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DEVELOPMENT OF A SIMPLE METHOD FOR THE SPECIFIC DETECTION OF ANTHRAX ANTIBODY IN SERA BY COLORED SLIDE AGGLUTINATION TEST

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ABSTRACT

This study was aimed to identify humoral immune response against anthrax vaccine in mice model by using colored slide agglutination test and detection of field infectivity of anthrax. The field isolates of *B. anthracis* (n=05) and F34 stern strain vaccine was isolated on agar plates in order to carry out the slide agglutination test. The field isolates of *B. anthracis* and vaccine bacteria grew on PLET agar medium produced roughly circular and creamy white colonies with ground glass appearance. The bacteria on sheep blood agar media produced rough, sticky, white-gray non hemolytic colonies. Colony polymerase chain reaction (PCR) protocol was adapted to detect fragments of pX01 (596bp) and pX02 (777bp) plasmid of virulent field isolates of *B. anthracis*. The fragment of pX01 plasmid was only detected in vaccine bacteria. Growth of a field isolate and a vaccine bacteria were colored with crystal violet and used in slide agglutination test to detect anthrax antibodies. The anti-anthrax antibody was prepared by immunizing female mice with 100ül anthrax vaccine through subcutaneous route. Tail bleed were collected on day 0, 30, 60, 120 and 180 of immunization. Cardiac bleed was collected on day 180 of immunization for extensive study. 25µl of diluted (1:10, 1:20, 1:50 and 1:100) antisera and 25µl colored antigen was mixed together onto a clean slide at room temperature and the results was red following 5min, 10mins, 15mins and 20mins of reaction. Unstained antigens and non-immunized sera from the mice were used as control. Results of slide agglutination test showed that the colored vaccine bacteria and field isolates clumped the mice anti-antisera (day 30, 60, and 120) at 1:20 dilution as seen in naked eye but the reaction was seen only at 1:10 dilution while colorless antigens were used. Under microscopic investigation of slide agglutination test, the reaction was read up to 1:100 dilutions with the sera collected at day 30, 60, 120 and 180 of immunization. The Anthrax Sterne strain vaccine induced anti-anthrax immunity in mice that was detected until day 180 of immunization. The clumping reaction was distinct while colored anthrax antigen was used in slide agglutination tests. The colored slide agglutination tests protocol developed in this study can be used to detect anti-anthrax immune response and anthrax bacteria in the field condition with minimum laboratory facilities.

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INTRODUCTION

Anthrax of mammals is caused by *Bacillus anthracis* (*B. anthracis*), a large gram positive rod, aerobic (facultative anaerobic), spore bearing soil bacterium, 1–1.5µm to 3–10 µm in size and the only obligate pathogen within the genus bacillus (Wang *et al.,* 2013). Anthrax popularly known as 'Torka' in animals in Bangladesh and is a disease of farm animals reported very often (Ahmed *et al.,* 2010; Saha et al., 2020). Report indicated that the disease is more prevalent in northern Bangladesh (Pabna, Sirajganj, and Tangail) where greater densities of cattle are reared (Chakraborty *et al*., 2012). There are sporadic repots of anthrax throughout the country but true scenario of anthrax in animals is not well studied in Bangladesh due to lack of technology applicable in the field. *B. anthracis* remains in nature as spore (endospore), the spores do not divide and are resistant to drying, heat, ultraviolet light, gamma radiation and many disinfectants (Watson, 1994). Spores do not form in host tissues unless the infected body fluids are exposed to air. The spores following entry into the host body germinate when exposed to a nutrient rich environment, such as the tissues or blood of an animal or human host. In infected blood or tissues, the bacilli are frequently present in short chains, surrounded by the polypeptide capsule, which can be visualized under the microscope while staining with polychrome methylene blue (McFadyean stain, Turnbull *et al.,* 1993; Leise *et al.,* 1959). Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar medium is widely used as selective for the isolation of *B. anthracis* (Fasanella *et al.,* 2008; Knisely, 1966), and used in this study. However, detection of anthrax in the field by growing them on culture is not a feasible technology. The application of molecularly based methods like polymerase chain reaction (PCR) has become increasingly important for the detection of *B. anthracis* (Daffonchio *et al*., 1999; Saha et al., 2020) and practicing globally. Detection of anti-anthrax antibodies on the other hand is required to know the vaccinal immunity and host response to previous infection if any.

