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Short Communication

EPIDEMIOLOGICAL INVESTIGATION OF ANTHRAX OUTBREAK IN WEST BENGAL BY MICROSCOPY, CULTURAL AND MOLECULAR TECHNIQUES

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ABSTRACT: Outbreak investigation of anthrax was reported by microscopic, cultural and molecular techniques from blood samples from the districts of Murshidabad, Nadia, and Hooghly in West Bengal. The Blood smear staining and molecular detection techniques revels that the repeated outbreak was confirmed at Hariharpara, Domkol, Beldanga-II, and Burwan block in the distict of Murshidabad as well as adjacent Kaliganj and Tehatta-II block in Nadia.

Key words: Anthrax, Blood, Molecular detection.

Outbreak of anthrax in bovine generally occurs by exposure to soil contaminated with spore of *Bacillus anthracis* in soil during post-rainy season (Ahsan *et al*. 2013). *Bacillus anthracis* is a gram positive, aerobic, endospore forming bacteria responsible for lethal disease anthrax which affects both humans and animals (Beyer and Turnbull 2009). Several environmental parameters like soil type, temperature, rainfall, relative humidity etc. potentiate the survival of spores and repeat the outbreak. The soil rich in organic matter and calcium promotes the survival of *B. anthracis* (Dey *et al*. 2012). Spores released into the soil from the carcasses of dead animals can survive for decades, even under adverse conditions serving as a source of infection for other animals (Beyer and Turnbill 2009). The pathogenesis of bacteria mainly depends on the presence of two virulence plasmids pXO1 (181 kb) having *pag, lef* and *cya* genes and pXO2 (96 kb) possessing *cap* gene. *Pag, lef* and *cya* genes encodes the anthrax toxin protein protective antigen (PA), lethal factor (LF) and edema factor (EF) respectively. The *cap* gene on pXO2 encodes for anti phagocytic poly D-glutamic acid capsule biosynthesis protein (Kolsto *et al*. 2009).

Commonly used laboratory techniques for diagnosis of anthrax in cattle includes microscopy, culture, animal inoculation and PCR assay. Blood smear stained with polychrome methylene blue, shows McFadyean reaction in positive cases characterised by the presence of large blue rods having truncated ends in short chains with purple/pink stained capsule. Colonies of *B. anthracis* isolates are white to grey, often looking like ground glass on nutrient agar. It is non- motile, non-haemolytic on sheep blood agar, grows rapidly at a temperature of 37°C and forms large colonies with irregularity tapered outgrowths in low power microscopy (Swartz 2001). A nested PCR targeting the genes of plasmids pXO1 and pXO-2 plasmids was developed to detect anthrax spores in natural soil and waste samples heavily contaminated by organic and inorganic compound from former tannery sites (Beyer *et al.* 1995). Molecular typing techniques like Multiple Locus VNTR (Variable Number Tandem Repeat) analysis (Keim *et al*. 2000), Amplified Fragment Length Polymorphism (AFLP) (Keim *et al.* 1997) and Single Nucleotide Repeat analysis (Stratilo *et al.* 2006) have been developed for distinguishing the isolates of *B. anthracis* and also to differentiate them from other bacilli.

STUDY AND DISCUSSION

After the death of animals in different districts, team of IAH&VB, Kolkata attended the outbreak sites for proper investigation and collection of samples for laboratory diagnosis. Whole blood, blood smear from ear vein and soil samples were collected, packed in ice cold boxes and transported to Research Laboratory Bacterial Disease (RLBD), Institute of Animal Health & Veterinary Biologicals (R&T), Belgachia, Kolkata. Sudden death within 24 hours, high rise of temperature abdominal pain,

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bloat and discharge of unclotted tarry coloured blood from natural orifices i.e. anus and nostrils were recorded during the investigation procedure. Susceptible animal population (bovines and caprines) were also included during this investigation for vaccination purpose.

