ROC) curve, and the performance of the test was evaluated based on the area under the ROC curve (AUC) (22).

**Diagnostic accuracy of ELISA.** 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 the Youden’s index (J) and calculated as [sensitivity + (specificity – 1)] (23). The presence of anti-LF IgG in false-positive sera was further confirmed by Western blotting.

**Statistical analysis.** A one-way analysis of variance (ANOVA) was performed to test the significance of the differences in mean anti-LF IgG titers in different groups. Tukey’s multiple comparison tests were applied to analyze pairwise comparisons between different groups by using GraphPad version 6.00 for Windows, GraphPad Software, La Jolla, CA.

**RESULTS**

Reactivity of human sera from different population groups with *B. anthracis* LF. The mean EU values of anti-LF IgG in different population groups are given in Table 1. The mean EU values (with overall 95% confidence interval [95% CI] in parentheses) in sera from groups I, II, III, and IV were 0.107 (0.093 to 0.121 EU), 0.184 (0.145 to 0.224), 0.169 (0.140 to 0.198), and 0.938 (0.834 to 1.041), respectively. The overall mean EU value (with overall 95% CI) of sera from all control groups (n = 153) was 0.136 (0.120 to 0.151). The cutoff EU was measured in different control population groups as the mean + 3SD (Table 1). The calculated cutoff values were 0.307 EU in the healthy control group (group I), 0.574 EU in the control population from the area of endemicity (group II), and 0.171 EU in the non-anthrax infection group (group III). The cutoff values for the overall control population were 0.422 EU (mean + 3SD) and 0.375 (by ROC). A scatter plot of the raw EU data from the groups is shown in Fig. 1.

**Detection sensitivity and specificity.** The sensitivity and specificity of the assay were determined at various cutoff values under

**TABLE 1 Comparison of anti-LF IgG titer expressed as ELISA units in sera from different groups**

| Parameter | Group I (n = 93) | Group II (n = 44) | Group III (n = 16) | Cumulative control population (n = 153) | Group IV (n = 50) |
|-----------|-----------------|------------------|-------------------|----------------------------------------|------------------|
| Minimum   | 0.012           | 0.022            | 0.082             | 0.012                                  | 0.400            |
| Median    | 0.097           | 0.158            | 0.181             | 0.113                                  | 0.878            |
| Maximum   | 0.35            | 0.683            | 0.257             | 0.683                                  | 1.746            |
| Mean      | 0.107           | 0.184            | 0.169             | 0.136                                  | 0.938            |
| SD        | 0.067           | 0.130            | 0.055             | 0.096                                  | 0.365            |
| SE        | 0.007           | 0.0197           | 0.014             | 0.008                                  | 0.052            |
| 95% CI     |                 |                  |                   |                                        |                  |
| Lower     | 0.093           | 0.145            | 0.140             | 0.120                                  | 0.834            |
| Upper     | 0.121           | 0.224            | 0.198             | 0.151                                  | 1.041            |
| Cutoff (mean + 3SD) | 0.307 | 0.574 | 0.171 | 0.422 |

* Group I, healthy controls; group II, controls from an area of endemicity; group III, non-anthrax infection; group IV, clinical anthrax infection.
the ROC curve (Table 2). The best combination of sensitivity and specificity was observed at a cutoff value of 0.375 EU (calculated from cumulative control population \( n = 153 \)). At this cutoff, the sensitivity and specificity of the ELISA were 100% (95% CI, 92.89 to 100%) and 97.39% (95% CI, 93.44 to 99.28%). The AUC index of the IgG test with a cutoff value of 0.375 EU by ROC curve analysis was 0.996 (95% CI, 0.992 to 1.000; \( P < 0.0001 \)). The likelihood ratio at this cutoff EU was 49.98 (Table 2).

Only 4 sera from endemic control group (group II) showed EU values higher than 0.375, while 100% of the clinical anthrax-infected subjects were above this threshold EU value (Fig. 2). The mean anti-LF EU value for clinical anthrax infection (group IV) sera was significantly higher (\( P < 0.0001 \), Tukey’s test) than the mean EU value for the three control groups. No significant differences were noted between the three control groups (\( P > 0.05 \)). The false-positive sera from group II and positive sera from group IV were reexamined by Western blotting and found to be negative for anti-LF IgG in false-positive sera from the control group and positive for sera from infected patients (data not shown).

**Characteristic features of anti-LF ELISA.** The sensitivity and specificity of the assay at a cutoff point of 0.375 were found to be 100% and 97.39%, respectively. The positive predictive value (PPV) of the assay was 92.5%, and the negative predictive value (NPV) was 100%. The efficiency or test accuracy in terms of all the tests that gave correct results was 98.02%. The \( J \) index for the reliability of the assay was 0.97. The corresponding false-positive rate (FPR) and false-negative rate (FNR) of the assay were 2.61% and 0%, respectively.