Vaccination is routinely practiced in Bangladesh using F34 Stern strain *B. anthracis* for more than five decades to prevent anthrax in animals. Stren strain is a live bacterial vaccine and found to be more effective than the killed bacteria (Roit *et al.,* 2001). The laboratory facility for the detection of antibodies to anthrax, either against the pathogen or vaccine strain is not available due to lack of suitable technologies. Therefore, it needs readily available protocols that can be used to detect the vaccine antibody in the field. This study was aimed to identify field infectivity due to anthrax and immunity against anthrax vaccine by developing a color antigen. The vaccine bacteria were used in mice model to raise anti anthrax antibodies. The antisera were also used in colored slide agglutination test to detect field isolates of *B. anthraces*.

MATERIALS AND METHOD

Isolation of *B. anthracis*

A total of thirteen (13) suspected field cases from various part of Bangladesh were investigated and collected samples for the isolation and identification of *B. anthracis*. Anthrax vaccine (Sterne strain F-34) was collected from local Upazila Veterinary Hospital, Kotoali Thana, Mymensingh city, Bangladesh. The suspected field isolates and vaccine vial were shifted to the laboratory, Department of Pathology with aseptic measures. The samples collected were from the soil (n=13) where the animal were died or disposed, turbinate bone (n=05) and spleen (n=05) from the dead animals, and cotton swab (n=05) from the discharges of suspected and dead animals. The suspensions from each of the soil, turbinate bone, cotton swab and spleen samples were processed to isolate *B. anthracis* in culture (Saha et al., 2020). For the isolation of vaccine bacteria, 1.5ml of vaccine preparation in adjuvant was taken into an appendorf tube and centrifuged at 10000g for 10mins. The supernatant was removed and the pellet was washed 3x in PBS through centrifugation at 10000g for 10mins. The pellet was diluted in 100µl PBS and transferred into a fresh appendorf tube. The 10µl bacterial suspension was added in 5ml of nutrient broth containing amphotericin b. Following overnight incubation at 37°C the growth of the bacteria in nutrient broth, PLET agar and blood agar plate were studied by staining smears onto clean slide with Grams iodine. The morphology of the isolated colonies on blood agar plates and bacterial smears in Grams staining were examined for the presence of Medusa head colonies and Gram +ve rods respectively. Gram positive bacilli appeared blue, whereas Gram negative bacilli and bacteria appeared pink in a contrasting background (Saha et al., 2020). The isolated colonies were smeared onto the clean slides and stained with polychrome methylene blue (Jorgensen et al., 2015). In positive cases, the capsule appeared pink around the blue staining bacilli (M'Fadyean Stain).

PCR detection of *B. anthracis*

Isolated colonies on to the surface of blood agar and PLET agar media showing Medusa head appearance was selected. The species of bacillus isolated was confirmed by using specific primers in colony PCR (Beyer *et al.,* 1995; Saha et al., 2020). Both the field isolates and vaccine bacteria were identified by analyzing the results of PCR. 50µl volume of reaction mixture was prepared consisting of 25µl PCR master mix, 2µl forward primer, 2µl reverse primer, 20µl nuclease free water and a tiny touch of isolated bacterial colony using sterile tooth pick. A total of 30 cycles of PCR amplification reaction was carried out with an initial denaturation at 94ºC for 5mins followed by denaturation at 94ºC for 1min, annealing at 52ºC for 1.5mins and extension at 72ºC for 1.5mins. The final elongation was carried out at 72ºC for 10mins and the reaction was held at 4ºC. The cDNAs as obtained from the PCR were detected by agarose gel electrophoresis and documentation of the images. The cDNAs as expected in PCR (Table 1) was consisting of 777bp for pX02 gene and 596bp for pX01 gene of *B. anthracis*. The morphological and biochemical tests used were unable to selectively identified *B. anthracis* from other bacilli.

