J Bangladesh Agril Univ 18(2): 405–414, 2020 https://doi.org/10.5455/JBAU.95978

ISSN 1810-3030 (Print) 2408-8684 (Online)

Journal of Bangladesh Agricultural University

Journal home page: http://baures.bau.edu.bd/jbau

The F-34 Stern strain Anthrax vaccine induced higher level of anti-anthrax IgG antibody response and appeared protective in mice model

Provat Chandra Saha[1], Tahomina Ruba[2], Umme Kulsum Rima[3], Mohammad Shahidul Islam[1], Golam Azam Chowdhury[1], Mohammed Ahasan Habib[1], Mohammad Zakir Hossain[1], Priya Mohan Das[2], Mohammad Abu Hadi Noor Ali Khan[2]✉

[1]Department of Livestock Services, Ministry of Fisheries and Livestock, Bangladesh
[2]Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[3]Department of Medicine, Surgery and Obstetrics, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh

ARTICLE INFO

Article history:
Received: 15 April 2020
Accepted: 21 May 2020
Published: 30 June 2020

Keywords:
B. anthracis,
Vaccine efficacy,
Immunity,
Sterne strain,
Experimental mice

Correspondence:
M. Abu Hadi Noor Ali Khan
✉: hadi.khan@bau.edu.bd

ABSTRACT

Anthrax is a fatal septicemic disease of warm-blooded animals including human and caused by *Bacillus anthracis*. Anthrax is common in farm ruminants, causes regular mortality and the disease is commonly prevented by using vaccine. The Sterne strain F-34 vaccine has been using in ruminants for more than five decades in Bangladesh but there is little work describing the efficacy of the vaccine in laboratory or farm animal's. This study was aimed to evaluate efficacy of F-34 Sterne strain anthrax vaccine in mice model and isolate *B. anthracis* in culture from field outbreaks to identify level of protection in vaccinated mice. Anthrax vaccine containing F-34 Stern strain was obtained from Livestock Research Institute (LRI), Mohakhali, Dhaka. Group C and D female mice were immunized subcutaneously with 0.1ml of vaccine and Group A and B mice were served as non-immunized control. Polymerase chain reaction (PCR) technique was carried out to detect pX01 (210bp) and pX02 plasmid (1035bp) of *B. anthracis* from the field isolates, vaccine bacteria and challenged mice. Out of 13 field samples tested, *B. anthracis* was isolated from 05 cases. Following 6 months of immunization the Group B and Group D mice were challenged intraperitoneally with $2x10^5$ colony forming unit (CFU) of virulent field isolate of *B. anthracis* (isolate of Shahjadpur Upazila, Sirajganj). The vaccine efficacy was evaluated in terms of anti-anthrax IgG antibodies responses in ELISA (A^{450}±SD) and protection to challenge *in vivo*. The early anti-anthrax IgG antibody response was detected in Group C and D mice following week 2 of immunization (0.501±0.167) and reached its peak in study week 4 (1.237±0.257). A steadily higher level of anti-anthrax IgG antibody response was detected until 180 days of study (1.269±0.217, group average±SD). *In vivo* challenge to vaccinated mice with the virulent *B. anthracis* bacteria found to confer solid protection following six months of immunization. Non-immunized mice challenged with field isolate of *B. anthracis* succumbed to death within 18-24hours of infection, showed characteristics lesions of anthrax including black berry jam spleen, wide spread congestion and hemorrhages and bleeding through natural opening after death. *B. anthracis* was re-isolated from the visceral organs of experimentally infected mice. This study provide evidence that the Sterne strain F-34 Anthrax vaccine is protective in mice model and generated higher level of anti-anthrax IgG antibody response until day 180 of immunization. It needs to study whether the vaccine is effective in farm animal model with longer duration.

Introduction

The causative agent of anthrax is *Bacillus anthracis*, a Gram positive, aerobic (facultative anaerobic), spore enduring soil bacterium of the genus bacillus (Baillie & Read, 2001; Wang *et al.,* 2013). *B. anthracis* is extremely a zoonotic pathogen, distributed globally and one of the pathogen which is convenient to class within the *B cereus* group (Turnbull, 1999). The organism possesses two virulence factors; the capsule and the anthrax toxin. Both virulent factors are encoded by genes located in plasmids; pX01 (toxin genes, a 185 kb plasmid, Mikesell *et al*. 1983); and pX02 (capsule genes, a 95kb plasmid, Green *et al*. 1985; Uchida *et al*. 1985). Therefore, the pX01 and pX02 genes are commonly targeted to achieve diagnosis by using polymerase chain reaction (PCR) technique. Vaccination is the best known and most successful principle by generating immunity against the virulent toxins to protect human and animal health. Different types of antigens were used as vaccines but the live vaccines found to be more effective than the killed formulation (Roit *et al.,* 2001). Cell free filtrate of the culture of *B. anthracis* while used to immunize rabbit with adjuvant, they produce higher level of humoral immunity and protect challenge to infection (Ionin *et al*., 2013). However, in experimental animals such vaccines are less protective than whole cell

Cite this article

Saha, P.C., Ruba, T., Rima, U.K., Islam, M.S., Chowdhury, G.A., Habib, M.A., Hossain, M.Z., Das, P.M., Khan, M.A.H.N.A. 2020. The F-34 Stern strain Anthrax vaccine induced higher level of anti-anthrax IgG antibody response and appeared protective in mice model. *Journal of Bangladesh Agricultural University*, 18(2): 405–414. https://doi.org/10.5455/JBAU.95978

vaccine. The formaldehyde inactivated spores (FIS) of *B. anthracis* elicited higher level of antibodies and yielded total protection against challenge with virulent *B. anthracis* strains in mice and guinea pigs. Acellular or subunit vaccine is less efficient than whole cell vaccine. Guinea pigs and mice had very different susceptibilities to infection with the nontoxigenic strain but are sensitive to toxigenic strain of *B. anthracis* (Brossier *et al*., 2002) highlighting the importance of verifying the pertinence of animal models for evaluating anthrax vaccines.

Several strains of Anthrax bacteria have been used in the field to protect animals from natural outbreaks of Anthrax, including 'Carbosap' vaccine strain, 'Pasteur' vaccine strain SS104 and 'Sterne' vaccine strain F-34 (Fasanella *et al*., 2001). Consequently, an improved vaccine was developed for livestock using Stern strain F-34, a live un-encapsulated avirulent variant of *B. anthracis*. Since then, this vaccine served as the principal veterinary vaccine to protect animals from anthrax (Sterne, 1959). Few of the reports describing immunity to Anthrax Stern strain vaccine in goats (Dipti *et al*., 2013) and cattle (Hassan *et al*., 2015; Zohora *et al*., 2012). The protective efficacy of the vaccine was evaluated in rabbit and mice model in abroad (Brossier *et al*., 2002) but their protective efficacy is not studied *in vivo* since its introduction in Bangladesh. Moreover, it seems necessary to characterize the vaccine strain by its morphological, biochemical and molecular means and compare the antigenic differences between the vaccine strain and field isolates of *B. anthracis*. This study was, therefore, aimed to isolate field pathogen and evaluate protective efficacy of the vaccine in mice model. The most sensitive animal model to study the protective efficacy of the vaccine are rabbit, guinea pig and mice (Ionin *et al*., 2013). As introduction of challenge infection in large animal model is a bit more difficult because the organism is extremely zoonotic, involving massive funding to maintain and dispose experimental animals, require to decontaminate wider areas, even the organism may persist in nature after decontamination and may act as source of infection. Therefore, this study was, designed to investigate experimental anthrax in mice model. The virulent *B. anthraces* from the infected and dead animals was isolated in culture and introduce challenge infection in mice model; an easy laboratory model to evaluate the pathogenicity of the isolates and protective efficacy of the vaccine *in vivo*.