Blood smear was prepared from each type of outbreak and stained with McFadyean stain. Briefly, film was prepared, air-dried and fixed by passing through flame. The slide was flooded with polychromemethylene blue stain for 2-3 minutes, washed with distilled water and excess water was dried. Finally, the slide was examined under oil immersion microscope. Mice inoculation was performed in suckling mice with 0.2 ml sample intramuscularly having at least 50 spores. Two mice were kept as controls and they were injected 0.2 ml normal saline solution intramuscularly. The death of all mice was recorded after 48 hours of inoculation in test group and heart blood was collected for cultural examination and molecular analysis, where control group remained normal. The heart blood from dead mice was streaked on 5% sheep blood agar and nutrient agar for isolation and characterization of *Bacillus anthracis* and the plates were incubated at 37°C overnight. After that, plates were removed for interpretation.

Fresh colony of *B. anthracis* grown on nutrient agar plate was used for genomic and plasmid DNA exraction using QIAamp DNA extraction kit (Qiagen, Germany). Firstly, bacterial cells were harvested and suspended in 180 µl of ATL buffer in 2 ml micro centrifuge tube and shaken vigorously. Then 20 µl of proteinase-K was added and incubated at 56°C in shaking water bath until cells were completely lysed. Upon complete lysis, 200 µl of AL buffer was mixed, subjected to pulse-vortexing for 15 seconds and incubated at 70 °C for 10 minutes. Subsequently 200 µl of ethanol (96-100%) was added to the sample and pulse vortexing was done for 15 seconds followed by brief centrifugation procedure to remove drops from the inside of the lids. The whole content thus obtained was transferred into the Mini spin column inside 2 ml collection tube. After closing cap, the content was centrifuged at 8000 rpm for 1minute. The tube containing filtrate was discarded and the column was transferred into another fresh collection tube. Then, 500 µl AW1 buffer was added to the column and centrifuged as stated earlier. Afterwards, the tube containing the filtrate was discarded and the column was transferred into another fresh collection tube. Second step washing was carried out by addition of 500 µl AW2 buffer into the column and centrifugation at 14000 rpm was done for 3 minutes. The QIAamp Mini spin column was placed in a new 2 ml collection tube and centrifugation at 14000 rpm is done for 1 min to remove any residual liquid in column. Finally, elution of DNA from column was performed using 100 µl of AE buffer, which was added and incubated for 5 minutes at room temperature. The content was centrifuged at 8000 rpm for 1 minute and stored at -70 °C for further use.

PCR was performed using pXO1 plasmid specific primers targeting the protective antigen gene as per OIE guideline, synthesized from private firm (GCC Biotech). The primers used for amplification were forward primer (PA5): 5' TCCTAACACTAACGAAGTCG 3' and reverse primer (PA8): 5' GAGGTAGAATATACGGT 3'. The PCR mixture was 2X Hot start buffer-25 µl, forward primer-0.25