Table 1. Oligonucleotide primers used in PCR amplification to detect the fragment of plasmids pX01 and pX02 genes of *B. anthracis* and synthesized from First BASE Laboratories Sdn Bhd, Malaysia

Genes targeted	Primers	Sequences (5'-3')	Expected Amplicon Size (bp)	Reference
Cap gene	CAP9 CAP102	atgtatggcagttcaacccg acccactccatatacaatcc	**777 (pX02)**	Beyer *et al.,* 1995
PA gene	PA8 PA5	gaggtagaaggatatacggt tcctaacactaacgaagtcg	**596 (pX01)**	

Production of hyperimmune serum

In order to evaluate efficacy of the colored antigens, female mice (n=05) were immunized once with 100µl anthrax Stern strain F-34 vaccine through s.c route. Tail bled was collected on day 30, 60, 90 and 180 of immunization. The vaccinated and control mice were deeply sedated through intraperitoneal injection of pentobarbital sodium (200mg/kg body weight, Zatroch et al., 2016), collected cardiac bleed in to the Eppendrof tube for extensive study. The collected blood was allowed to clot at room temperature and incubate overnight at 4ºC temperature. The anti-serum was separated by centrifugation of the blood at 2000g for 5mins, diluted to 1:10, 1:20, 1: 50, 1:100, 1:200 with PBS, pH 7.4 and used in slide agglutination test.

Development of colored antigen test

Antigen stock was prepared from pure culture of field isolate of *B. anthracis* and Sterne strain F-24 Anthrax vaccine grown on sheep blood agar. Overnight growth of 50 isolated bacterial colonies was collected in screw capped Pyrex test tube using a bacteriological loop and suspended in 10ml PBS. The test tube containing bacterial suspension was heated in boil water for 20 minutes to kill the bacteria. Following thermal killing, 100µl of the bacterial suspension was smeared onto the sheep blood agar and incubated 18 hours at 37ºC to observe the bacterial growth if any. The killed bacterial suspension was, therefore, taken in the appendorf tube, centrifuged at 10,000g for 10mins.The supernatant was discarded, 20µl crystal violet working solution and 1ml PBS per tube were added, incubated at room temperature for 5mins and vortexed. The bacteria containing appendorf tube was centrifuged at 10,000g for 10mins. The supernatant was discarded; 500µl PBS was added and centrifuged at 10,000g for 10mins to eliminate unbound color. The supernatant was discarded and 100µl of PBS was added and preserved at 4°C until slide agglutination test was carried out.

Development of colored slide agglutination test

The slide agglutination test was performed by adding 25µl of colored anthrax antigen and 25µl of diluted serum. The mixture was tilted slowly at room temperature and examined at every 5mins to observe the appearance of clumping. The appearance of clumping through naked eye and under microscope (4x objective) was recorded to analyze the end point titer of the serum yielded positive (clumping) reaction. As negative control non immunized mice serum was used in clumping trials. Both the field isolate and vaccine bacteria were used in color antigen formulation and slide agglutination test.

RESULTS

Anthrax is an economically important disease of ruminant especially in dairy cattle characterized by a rapid fatal course and a sudden death. This study was, aimed to detect field cases of anthrax, isolate *B. anthracis* in culture from field outbreaks and developed colored anthrax antigen to detect anthrax antibodies using slide agglutination test.

Isolation and identification of *B. anthracis*

In PLET agar medium the bacteria produced typical growth, which was roughly circular and creamy white with ground glass appearance, characteristics of *B. anthracis* (Figure 1a). The isolated bacterial colonies on PLET agar was stained with Gram's stain and characteristics bacillus bacteria was sub-cultured onto sheep blood agar plate (Figure 1a). Out of 13 field samples examined, 07 field samples and the vaccine vial showed growth of rod shaped bacteria on PLET and blood agar plate (Figure 1b). On sheep blood agar plate the bacteria produced typical rough, sticky, white-gray non hemolytic colonies (Figure 1c) with a characteristic "Medusa head pattern". The physical properties of the bacteria from culture were examined by staining of the smears by Gram's staining and polychrome methylene blue staining. Finally, the bacteria were identified by using PCR.