Materials and Methods

Isolation of B. anthraces in culture and preparation of antigens

Isolation and identification of field isolates of *B. anthracis* was carried out from a total of 13 suspected animal cases in Bangladesh during the period between May 2013 to April 2015. The samples was collected from two infected areas of Tangail sadar, Tangail, Shahjadpur Upazila, Sirajganj and Srimongal Upazila, Moulvibazar. Samples were collected from an infected area of Madhupur Upazila, Tangail, Gopalpur Upazila, Tangail, Ghatail Upazila, Tangail, Dhanbari Upazila, Tangail, Belkuchi Upazila, Sirajganj, Barisal sadar, Barisal and Moulvibazar sadar, Moulvibazar. Cattle suddenly died and discharging tarry colored blood through the natural opening constituted the study materials. The turbinate bone and discharges through rectal opening was collected in sterile vials and transported to the laboratory, Department of Pathology, Bangladesh Agricultural University, Mymensingh. About 1.0gm of turbinate bone or discharges from the dead cattle was crushed on sterile pestle and mortar with 5ml sterile distilled water under a biosafety cabinet level 2 (BSL-2). The suspension was heated in a thermal block at 90℃ for 05mins to kill contaminating bacteria and other vegetative pathogens. 100µl suspension in 10ml nutrient broth containing 0.05mg/ml Fungizone (amphotericin B, Thermo Fisher Scientific INC, NY) was incubated at 37°C for 12hrs with shaking (160rpm). About 1ml of bacterial growth in nutrient broth in eppendorf tube was centrifuged at 10000 x g for 5 minutes and sediment was smeared on glass slides, stained with Gram's iodine to visualize the growth of bacteria. In positive cases the bacterial growth in nutrient broth was, therefore, used to isolate *B. anthraces* on polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar and sheep blood agar media. To isolate vaccine strain of *B. anthracis* (Sterne strain F-34), a vaccine vial (100ml) was collected from the Livestock Research Institute (LRI), Mohakhali, Dhaka, Bangladesh on July 2013. About 1.0ml of vaccine formulation was taken into an eppendorf tube and centrifuged at 10000 x g for 5mins. The supernatant was discarded and the pellet was washed twice with sterile distilled water by centrifugation at 10000 x g for 5mins. The supernatant was discarded and 100µl sterile distilled water was added to the tube. Using a bacteriological loop the broth was used to grow on sheep blood agar plate at 37°C for 12 hrs. Once the bacterial growth was seen on culture, the bacterial colony was stained with Gram's iodine (Luna, 1968) and examined under microscope to observe the morphology of the bacteria. PX01 and PX02 plasmid specific PCR was carried out to identify species of *B. anthraces*. The field bacterial isolates of *B. anthraces* was used in antigen preparation and to evaluate protective efficacy of the vaccine against challenge infection in mice model. The name of the isolates designated as BD_Vac_2014 (vaccine bacteria, F-34 Stern strain) and FI SPUR_2014 (virulent field isolate of *B. anthracis*) with the Genbank accession number of KT995458 and KT893481 respectively. The virulent field isolate used in this study was collected and characterize from Shahjadpur Upazila, Sirajganj district, Bangladesh.

Immunization of mice

A total of 20 apparently healthy 6-week old female mice obtained from icddr,b Mohakhali, Dhaka, Bangladesh was used in this experiment. They were divided into groups of four; Group A (Negative Control), Group B (non-immunized & Challenged), Group C (immunized

& Un-challenged) and Group D (immunized & Challenged). The mice were fed with pelleted feed and fresh drinking water ad libitum. The freshly prepared anthrax vaccine (100ml) bottle was collected from the Vaccine production unit, LRI, Mohakhali, Dhaka, Bangladesh. Group C and D mice were immunized with 0.1ml Sterne strain vaccine anthrax vaccine through subcutaneous (s.c) route; Group A and B mice served as non-immunized control. Tail bleed (100µl/mice) from the vaccinated and control mice were collected on day 0, 15, 30, 60 and 180 of immunization in the heparinized capillary tubes, plasma was separated by centrifuging the tube at 3000rpm for 5mins. The plasma was collected in the eppendorf tubes, preserved at -20ºC before an indirect ELISA was carried out.

Protective efficacy of the vaccine

Following six months of immunization, Group B and D mice were challenged intra-peritoneally with $2x10^5$ CFU (Weiner *et al*., 2012) of the virulent *B. anthracis* (isolated from Shahjadpur Upazila, Sirajganj) and examined for the morbidity and mortality. The infected and dead mice obtained within 18-24hours of infection, were necropsied immediately after death of the mice. The non-infected and healthy mice and vaccinated mice challenged with the virulent field isolate of *B. anthracis* were sacrificed, examined at necropsy following 15 days of challenge. Detailed histopathological examination of the visceral organs of all mice was carried out. The clinical signs and morbidity and mortality rate seen were examined and documented with care.

Immunoefficacy of anthrax vaccine

Plasma anti-anthrax IgG antibody response was detected in this study using an indirect enzyme linked immuno sorbent assay (iELISA). The plate coating antigen was prepared with the bacterial isolates grown on sheep blood agar plate. Briefly 12-15 loop-full isolated colonies of *B. anthraces* from blood agar plate (18hrs of culture) was collected and suspended in 7ml PBS, pH 7.4 in an Eppendorf tube. 20 sterile cover slips was crushed on pestle and mortar and the crushed cover slips was added in the tube containing bacterial suspension. Tube containing Anthrax bacteria and coverslips were slowly vortexed for 30 minutes to lyse the cells, help liberating bacterial antigens and allowed to stand for a minute. The supernatant was used as Anthrax plate coating antigen in an iELISA. The plate coating antigen 100µl/ well was added in the 96 well tissue culture plate (Cell star, Greiner bio-one, Austria). The plate was incubated at 37°C for an hour and the plate was washed 3x with PBS (pH7.4) Tween 20 (0.05%). The left over charged sites in the well was blocked by incubating 90mins at 37°C with 150µl/well of 3% Bovine serum albumin containing 0.05% Tween-20. The plate was washed 3x with PBS Tween 20 (0.05%) and incubated at 37°C with 100µl/well, 1:500 dilution (in PBS) of anti-sera. As control, non-immunized diluted sera (100µl/well) was added in the first three antigen coated well of 96 well cell culture micro-plate. The plate was washed 3x with PBS and incubated at 37°C for 45mins with 100µl/well 1:500 dilution of goat anti-mouse IgG (H and L) tagged with HRP (BIO-RAD, UK). The plate was washed 3x with PBS, enzyme reactivity was developed with 100µl per well TMB solution (Zohora *et al*., 2012), the reaction was terminated with 50µl/well 10% H_2SO_4 and the reaction was read at 450nm in an ELISA plate reader. A 450nm read of 0.239±0.093 was consider as base line value and OD value at or above 0.400 was considered as positive response. The repeated measures ANOVA followed by Duncan's Multiple Range Test was used to analyze difference of responses (OD values) between and among the groups of mice investigated. A p-value less than 0.05 (typically ≤ 0.05) was considered as statistically significant and used in this study.