**DISCUSSION**

Cutaneous anthrax is a public health problem in several agricultural countries, including India. Recently, several cases of cutaneous anthrax were reported in Bangladesh \( (12, 24, 25) \). The incidence of anthrax outbreaks in the United States and Europe over the past 10 years \( (26, 27) \) have emphasized the health threat that this infection poses. The pathogenicity of *B. anthracis* is primarily due to the action of its three main virulence factors, the \( \gamma \)-linked poly-\( \delta \)-glutamic acid capsule and the two exotoxins, LT and ETx. Anthrax LT and ETx are A-B-type exotoxins composed of 2 proteins each, the A component being LF and/or EF and the B-component, common to both, being PA. Detection of anti-PA IgG in serum has been preferred for serodiagnosis of anthrax. However, in this study, we have developed a serological test for diagnosis of cutaneous anthrax on the basis of anti-LF IgG antibodies in human serum samples.

The lethal factor of *B. anthracis* toxin is a critical component and potentially participates in the pathogenesis leading to shock and injury during anthrax infections by suppressing host microbial clearance and contributing to the very high bacterial load, as observed in subjects dying with infections \( (28) \). Previously, it has been shown that detection of anti-LF response could be a better diagnostic marker of infection than the anti-PA response \( (16) \). It was also found that the class switching from IgM to IgG occurs early in the anthrax infection. Only 24% and 18% of patients produced an IgG response against EF and PA, respectively, while 65% of patients produced an IgG response against LF \( (16) \). It was also found that LF elicits higher IgG antibody titers than PA in early stages of infection. It was observed that individuals with inhalational anthrax and cutaneous anthrax produced an IgG response to PA not before 11 days and 21 to 34 days, respectively, following the onset of symptoms \( (16) \). However, LF-specific IgG could be detected as early as day 4 after the onset of symptoms, and the titer reached a peak by day 15, compared to the anti-PA IgG titer, which could be detected on day 13 after the onset of symptoms and reached the peak after 30 to 60 days. Hence, the kinetics of the immune response were found to be rapid for anti-LF IgG compared to those of anti-PA IgG. Therefore, determination of anti-LF IgG titer in serum can be a good diagnostic marker for anthrax infection.

In this study, an anti-LF ELISA was optimized to detect the level of IgG in the serum of individuals with cutaneous anthrax. The mean anti-LF IgG titers were determined in various groups of the population (Table 1). The mean anti-LF EU (overall 95% CI shown in parentheses) in sera from groups I, II, III, and IV were 0.107 (0.093 to 0.121), 0.184 (0.145 to 0.224), 0.169 (0.140 to 0.198), and 0.938 (0.834 to 1.041), respectively, Tukey’s multiple comparison by ordinary one-way ANOVA showed nonsignificant difference (\( P > 0.05 \)) between mean EU values of different control groups. However, a significant difference (\( P < 0.0001 \), Tukey’s test) was found between mean EU values of different control population groups and clinical anthrax infection sera (group IV). The cutoff EU value was decided from the area under the ROC curve

**TABLE 2** Comparison of sensitivities and specificities with 95% CIs at various cutoffs for the anti-LF ELISA

| Cutoff (EU) | % sensitivity (95% CI) | % specificity (95% CI) | Likelihood ratio |
|------------|------------------------|------------------------|-----------------|
| >0.240     | 100 (92.89–100.0)      | 92.16 (86.70–95.88)    | 15.3            |
| >0.243     | 100 (92.89–100.0)      | 92.81 (87.50–96.36)    | 17              |
| >0.251     | 100 (92.89–100.0)      | 93.46 (88.31–96.82)    | 19.13           |
| >0.263     | 100 (92.89–100.0)      | 94.12 (89.13–97.28)    | 21.86           |
| >0.281     | 100 (92.89–100.0)      | 94.77 (89.96–97.72)    | 25.53           |
| >0.303     | 100 (92.89–100.0)      | 95.42 (90.80–98.14)    | 30.63           |
| >0.322     | 100 (92.89–100.0)      | 96.08 (91.66–98.55)    | 38.25           |
| >0.341     | 100 (92.89–100.0)      | 96.73 (92.54–98.93)    | 37.49           |
| >0.375     | 100 (92.89–100.0)      | 97.39 (93.44–99.28)    | 49.98           |
| >0.421     | 98 (89.35–99.95)       | 97.39 (93.44–99.28)    | 48.96           |
| >0.444     | 98 (89.35–99.95)       | 98.04 (94.38–99.59)    | 73.44           |
| >0.457     | 96 (86.29–99.51)       | 98.04 (94.38–99.59)    | 71.91           |

*Note that the best combination of sensitivity, specificity, and likelihood ratio was obtained at a cutoff of 0.375 EU (highlighted in boldface).
TABLE 2. The best combination of sensitivity and specificity as well as likelihood ratio was obtained at a cutoff EU value of 0.375. The observed sensitivity and specificity of the ELISA were 100% and 97.39%, respectively, with narrow 95% CIs for both sensitivity and specificity and a likelihood ratio of 49.98. Only four false-positive serum samples were observed from areas of endemicity. The PPV, NPV, efficiency, and J index of the assay were found to be 92.5%, 100%, 98.02%, and 0.97, respectively. The false-positive predictive rate and false-negative predictive rate of the assay were 2.61% and 0%, respectively. Therefore, the present anti-LF ELISA could serve as the best serological assay for diagnosis of anthrax infections in human serum samples.

To the best of our knowledge, this study presents the first optimized assay from India that could detect anti-LF antibodies in cutaneous anthrax. The assay can be a very useful tool for rapid, antemortem diagnosis of cutaneous anthrax as well as for serosurveillance of anthrax cases.

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