Morphological characterization of the isolates

The bacterial grown in nutrient broth smeared on clean slides and stained with Gram's iodine showed rod shaped bacteria in 07 cases. The isolated colonies while grew on PLET agar and sheep blood agar, two of them showed different colony morphology and were hemolytic on sheep blood agar plate, they were suspected as non *B. anthracis* and were discarded. Five field isolates were studied extensively. Impression smears prepared from the selected five isolates stained with Grams iodine and examined under high power microscopic field the bacteria appeared as gram-positive, rod-shaped, arranged in single to short-chained with square or truncated ends typical morphology of *B. anthracis*. The smears from the five isolates on to the clean slide stained with Polychrome methylene blue showed McFadyean reaction. The bacilli were stained blue and the capsule appeared as red amorphous material (McFadyean reaction) around the chains of bacilli. These five isolates were Gram +ve, non-hemolytic on sheep blood agar. The vaccine bacteria isolated in nutrient broth, PLET agar and sheep blood agar shower typical colony characteristics similar to the field isolates. They were Gram positive rod but lacking McFadyean reaction while staining the smears with polychrome methylene blue.

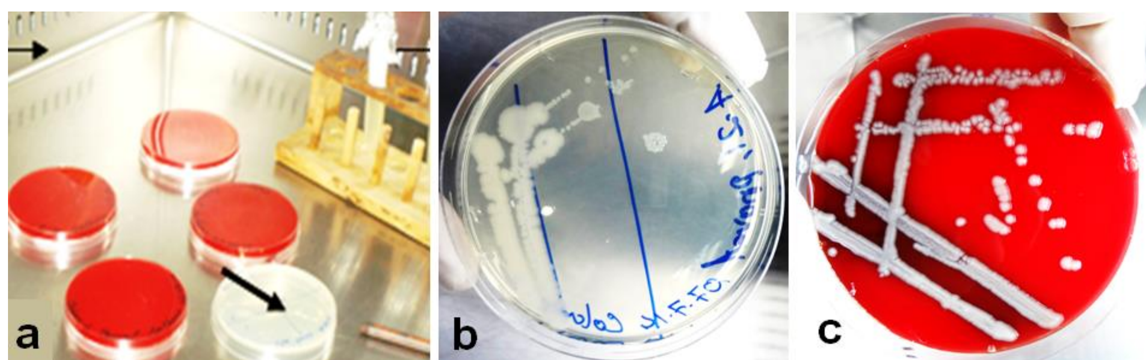

Figure 1. *B. anthraces* bacteria grown on PLET agar (a, white plate) and sheep blood agar (a, red plate) medium. The bacteria grown on PLET agar (b) produced typical growth, which was roughly circular and creamy white with ground glass appearance. Bacteria grown on blood agar plate (c) produce colonies that were non-hemolytic, slightly convex, ground-glass in appearance and showed a characteristic "Medusa head" pattern of growth

Detection of *B. anthracis by PCR*

Colony PCR was carried out with the isolated bacterial colonies using pX01 and pX02 genes specific primers of *B. anthracis*. Out of 13 field samples tested, seven samples showed growth of rod shaped bacteria and pX01 gene specific amplification (596bp) was seen in five field cases and vaccine bacteria (Figure 2a). The pX02 gene specific amplification (777bp) was only seen in 5 field isolates (Figure 2b) but was absent in the vaccine bacteria. Results of PCR showed that animals in five Upazilas (Sahjadpur Upazilla, Sirajganj, Tangail sador, Tangail, Dhanbari, Tangail, Sreemangal Upazila, Moulvibazar and Barisal sador) were infected with anthrax. Two samples although showed similar growth pattern like anthrax, they were non *B. anthracis* and were discarded.