Necropsy of mice and detection of experimental anthrax

Mice belonging to Group B were died following 18 to 24 hours of challenge and a systemic investigation was carried out. Mice belonging to Group A, C and D remain unaffected and sacrificed following day 15 of challenge. A systemic examination was carried out and characteristics changes observed were discharges of tarry color blood through natural orifices after death. Smears with the peritoneal fluid and from the liver and spleen of infected mice were made on to clean slide and stained with Gram's iodine to visualize the bacteria (Luna, 1968). Representative tissues from various organs were collected in 10% buffered neutral formalin to study the pathology produced in tissue using H&E and Grams staining (Jahan *et al*., 2019; Luna, 1968). Part of liver and spleen was collected aseptically and preserved at -20^0C to extract genomic DNA and PCR detection of *B. anthracis*. Tissues of the Group A and C mice were also evaluated to compare the differences. The experimental mice after infection was disposed by decontaminating in 10% formalin and the cages and sheds were decontaminated twice by spraying formalin and potassium permanganate solution (35mL of 40 % formaldehyde to 10g potassium permanganate for per cubic meter of space). The experimental room was allowed 24 hours for the vapor to penetrate all the surfaces.

Goldner's trichrome staining of tissue section

For better demonstration of organism in tissues Goldner's trichrome staining was carried out. Paraffin embedded sections of lungs, liver, kidney, spleen, heart, stomach, intestine, and muscle of infected and control mice were deparaffinized and stained with Goldner's trichrome staining (Khan *et al*., 2008). The stained sections were cleared in xylene and mounted using DPX. Changes in the tissues and identification of Gram +ve rod was identified under low (10x) and high powers (40x and 100x) microscopic fields.

PCR detection of B. anthraces

A known positive sample obtained from the central diseases investigation laboratory (CDIL), Kazi Alauddin Road, Fulbaria, Dhaka was used in this study. The bacteria was isolated from the known positive sample, characterized, genomic DNA from the isolated bacteria

and tissues were extracted and pX01 and pX02 gene specific PCR was carried out to ensure the specific cause of anthrax (*B. anthracis*). This sample was used as known positive control in PCR settings. Genomic DNA from the isolated bacteria and from the spleen, liver, heart and lungs of experimental mice was extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). Qualitative and quantitative assessment of the extracted DNA were made out by using 1.5% agarose gel electrophoresis and spectrophotometry (A^{260}/A^{280}). The concentration of the extracted DNA was measured using standard protocol (Bhat *et al*., 2010) and the concentration of extracted DNA detected were between 200 to 250ng/µl. The DNA solution was stored at -20°C until used in PCR. The PCR was carried out with 25µl reaction volume consisting of 2x PCR Master Mix (Promega Corporation, USA), 20pmol primers of each, 250-300nmol DNA template and nuclease free H_2O. PCR amplification was carried out in a thermal cycler (Master Cycler Gradient, Eppendorf, Germany) with an initial denaturation at 95°C for 5min followed by 30cycle of amplification reactions including denaturation at 94°C for 1min, annealing at 52°C for 90secs and extension at 72°C for 90secs. The final extension was carried out at 72°C for 10min and the program was held at 4°C. The amplified product were analyzed in 1.5% agarose gel, visualized in an image documentation system (Cell Biosciences, Alphalmager HP, USA) and the images were captured.

Results

Mice tolerated well the immunization and did not suffer from agitation, distresses and or off feed. The vaccinated mice receiving challenged infection with the field isolate of virulent *B. anthracis* appeared to feed and drink well. The vaccine bacteria and field isolates of *B. anthracis* used in this study were characteristics. None of the mice appeared dead during the vaccination and infection trials.

Characterization of field isolates of B. anthracis

Out of 13 field samples collected and tested, *B. anthracis* was isolated from five cases (Shahjadpur Upazila, Sirajganj, Tangail sadar, Tangail, Dhanbari Upazila, Tangail, Ghatail Upazila, Tangail and Srimongal Upazila, Moulvibazar). The bacteria was isolated from five turbinate bones and three natural discharges of suspected cattle. Prior incubating the inoculum in nutrient broth, the samples was preheated at 90^0C for 05mins that allowed survival and growth of spore forming microbes only. In nutrient broth and blood agar plate antifungal agent was used, that prevent growth of contaminating fungus, without which, fungal growth was seen in four cases. The bacterial growth in nutrient broth were further grown on PLET agar and sheep blood agar medium.

Table 1. Oligonucleotide primers used in PCR amplification and detection of the fragment of Cap and PA genes present on pX01 and pX02 plasmid of *B. anthracis* (Beyer *et al.,* 1995)

Gene	Primers	Sequences (5´-3´)	Expected Amplicon Size (bp)
Cap gene	CAP6	TACTGACGAGGAGCAACCGA	1035
	CAPI03	GGCTCAGTGTAACTCCTAAT	
PA gene	PA7	ATCACCAGAGGCAAGACACCC	210
	PA6	ACCAATATCAAAGAACGACGC	

Fig. 1. *B. anthraces* bacteria grown on PLET agar and blood agar (a) medium. The bacteria on blood agar plate produce colonies that were non-hemolytic, slightly convex and were ground-glass in appearance (b). Following Gram's staining, the bacteria stained blue (Gram +ve, 40x), arrange in single or in short chain (c, inset, 100x).

In PLET agar medium a typical growth of the organism was seen, which was roughly circular and creamy white with ground glass appearance, characteristics of *B. anthracis* (Fig. 1a). On sheep blood agar plate, typical rough, sticky, white-gray non hemolytic colonies (Fig. 1b) were produced. The isolated bacterial smear onto slides was stained with Gram's iodine showed violet color bacteria (Gram +ve) containing spores (Fig. 1c). The bacteria arranged in short chain consisting mostly of 2 to 3 rods and individual rod showed characteristics "comma in appearance" (Fig. 1c, inset).

Anti-anthrax antibody response

Results of iELISA showed an early anti-anthrax IgG antibody response (OD value) in Groups C (0.441±0.135) and Group D (0.501±0.167) mice following week 2 of immunization. Anti-anthrax IgG antibody response reached its peak in Group C (1.139±0.215) and D (1.237±0.257) mice following

week 4 of immunization. The steadily higher level of anti-anthrax IgG antibody response was detected until day 180 in Group C and D mice (Fig. 2).

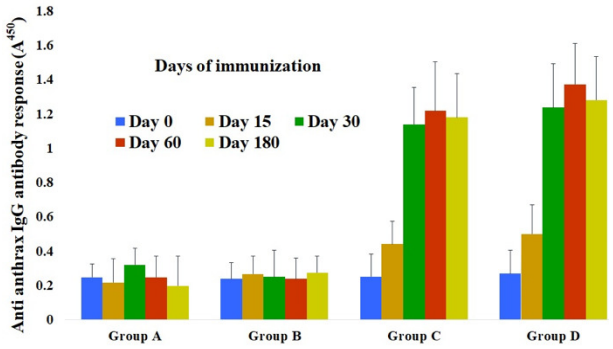

Fig. 2. IgG anti-anthrax antibody response in mice immunized with the F-34 stern strain of anthrax vaccine. The early anti-anthrax IgG antibody response was detected in Group C and D mice (0.501±0.167) following 2 weeks of immunization and reached its peak in study week 4 (1.237±0.257). A steadily higher level of anti-anthrax IgG antibody response 1.239±0.197 and 1.297±0.134) were maintained until 180 days of immunization in Group C and D mice respectively. The non-immunized mice did not develop any anti-anthrax IgG antibody response (Group A and B) throughout the study.

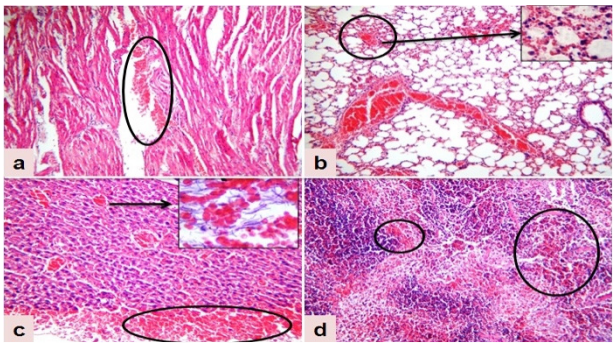

Fig. 3. Sections of heart (a), lungs (b), liver (c) and spleen (d) of experimentally infected mice and staining with H&E (10x). Wide spread congestion and hemorrhages (black circle) was seen throughout the visceral organs.

The iELISA carried out with the un-vaccinated plasma lacked anti-anthrax antibody response throughout the study and the OD value observed in iELISA remained below the base line value. There was significant difference of response ($p< 0.001$, 0.000, 0.000 and 0.000) between the vaccinated and control groups of mice following day 15, 30, 60 and 180 days of immunization, respectively.

Protective efficacy of the vaccine

Non-immunized mice (Group B) challenged with $2x10^5$ CFU of the virulent field isolates of *B. anthracis* were died within 18-24hours of infection. Mice showed per-acute to acute illness and clinical signs observed were staggering gait, decreased mobility and tachypnea and ending with deep agonal breathing. Smears on to the clean slides stained with Gram's iodine showed Gram +ve rod but the densities were higher in spleen followed by lungs, liver, heart and peritoneal fluid. On the other hand, vaccinated mice (Group D) challenged with virulent field isolate of *B. anthraces* remained healthy until day 15 of challenge. Non-infected control (Group A) and immunized but unchallenged (Group C) mice were also sacrificed following day 15 of challenged to compare the differences. From the visceral organ (spleen) of control, infected and dead mice, DNA was extracted and detected *B. anthracis* by using PCR.

Necropsy of experimental anthrax in mice

At necropsy, infected mice (Group B) revealed wide spread congestion and hemorrhages throughout the body but the intensity was higher in the lungs, meninges, gastrointestinal tract, liver, kidney and spleen. Noticeable splenic changes observed was overall enlargement with rounded edges, giving it a plump appearance, dark red to black color, and a friable parenchyma on sectioning (historically referred to as blackberry jam spleen). Wide spread hemorrhages and necrosis was seen in the intestinal mucosa and the lumen of intestine was filed up with blood. Changes were not seen in the visceral organs of non-infected mice (Group A), vaccinated but un-challenged (Group C) and vaccinated and challenged (Group D) group of mice.

Microscopic changes of anthrax in tissue

Microscopically, infected mice displayed severe bacteremia, with bacilli frequently noted within blood vessels of the lung. Widespread congestion and hemorrhages was seen in heart muscle (Fig. 3a). The extensive changes were seen in the interstitial tissue of lungs, the lungs alveoli were distended and ruptured. There was massive congestion and hemorrhages in the lung parenchyma with infiltrations of neutrophils and macrophages (Fig. 3b). In liver, the sinusoids and central veins were drawn with blood and exudates. Rod shaped bacilli was seen in the central veins and sinusoids of liver (Fig. 3c). Numerous bacilli and hemorrhages were present within glomeruli and interstitial tissues of kidney. Splenic lesions showed massive congestion and hemorrhages (Fig. 3d) with lymphocytic destruction, tensed capsule with anthrax bacilli within hemorrhagic masses. Wide spread congestions, hemorrhages and necrosis was seen in the gastro intestinal mucosa. Section of the liver, spleen, heart, kidney, lungs stained with Grams iodine showed blue color gram +ve rod (Fig. 4) in Group C mice, which however was absent in other groups of mice. Section of the visceral organs while stained with Goldner's trichrome stain, the bacteria stains lightly but the capsule stained intensely (Fig. 5b and c) and such staining can be used in the detection of anthrax bacilli in tissues.

PCR detection of B anthraces

Plasmid DNA from the vaccine and field isolates and DNA from experimentally infected mice (spleen, liver, heart and lungs) were used to amplify fragments of

pX01 and pX02 plasmid of *B. anthraces*. Out of 13 field isolates and vaccine strain tested in PCR, pX01 plasmid specific amplification (210bp) was seen in 05 field cases (Fig. 6a) and from a vaccine bacteria.

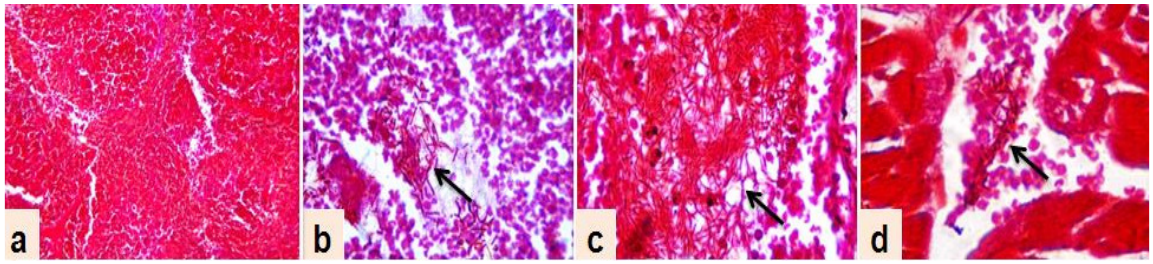

Fig. 4. Section of spleen (b), liver (c) and heart (d) from experimental anthrax of mice stained with Gram's iodine (40x). Violet color rod shaped bacteria (black arrow) was seen in the sections of spleen, liver and heart of infected mice. Gram +ve rod was absent in the control section (a).