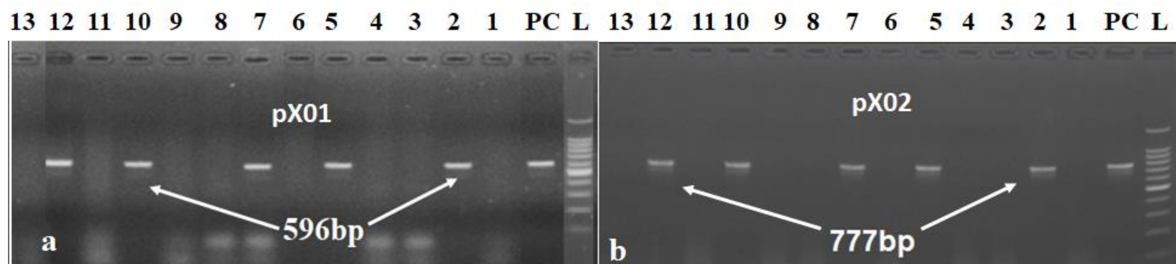

Figure 2. Results of PCR with the field samples (n=13) to detect the fragments of pX01 (a) and pX02 (b) genes of *B. anthracis*. Lane 1 is for 100bp ladder, lane PC is for the +ve control and lanes 1 to 13 are for test samples. Out of 13 field samples tested in colony PCR five samples (lane 2, 5, 7, 10 and 12) yielded amplicons specific to pX01 (596bp) and pX02 (777bp) genes of *B. anthracis*

Colored slide agglutination test

Both the field isolates and the vaccine bacteria were stained with crystal violet and tested in slide agglutination test to visualize the reactivity in naked eye and under microscope. Results of slide agglutination test showed that the colored vaccine bacteria and field isolates clumped with 1:20 dilution of antisera, as seen in naked eye. Under microscopic investigation of slide agglutination test, the reaction was read up to 1:100 dilutions (Figure 3). *B. anthracis* bacteria stained with crystal violet showed visible slide agglutination test than unstained bacteria and control sear. At room temperature, the clumping was graded as +++ = agglutination within 5mins, ++ = agglutination within 10mins, + = agglutination within 15mins, - = absence of agglutination even after 15mins. The agglutination reaction was seen in naked eye up to 1:20 dilution and under microscopic fields up to 1:100 dilutions (Table 2). Each of the test reaction was compared with negative test a serum which was collected from non-vaccinated control mice. There was absence of clumping of the bacteria while tested with negative test sera. This agglutination test proved that the test sera contained specific antibody against anthrax antigen which is absent in negative test sera. This study also provided evidence that the antibody maintained in mice against the vaccine strain reacts equally with the vaccine strain and field isolates of anthrax antigen. Interestingly, the bacteria forms spore while stained with crystal violet and used in slide agglutination test.

DISCUSSIONS

This research work was designed to develop color antigen of *B. anthracis* and anthrax bacteria specific antisera in mice. Both the colored anthrax antigen and serum was used in slide agglutination test to enhance naked eye and microscopic visualization of *B. anthracis* in the field where most sophisticated laboratory facilities are absent to detect field cases of anthrax. It is not difficult to grow *B. anthracis* from field and biological samples but to achieve confirmatory detection, the diagnosis requires specialized techniques.