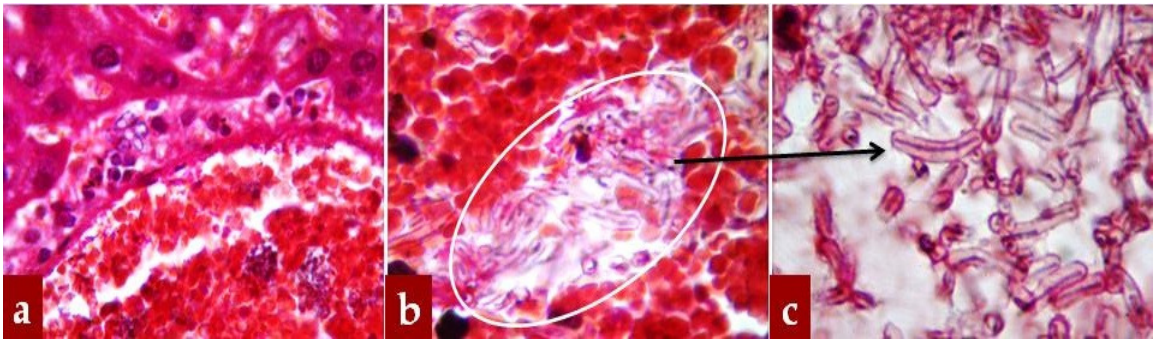

Fig. 5. Goldner's trichrome staining of the sections of liver from experimentally infected mice (Group B). Rod shaped *B. anthracis* bacteria was seen in liver (b, circle, 40x) which however was absent in control section (a, 40x). The bacteria showed light purple color capsules (c, black arrow) as seen under high power microscopic field (200x).

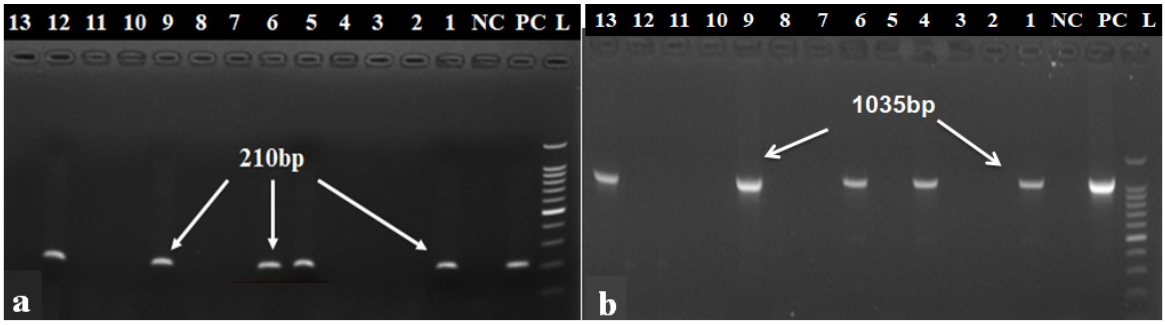

Fig. 6. The uniplex PCR detection of pX01 (210bp) and pXO2 (1035bp) genes of *B. anthracis* (lane 1 to 13). The lane L1= 100bp DNA ladder; Lane PC= Positive control; Lane NC= Negative control. PCR amplification of the fragment of pX01 and pX02 gene were seen in lane 1, 4, 6, 9 and 13 indicating positive field samples containing *B. anthracis* bacteria.

The pX02 plasmid specific amplification (1035bp) was only seen in 05 field isolates (Fig. 6b) and was absent in the vaccine bacteria. The pX01 and pX02 plasmid specific amplicons in PCR were also detected from the visceral organs of experimentally infected mice (Group B).

Discussion

Anthrax is an economically important disease of ruminant especially in dairy cattle and is characterized by a fatal course leading to sudden death. The bacterial spores are highly resistant, surviving in extreme environmental temperature, has got very low-nutrient environments, protected in harsh chemical exposure and survive in nature over decades. Anthrax spores are extremely pathogenic and upon ingestion, the vegetative bacteria emerged, invade inner tissues and cause maladies (Bartlett *et al.,* 2002). During August 2009–October 2010, a multidisciplinary team investigated a total of 14 outbreaks of animal and human anthrax in Bangladesh. A total of 140 animal cases of anthrax and 273 human cases of cutaneous anthrax were investigated (Chakraborty *et al*., 2012). Since then lots of outbreaks of anthrax in man and animals were reported in Pabna and Sirajgonj districts, important milk zone areas of Bangladesh. The treatment of anthrax in animals is useless in most of the cases and prevention is the choice to control field infectivity. Routinely the disease in

animals is prevented by using bacterial vaccine. There are subunit vaccines in clinical or laboratory trials but their application at field level is yet to accept (Luxembourg *et al*., 2008; Vance *et al*., 2015). The Sterne F-34 strain is the prevailing vaccine, has been using in Bangladesh more than five decades but literature is scanty about their efficacy in vivo. The definite tool(s) require to evaluate vaccine efficacy is also not standardized for anthrax in Bangladesh. There are scanty of literature describing the pathology, serology, immunology, protective efficacy of Sterne F-34 vaccine in Bangladesh (Hassan *et al*., 2015; Zohora *et al.,* 2012), therefore, it needs to evaluate the efficacy of the vaccine in terms of inducing immunity and level of protection following challenge. To evaluate protective efficacy of the vaccine it needs to isolate *B. anthracis* from field cases but it is difficult to isolate due to presence of similar bacteria.

The bacilli similar to *B. anthracis* in nature often creating confusion at diagnostic valley and health hazard to man and animals and hence require differential diagnosis. *B. anthracis* mostly confused with *B. cereus* and *B. thuringiensis,* member of the *Bacillus cereus* group. *B. thuringiensis* is an insect pathogen, and *B. cereus* is known mainly as a food poisoning pathogen characterized by toxin-induced emetic and diarrheic syndromes. More severe infections in human due to *B. cereus* is developed mainly in immunocompromised patients or patients having weaker immune system (Drobniewski, 1993). The life-threatening and fatal cases of pneumonia, enteritis and septicemia can be developed in man and animals following infection with *B. anthracis* (Hoffmaster *et al.,* 2004). The vaccine bacteria is also belonging to bacillus group and bears similar biochemical and physiological properties but incapable of producing disease. The virulent field isolates and vaccine bacteria are not always available to study and evaluate vaccine efficacy due to contagiousness and zoonotic potentiality. Moreover, it requires higher laboratory facilities to isolate and maintain the culture and facilities are not prevailing in most of the labs. Hence field isolate of the bacteria were collected from natural course of infection and grown in culture for the production of experimental diseases in mice model. During the investigation, out of 13 samples tested, two case showed the growth of Gram +ve rod but was hemolytic on sheep blood agar media, this bacteria was suspected as non *B. anthracis* and was discarded.