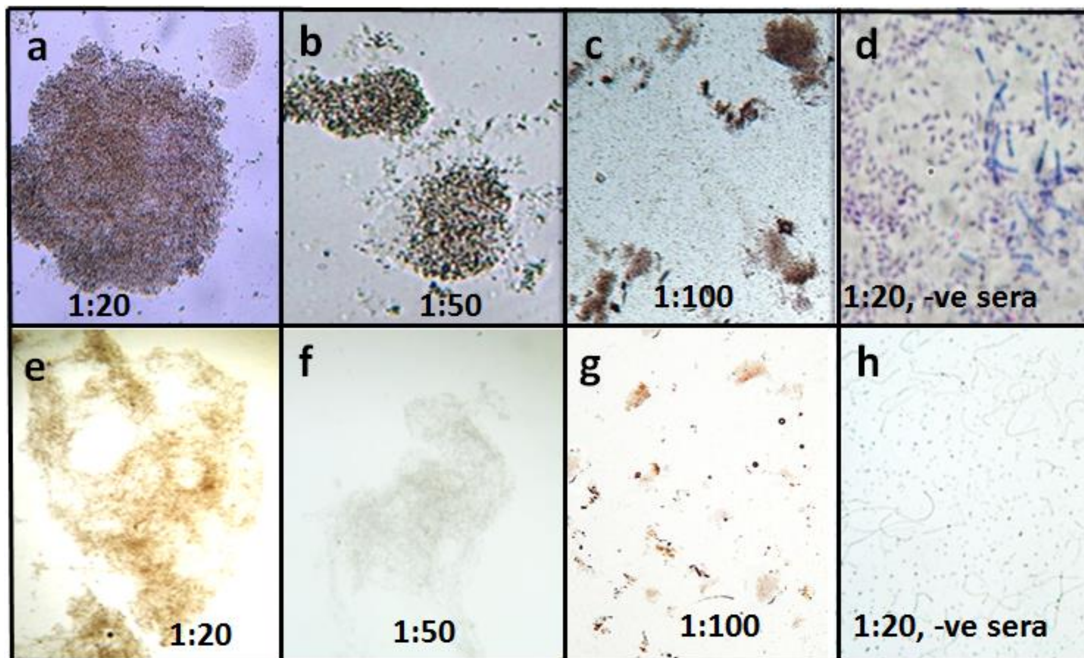

Figure 3. Results of slide agglutination tests with the antisera collected on day 30 of immunization and clumping reaction was detected under a microscope (10x). The colored anthrax antigens (a, b, and c) were clumped with bovine anti-sera at 1:20, 1:50 and 1: 100 dilutions but the intensities was higher at lower dilution. The anthrax antigens lacking crystal violet color (e, f and g) clumped with the mice antisera but the reaction was less visible under microscope compared to colored antigens. The colored (d) and non-colored (h) anthrax antigen did not clump with the control mice sera.

Table 2. Dilution of mice antisera found to clump colored *B. anthracis* in slide agglutination test. 1:100 dilution of antisera yielded slide agglutination test up to day 180 of immunization. Both the colored vaccine bacteria and field isolates showed similar slide agglutination properties while examined under microscope. The results were interpreted by using + or – symbols. The black + results are for naked eye observation and red + for microscopic observation. +++ is indicative for agglutination reaction within 5mins of reaction, ++ is indicative for agglutination reaction within 10mins, + for the agglutination reaction within 15mins and – is indicative for the absence of agglutination even after 15mins

Dilution of Sera	Days of immunization					
	Day 0	Day 30	Day 60	Day 120	Day 240	Day 365
1:10	-	+	+++	++	+	+
1:20		+	+++	++	+	+
1:50	-	+	++	++	+	+
1:100	-	+	+	+	+	-
1:200	-	-	+	-	-	-
1:500	-	-	-	-	-	-

Interpretation of results:
+++ = agglutination within 5mins; **++** = agglutination within 7mins; **+** = agglutination within 10mins; **-** = absence of agglutination even after 15mins; **+** = indicate positive during naked eye observation; **+** = indicate positive test result under microscopic observation