Isolation of virulent and vaccine bacteria

This study successfully adapted method for the isolation of *B. anthracis* from suspected field samples using standard protocol of Ramisse *et al*. (1996). The bacteria grew slowly on PLET agar medium but grew rapidly on sheep blood agar. The isolated colonies appeared white, slightly opaque, showed a pasty consistency, non-hemolytic and margins were slightly indented gave the typical appearance of "*caput medusae*" (Fasanella *et al.*, 2008). A heat treatment was given to the suspension before culture that appeared effective to remove microbes other than spores of *B. anthracis*. The "PLET medium" and the Anthracis Chromogenic Agar (CHRA, Marston *et al.,* 2008) was suggested as semi-selective media as those were able to limit the growth of several saprophytic rods and encourage those bacilli belonging to the Cereus group. The study used PLET agar as selective media to isolate *B. anthraces* in culture (Fasanella *et al.,* 2008; Knisely, 1966) but the PLET agar did not appear truly selective for *B. anthracis* as some other microbes found to grow during isolation procedures. Out of 13 field samples grown on PLET agar, *B. anthracis* was isolated from 05 infected cattle and Sterne F-34 strain of *B. anthracis* from a vaccine vial. Few other microbes were grown on PLET agar media with *B. anthracis*, these colonies were discarded. Out of five isolates of *B. anthracis* obtained, an isolate of *B. anthraces* bacteria (Shahjadpur Upazila, Sirajganj) was used in experimental disease production in mice model. The isolates were further grown on blood agar medium containing carbonate buffer, the bacteria acquired capsule and the detection of capsule appeared supportive to differentiate *B. anthracis* from *B. cereus* group of bacteria. However, rapid detection of *B. anthracis* was carried out using PCR (Berg *et al.,* 2006; Beyer *et al.,* 1995).

Identification of *B. anthracis* by PCR reported to be a rapid and sensitive molecular test protocol (Ellerbrok *et al*, 2002; Radnedge *et al*, 2003). Both the pX01 (210bp) and pX02 (1035bp) plasmids were targeted and fragments were amplified from five field isolates. The pX01 gene (210bp) alone was amplified from the vaccinal strain. Virulent isolates of *B. anthracis* containing both the pX01 and pX02 plasmids with unique targets that allow the rapid and specific identification of *B. anthracis* in PCR. Similar observation was also reported earlier by Lyons *et al.* (2004). They have used multiplex PCR and detected pXO1 and pXO2 plasmids and the species identified was *B. anthracis*. The CDC and OIE terrestrial manual recommended to use direct challenge infection in laboratory condition and reisolate the bacteria with characteristic pathology from the infected animals. Vaccinated and control mice were injected with $2x10^5$ CFU of field isolates. The non-immunized mice challenged with the bacteria were died within 18-24hours of injection indicating that the isolate of *B. anthracis* was virulent. *B. anthracis* was also reisolated in culture from experimentally infected mice that also supported the recommendation of CDC and OIE. The isolated organism was proven very virulent and used to evaluate vaccine efficacy by injecting virulent bacteria in vaccinated mice.

Immunoefficacy of anthrax vaccine

As with most vaccines, measuring anthrax antibody response provide a direct evidence of vaccine potency but evaluation of antibodies response is an indirect

evidence of the vaccine's ability to prevent disease. Literature available indicated that about 91% of adults receiving two or more doses of Anthrax vaccine showed an immune response and 95% have a four-fold increase in antibodies after three doses. The higher level of anthrax antibodies are thought to protect the vaccine recipient against anthrax, though scientists do not know the precise level of antibody at which protection against anthrax can be assured (CDC, 2000). Virulent *B. anthracis* strains and two animal models were used in immunization and protection trials; (i) guinea pigs, (ii) mice, they were very sensitive to virulent *B. anthracis* and particularly difficult to protect. Mice was, therefore, used in immunization and protection trial (Brossier *et al.,* 2002).

The Anthrax vaccine is an aluminum-containing vaccine that is given under the skin (subcutaneous administration), and hence this route was used in this study. The efficacy of the vaccine in mice was evaluated in terms of anti-Anthrax IgG antibody response and protective efficacy against challenge infection with the virulent field isolate of *B. anthracis*. In this study, anti-anthrax IgG antibody response was detected following week 2 of immunization and a steadily higher level of antibody response was detected until day 180 of immunization. There was insignificant differences in OD values within groups at day 0 of immunization but at Day 15, 30, 60, and 180 of immunization significant differences in OD values between groups of mice was seen. The mean OD values in groups C and D were significantly higher ($p<0.001$ to 0.000) compared with OD values of groups A and B. However, an injection of anthrax vaccine found to confer solid and longer immunity but the boosting was not practiced in this study as the vaccine is routinely used in animal in Bangladesh once in a year. However, it needs extensive study combining the level of humoral and cell mediated immune response and duration of immune response to set up standard protocol to evaluate immunoefficacy of the vaccine, understanding vaccine physiology and its safe use.

Protective immunity

Researchers demonstrated that inoculation of animals with attenuated strains of *B. anthracis* led to protection (Pasteur, 1881; Greenfield, 1880) even before the mechanisms of humoral and cellular immunity were understood. Subsequently, an improved vaccine for livestock, based on a live un-encapsulated avirulent variant of *B. anthracis*, was developed (Sterne, 1939, 1942). Since then, this vaccine has served as the principal veterinary vaccine in the Western Hemisphere. The use of livestock vaccines was associated with occasional animal casualties, and live vaccines were considered unsuitable for humans. In 1904, the possibility of using acellular vaccines against *B. anthracis* was first suggested by investigators who discovered that injections of sterilized edema fluid from Anthrax lesions provided protection in laboratory animals (Bail, 1904; Salsbery, 1926) but the level of either humoral or cell mediated immunity was not studied. Evidence suggests that a humoral and cellular response is critical for protection against disease following exposure (Mahlandt *et al*,. 1966). This study evaluated efficacy of F-34 anthrax stern strain vaccine in mice in terms of humoral immunity. Following six months (day 180) of immunization, the vaccinated mice were challenged with $2x10^5$ CFU of virulent field isolate of *B. anthracis* protect infection but the unvaccinated mice succumbed to death indicated that the vaccine is protective in mice model.

Pathology of experimental anthrax

Gross pathological changes observed in the unvaccinated but challenged group includes severe congestion and hemorrhages in the lungs, liver, spleen, kidney, thymus and heart. Grams staining of tissue section and impression smears detected *B. anthracis* in tissues and body cavities. Tissue section stained with Goldner's trichrome stain found to detect capsule of the organism distinctly, which however was not seen in Grams staining and H&E staining. The damage of the small vasculature was common throughout the body indicating toxic effect of the bacteria *in vivo* and causes of wide spread hemorrhages. Such systemic effect was absent in vaccinated mice challenged with the virulent field isolate of *B. anthracis* indicating unaffected visceral organs following challenge infection. However, it would have been value if we could evaluate vaccine efficacy and challenge infection of vaccinated mice following 2-3 days of infection; mild to significant level of host response may be observed. However, rod shaped *B. anthracis* bacteria was successfully isolated from the visceral organs of Group B mice (unvaccinated but challenged) following experimental infection but was lacking in Group D mice. Histopathological study of those organs revealed widespread congestion and hemorrhage, infiltration of neutrophils, macrophages in all organs. There was lymphoid depletion in the spleen, tensed capsule and compressed trabeculi, such changes were typical for experimental anthrax in mice (Duong *et al.,* 2006). Visualization of *B. anthracis* with Goldner's trichrome staining on those tissue sections was characteristics and can be used in routine investigation of anthrax in animals.