Characterization of field isolates of *B. anthracis* and F-34 strain

The method used for the isolation of Anthrax spores from environmental and infected samples in this study, was that described in OIE Terrestrial Manual (OIE, 2012) with required modifications. For culturing and isolation of *B. anthracis* bacteria the PLET medium and sheep blood agar plate were used. The bacteria grew slowly on PLET agar and rapidly on sheep blood agar. The colonies were white, slightly opaque, a pasty consistency, non-haemolytic and margins slightly indented give the typical appearance to "*caput medusae*" (Fasanella *et al*., 2008). However, the isolation of the bacteria from the soil is much more difficult than text books recount due to the presence of telluric contaminants such as yeasts and bacteria, especially spore-formers, closely related to *B. anthracis,* such as *B. thuringiensis, B. cereus, B. mycoides (Saha et al., 2020;* Turnbull, 1999*)*. The conflicting presence of contaminating bacteria makes it necessary to heat treat a sample to reduce the vegetative forms of this microbial load (Dragon and Rennie, 1995). However, heat treatment is ineffective against spores closely related to *B. anthracis*, and this necessitates the use of selective medium (Marston *et al.,* 2008). Dragon and Rennie (2001) have shown that a selective culture medium is crucial when isolating *B. anthracis* from environmental samples. The "PLET medium" (Polymyxin, lysozyme, EDTA, thallous acetate) and subsequently the Anthracis Chromogenic Agar (CHRA, Marston *et al.,* 2008) were considered as semi-selective media as those were able to inhibit the growth of several saprophytic rods and encourage those bacilli belonging to the Cereus group.

In this study, *B. anthracis* like bacteria were isolated on PLET agar medium in two cases and that was hemolytic in blood agar plates, non *B. anthracis* and was discarded. This study revealed that the PLET agar medium is not truly selective for *B. anthracis* allow some other bacteria to grow and were semi-selective (Fasanella *et al.,* 2008). Out of 13 field samples grown on PLET agar, *B. anthracis* was isolated from 5 infected cattle and Sterne F-34 strain of *B. anthracis* from the vaccine vial. All of the 13 samples showed bacterial growth on PLET agar medium. The field sample while directly grown on PLET agar medium, streptococcal and staphylococcal growth was observed in all 13 cases. The physical properties of the bacteria from culture were demonstrated by using Gram's staining and polychrome methylene blue staining. A special isolation technique was, therefore, adapted to grow *B. anthracis* on culture (Saha et al., 2020).

The anthrax bacteria grown on medium containing carbonate buffer help to acquire capsule and the detection of capsule could be a supportive evidence of differential diagnosis of *B. anthracis* from *B. cereus* group of bacteria. The virulent strains of *B. anthracis* are encapsulated and causes death of humans and animals by producing capsule labeled various toxins, including the lethal factor, the protective antigen, and the edema factor (Relf *et al.,* 1994). The poly-D-glutamic acid capsule and the toxins are encoded by genes present on two large plasmids, designated pXO2 and pXO1, respectively. Once the bacterial growth resembles *B. anthracis* type, they were further confirmed by PCR techniques.

PCR detection of *B. anthracis*

Identification of *B. anthracis* by molecular based methods including PCR reported to be a rapid and sensitive molecular test protocol (Daffonchio *et al.* 1999; Ellerbrok *et al*, 2002; Patra *et al*, 1996; Radnedge *et al*, 2003). In this study, a total of 13 field samples and one vaccinal strain grown on agar medium were identified using PCR. Both the fragments of pX01 gene (596bp) and pX02 gene (777bp) and were amplified from five field isolates. Rest of the field samples did not amplify any fragment of pX01 and pX02 gene in PCR. pX01 gene (596bp) alone was amplified from the vaccinal strain (Figure 2). The PCR protocols adapted in this study appeared very sensitive and specific to detect field isolates of B. anthrais (Saha et al., 2020). According to Kolsto *et al.,* 2009, *B. anthracis* containing two large plasmids that are essential for toxicity: pXO1, which contains the toxin genes, and pXO2, which encodes a capsule. The vaccinal strain lacking capsule and therefore pX02 gene, that's why the test protocol did not amplify any fragment of pX02 gene in PCR. The bacteria identified as *B. anthracis* was therefore used in colored slide agglutination test using known and control sera in mice model.