Conclusion

Out of 13 field samples tested, *B. anthracis* bacteria was isolated from five cases. The cultural, morphological and PCR assay (targeting pX01 and pX02 plasmids) confirmed that the species of the bacteria isolated in culture was *B. anthracis*. In two cases bacillus bacteria were isolated, appeared motile, hemolytic in sheep blood agar, were non *B. anthracis* and thus discarded. The mice model used in immunoefficacy trial, showed fairly a significantly higher level of anti-anthrax IgG antibody response and the response persisted until the end of study (day 180 of immunization). The protective efficacy of anthrax Sterne strain F-34 vaccine was

evaluated in mice model. The vaccinated mice were challenged with $2x10^5$ CFU of virulent field isolate of *B. anthracis* (isolate from Shahjadpur Upazila, Sirajganj district) following 180 days of immunization withstand challenge. The non-vaccinated mice challenged with $2x10^5$ CFU of the virulent field isolate of *B. anthracis* were died within 18-24hours of infection. The Sterne strain F-34 vaccine appeared protective in mice model following a single injection and provide protection up to six months of immunization. It needs to study whether the vaccine is effective for a year and effective in large ruminants as well.

Acknowledgement

Project implementation unit (PIU), Bangladesh Agricultural Research Council (BARC), NATP-Phase 1 for funding the research.

Ethical Statement

All the authors have agreed for authorship, read and approved the submission for publication. As the study was conducted onto a deadly pathogen, and live mice model was used, the infectious object was killed by dipping the research materials in 10% formalin solution. The mice used was fed with optimum food and drinking water and were sacrificed following deep sedation and cervical dislocation as require for the Animal Welfare Committee, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

References

Bail, 0. 1904. Research into natural and artificial Anthrax immunity. *Zentralb. Bakteriol Parasitenk Infectionskr*, **47:** 270-272.

Baillie, L., and Read, T.D. 2001: *Bacillus anthracis*: a bug with attitude. *Current Opinion* in *Microbiology,* **4:** 78–81. https://doi.org/10.1016/s1369-5274(00)00168-5

Bartlett, J., Inglesby, T. and Borio, L. 2002. Management of Anthrax. *Clinical Infectious Diseases,* **35:** 851-857. https://doi.org/10.1086/341902

Berg, T., Suddes, H., Morrice, G. and Hornitzky, M. 2006. Comparison of PCR, culture and microscopy of blood smears for the diagnosis of anthrax in sheep and cattle. *Letters In Applied Microbiology,* **43:** 181-186. https://doi.org/10.1111/j.1472-765X.2006.01931.x

Beyer, W., Glöckner, P., Otto, J. and Böhm, R. 1995. A nested PCR method for the detection of *Bacillus anthracis* in environmental samples collected from former tannery sites. *Microbiological Research,* **150** (2): 179-86. https://doi.org/10.1016/S0944-5013(11)80054-6

Bhat, S., Curach, N., Mostyn, T., Bains, G.S., Griffiths, K.R. and Emslie, K.R. 2010. Comparison of Methods for Accurate Quantification of DNA Mass Concentration with Traceability to the International System of Units. *Analytical chemistry*, 82(17): 7185-7192. https://doi.org/10.1021/ac100845m

Brossier, F., Martine Levy, M. and Mock, M. 2002. Anthrax Spores Make an Essential Contribution to Vaccine Efficacy. *Infection and immunity,* 70(2): 661–664. https://doi.org/10.1128/IAI.70.2.661-664.2002

CDC, 2000. Surveillance for adverse events associated with anthrax vaccination, U.S. Department of Defense, 1998-2000. *Morbidity and Mortality Weekly Report,* **49:** 341-345.

Chakraborty, A., Khan, S.U., Hasnat, M.A., Parveen, S., Islam, M.S., Mikolon, A., Chakraborty, R.K., Ahmed, B.N., Ara, K., Haider, N., Zaki, S.R., Hoffmaster, A.R., Rahman, M., Luby, S.P., and Hossain, M.J. 2012. Anthrax Outbreaks in Bangladesh, 2009–2010. *The American Journal of Tropical Medicine and Hygiene*, 86(4): 703–710. https://doi.org/10.4269/ajtmh.2012.11-0234

Dipti, M., Rashid, M. M., Ferdoush, M. J., Roy, P., Khan, MAHNA and Hossain M.M. 2013. Morphological and immunological characterization of Anthrax vaccine in goats. *Bangladesh Journal of Veterinary Medicine,* 11 (1): 43-49. https://doi.org/10.3329/bjvm.v11i1.17732

Drobniewski, F.A. 1993. *Bacillus cereus* and related species. *Clinical Microbiology Reviews,* **6:** 324-338. https://doi.org/10.1128/cmr.6.4.324

Duong, S., Chiaraviglio, L. and Kirby, J.E. 2006. Histopathology in a murine model of anthrax. *International Journal of Experimental Pathology,* **87:** 131–137. https://doi.org/10.1111/j.0959-9673.2006.00473.x

Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B. and Pauli, G. 2002. Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. *FEMS Microbiology Letters,* **214:** 51–59. https://doi.org//10.1111/j.1574-6968.2002.tb11324.x

Fasanella, A., Losito, S., Trotta, T., Adone, R., Massa, S., Ciuchini, F. and Chiocco, D. 2001. Detection of Anthrax vaccine virulence factors by polymerase chain reaction. *Vaccine,* **19:** (30): 4214–4218. https://doi.org/10.1016/s0264-410x(01)00159-1

Fasanella, A., Tonello, F., Garofolo, G., Muraro, L., Carattoli, A., Adone, R. and Montecucco, C. 2008. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. *Vaccine,* **26** (45): 5684-5688. https://doi.org/10.1016/j.vaccine.2008.08.026

Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B. and Ivons, B.E. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infection and Immunity,* **49:** 291-297. https://iai.asm.org/content/49/2/291

Greenfield, W.S. 1880. Lectures on some recent investigations into the pathology of infective and contagious diseases. Lecture Ill. Part I. Anthrax and anthraco'ld diseases. *Lancet,* **1:** 865-867. https://doi.org/10.1016/S0140-6736(02)42734-1

Hassan, J., Rahman, M.B., Chowdhury, S.M.Z.H., Rabidas, S.K., Parvej, M.S. and Nazir, K. H. M.N.H. 2015. ELISA Based Anthrax Antibody Titer in Cattle Induced by Locally Prepared Anthrax Vaccine Originated from Sterne F-24 Strain in Bangladesh. *Microbes and Health,* 4(1): 36-3. https://doi.org/10.3329/mh.v4i1.23104