Slide agglutination test

In this study, colored slide agglutination test was used to detect anti-anthrax antibodies and *B. anthracis*. Slide agglutination tests were designed with the sera from mice raised against anthrax Sterne strain F-34 vaccine. The slide agglutination tests of the isolated bacteria were carried out with 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500 dilutions of antisera in PBS. Both the vaccine strain and virulent *B. anthracis* (n=05) bacteria were used in slide agglutination test. The clumping of bacteria with the antisera as seen in naked eye was at 1:10 and 1:20 dilutions and under microscope up to 1:200 dilution. Mice immunized with the vaccine were maintained until six-month post immunization and the anti-anthrax antibodies were detected until six months of immunization (Table 2). Such response was not seen with the sera collected from non-immunized mice sera. Previously anti-anthrax sera raised in cattle were used to detect *B. anthracis* vaccine bacteria. The positive and control sera were also collected from cattle and used in slide agglutination test. About 30% of control sera showed positive reactivity to the vaccine strain (Zohora *et al.,* 2012) and field isolates, indicated non-specific reactivity of polyclonal antisera. This may raise issues whether the anti-anthrax antibody response seen in non-immunized cattle was due to anti-anthrax antibodies or antibodies against *B. cereus* group of bacteria; the specificity of slide agglutination or ELISA test with the polyclonal sera, therefore, required further validation. In this study the vaccinated and control mice was used in laboratory condition, where there was no opportunity to get other bacillus group of bacteria and development of bacillus group specific antibodies, thus the antisera raised were selective for *B. anthracis*. Vaccinated dairy cattle and other farm animals raised in open grazing land reasonably pick up non bacillus bacteria thus there is the development of polyclonal antibodies against bacillus and non-bacillus as well.

CONCLUSIONS

A total of 13 field outbreaks of anthrax were investigated including a vaccine bacteria. Out of 13 suspected field outbreaks investigated, bacillus type of bacteria was isolated in PLET medium from seven cases. Results of morphological and PCR assay revealed that five cases were *B. anthracis* and two were non *B. anthracis*. PLET agar medium appeared semi-selective to isolate *B. anthracis* in culture. Results of PCR revealed that the field bacteria containing both the pX01 and pX02 genes and the vaccine bacteria containing pX01 gene. Anthrax vaccine bacteria was successfully isolated in culture, the vaccine was used to raise anti-anthrax antibodies in mice model. The immunogenicity of the Anthrax vaccine Sterne strain F-34 was evaluated in mice model. The duration and level of immune response (OD value) in mice was evaluated by using slide agglutination test. The *B. anthracis* specific antibody response was detected following 30 days of immunization, reached its peak in day 60 and maintained until six months of immunization. Mice anti-anthrax sera found to clump field isolates and vaccine bacteria, indicative an effective anti-anthrax response. The colored antigen used in slide agglutination test revealed naked eye clumping reaction at a dilution of 1:20, whereas, colorless *B. anthracis* antigen failed to demonstrate any clumping reaction in naked eye observation. The clumping reaction using the colored and colorless antigen under microscopic fields was observed at the dilution of 1:100 and 1: 50 dilution respectively. The colored antigen we have developed can be used to detect host immunity in field condition under naked eye and light microscopic observation. However, nonspecific reactivity of the anti-anthrax antibodies due to other Bacillus group of bacteria need to evaluated further before field applicability of the test protocol.

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CONFLICT OF INTEREST

The authors have no affiliations with or involvement in any organization or entity with any financial and non-financial interest in the subject matter or materials stated in this manuscript.

AUTHOR'S CONTRIBUTION

P.C.S.: Carried out the whole experiment for his PhD and writing up the draft manuscript. **H.E.J**. Help to carry out research works and writing the manuscript **T.R**.: Help to carry out the research work, analyzing data and writing the manuscript. **U.K.R**.: Help to carry out research work, and writing the manuscript. M.S.I.: Collection of samples from the field, writing up the manuscript. **G.A.C**.: Collection of samples from the field, processing of tissues for analysis. **M.A.H**.: Contributed to sample collection and shipment to the laboratory of Pathology, BAU. **M.Z.H**.: Analyzing research materials in the lab, writing up the manuscript. **P.M.D**.: PhD Co-Supervisor of PCS, **M.A.H.N.A.K**.: PhD supervisor, Hunt the research grant, design and help in implementing the research work, final manuscript writing and completing.

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