Hoffmaster, A.R., Ravel, J., Rasko, D.A., Chapman, G.D., Chute, M.D., Marston, C.K., De BK, Sacchi, C.T., Fitzgerald, C., Mayer, L.W., Maiden, M.C., Priest, F.G., Barker, M., Jiang, L., Cer, R.Z., Rilstone, J., Peterson, S.N., Weyant, R.S., Galloway, D.R., Read, T.D., Popovic, T. and Fraser, C.M. 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proceedings of the National Academy of Sciences of the United States of America,* **101:** 8449-8454. https://doi.org/10.1073/pnas.0402414101

Jahan, A.A., Ruba, T., Mumu, T.T., Rana, M.S., Islam, M., Belal, S.M.S.H., Khan, M.A.H.N.A., and Bari, A.S.M. 2019. Pathological and molecular detection of diseases of cattle at slaughter**.** *Bangladesh Journal of Veterinary Medicine*, 16 (2): 213–222. https://doi.org/10.33109/bjvmjd1811

Ionin B, Hopkins RJ, Pleune B, Sivko GS, Reid FM, Clement KH, Rudge Jr. TL, Stark GV, *et al*. 2013. Evaluation of Immunogenicity and Efficacy of Anthrax Vaccine Adsorbed for Postexposure Prophylaxis. *Clinical and Vaccine Immunology,* 20(7): 1016 –1026. https://doi.org/10.1128/IAI.70.2.661–664.2002

Khan, M.A.H., Ogita, H., Ferro, V.A., Kumasawa, K., Tsutsui, T. and Kimura. 2008. Immunisation with a plasmid DNA vaccine encoding gonadotrophin releasing hormone (GnRH-I) and T-helper epitopes in saline induces an anti-GnRH-I antibody response and suppresses rodent fertility. *Vaccine,* 26(10): 1365-1374. https://doi.org/10.1016/j.vaccine.2007.12.052

Knisely, R.F. 1966. Selective medium for *Bacillus anthracis*. *Journal of Bacteriology,* **92:** 784–786. https://doi.org/10.21236/ad0479102

Luna, L.G. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill, New York.

Luxembourg, A., Hannaman, D., Nolan, E., Ellefsen, B., Nakamura, G., Chau, L., Tellez, O., Little, S. and Bernard, R. 2008. Potentiation of an anthrax DNA vaccine with electroporation. *Vaccine*, 26(40): 5216-5222. https://doi.org/10.1016/j.vaccine.2008.03.064

Lyons, C.R., Lovchik, J., Hutt, J., Lipscomb, M.F., Wang, E., Heninger, S., Berliba, L. and Garrison, K. 2004. Murine Model of Pulmonary Anthrax: Kinetics of Dissemination, Histopathology, and Mouse Strain Susceptibility. *Infection and Immunity*, 72(8): 4801–4809. https://doi.org/10.1128/IAI.72.8.4801-4809.2004

Mahlandt, B.G., Klein, F., Lincoln, R.E., Haines, B.W., Jones, W.I. Jr. and Friedman, R.H. 1966. Immunologic studies of Anthrax: IV. Evaluation of the immunogenicity of three components of Anthrax toxin. *Journal of Immunology,* **96:** 727-733. https://doi.org/10.21236/ad0640357

Marston, C.K., Beesley, C., Helsel, L. and Hoffmaster, A.R. 2008. Evaluation of two selective media for the isolation of *Bacillus anthracis*. *Letters In Applied Microbiology,* **47:** 25-30. https://doi.org/10.1111/j.1472-765X.2008.02375.x

Mikesell, P., Ivins, B.E., Ristroph, J.D. and Dreier, T.M. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infection and Immunity,* **39:** 371–376.

Pasteur, L. 1881. On the attenuation of viruses and on it's return to virulence [French]. *Comptes Rendus de l'Académie des Sciences,* **101 :** 429-435.

Radnedge, L., Agron, P.G., Hill, K.K., Jackson, P.J., Ticknor, L.O., Keim, P. and Andersen, G.L. 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and Bacillus thuringiensis. *Applied and Environmental Microbiology,* **69:** 2755–2764. https://doi.org/10.1128/AEM.69.5.2755-2764.2003

Ramisse V., Patra G., Garrigue H., Guesdon J.L. and Mock M. 1996. Identification and characterization of Bacillus anthracis by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiol Lett 145: 9–16. https://doi.org/10.1111/j.1574-6968.1996.tb08548.x

Roit, I., Brostoff, J. and Male, D. 2001. Immunology (6th Edn.), Mobsly. London. England.

Salsbery, C.E. 1926. Anthrax aggressin. *Journal of the American Veterinary Medical Association,* **68:** 755-757.

Sterne, M. 1939. The immunization of laboratory animals against anthrax. *Onderstepoort journal of veterinary science and animal industry,* **13:** 315 - 317.

Sterne, M. 1942: The immunization of laboratory animals against Anthrax. *Journal of the South African Veterinary Medical Association,* **13:** 53-57.

Sterne, M. 1959. Anthrax. In: Stableforth AW and Galloway IA (Editors) Infectious Diseases of Animals. Diseases due to Bacteria, Butterworths, London 16-52.

Turnbull, P.C.B. 1999. Definitive identification of *Bacillus anthracis*—a review. *Journal of Applied Microbiology,* **2:** 237–240. https://doi.org/10.1046/j.1365-2672.1999.00876.x

Uchida, I., Sekizaki, R., Hashimoto, K. and Terado, N. 1985. Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *Journal of General Microbiology,* **131:** 363-367.

Vance, D.J., Rong, Y., Brey, R.N. and Mantis, N.J. 2015. Combination of two candidate subunit vaccine antigens elicits protective immunity to ricin and anthrax toxin in mice. *Vaccine*, **3**(9): 417-421. https://doi.org/10.1099/00221287-131-2-363

Wang, D.B., Tian, B., Zhang, Z.P. Deng, J.Y., Cui, Z.Q., Yang, R.F., Wang, X.Y., Wei, H.P. and Zhang, X.E. 2013. Rapid detection of *Bacillus anthracis* spores using a super-paramagnetic lateral-flow immunological detection system. *Biosens Bioelectron,* **42:** 661–667. https://doi.org/10.1016/j.bios.2012.10.088

Weiner, Z.P., Boyer, A.N., Gallegos-Candela, M., Cardani, A.N., Barr, J.R. and Glomski, I.J. 2012. Debridement Increases Survival in a Mouse Model of Subcutaneous Anthrax. PLoS ONE 7(2): e30201. https://doi.org/10.1371/journal.pone.0030201

Zohora, F.T., Hossain, M.M., Islam, M.S., Rahman, M.G.U.I., Saha, P.C. and Khan, M.A.H.N.A. 2012: Standardization of an ELISA protocol for the detection of IgG antibodies in cattle against Anthrax vaccine. *Bangladesh Journal of Progressive Science and Technology,* **1**(1): 073-076.