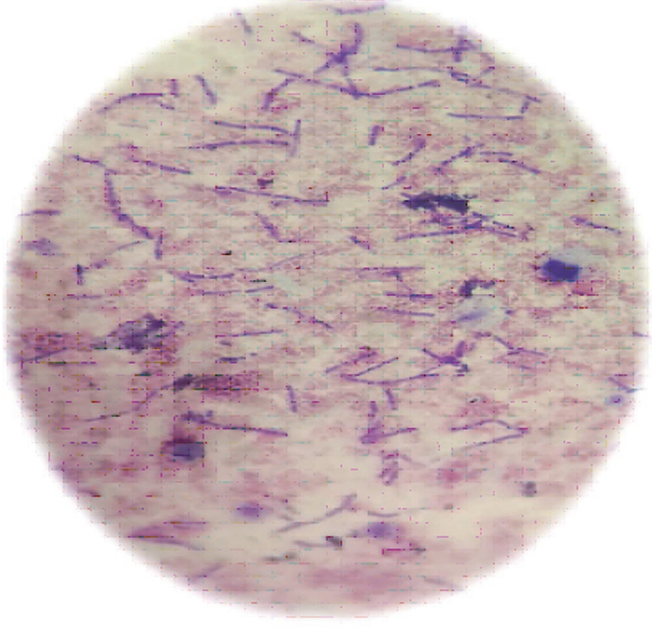

Fig. 1. Microscopic observation of *B. anthracis* bacteria

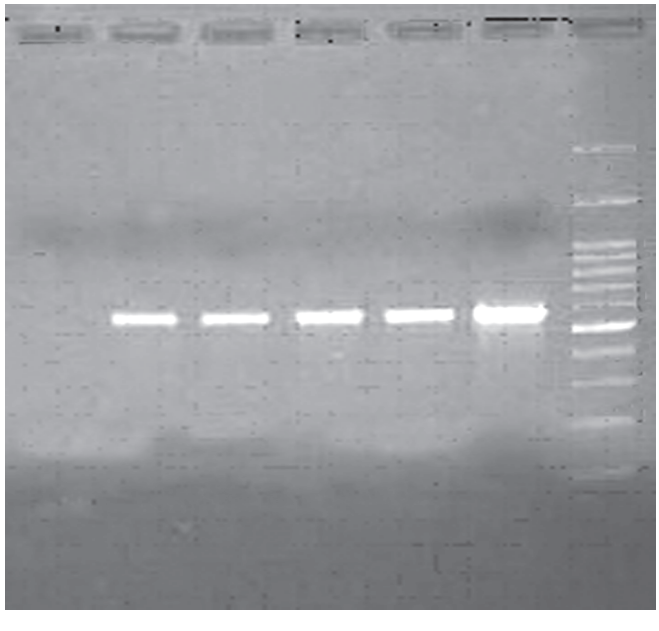

Fig. 2. PCR amplification of 596 bp of *B. anthracis* (L-ladder, PC-positive control, 1-4- samples, NC-negative control).

Table 1. Investigation of anthrax outbreak in different districts In West Bengal during 2016-19.

Out break	Place	Date of onset	Species affected	No of animal affected/die	Clinical signs	Materials collected/sent
1.	Hariharpara, Murshidabad	18.07.2016	Cattle	20/05	sudden rise of temperature, restlessness, bloat and death	blood smear and whole blood
2.	Hariharpara, Murshidabad	20.07.2016	Cattle	04/02	pyrexia, sudden death, oozing out blood from natural orifice	blood smear and whole blood
3.	Burdwan, Murshidabad	20.07.2016	Cattle	09/08	pyrexia, sudden death, oozing out blood from natural orifice	blood smear and whole blood
4.	Tehatta, Nadia	09.08.2016	Cattle	05/05	pyrexia, sudden death, oozing out blood from natural orifice, no rigor mortis	blood smear, whole blood, contaminated soil and ear tip
5.	Domkol, Murshidabad	26.09.2016	Caprine	03/03	pyrexia, sudden death, oozing out blood from natural orifice	blood smear and whole blood
6.	Beldanga-II, Murshidabad	17.05.2017	Cattle	01/01	temperature-106°F, sudden death, oozing out blood from natural orifice, no rigor mortis	blood smear, whole blood from ear vein
7.	Kalijang, Nadia	02.06.2017	Cattle	03/03	abdominal pain, sudden death within 24 hours, oozing out blood from natural orifice	blood smear, whole blood
8.	Pandua, Hooghly	11.06.2018	Cattle	04/03	sudden death, oozing out blood from natural orifice, enlarged abdomen, bloat	blood smear, whole blood
9.	Domkol, Murshidabad	24.07.2018	Cattle	01/01	pyrexia, bloat, bleeding from natural orifice	blood smear, whole blood from ear vein
10.	Hariharpara, Murshidabad	18.08.2018	Cattle	01/01	respiratory distress, sudden death, oozing out blood from anus	blood smear, whole blood and affected soil

µl, Reverse primer-0.25 µl, Template-05 µl and nuclease free water-19.5 µl in a 50 µl reaction mixture. The amplification of genomic DNA was carried out in thermal cycler (Applied Bio-systems, USA) and programme was set up at 95° C for 4 minutes; 40 cycles of 95°C for 1 minute, 50°C for 30 seconds, 72°C for 1 minute followed by final extension step at 72°C for 10 minutes. Immediately the samples are held at 4°C and the amplified PCR product was visualized and recorded using gel documentation system (Biorad).

Epidemiological study of the outbreak with sudden death of cattle and goat were recorded from Hariharpara, Domkol, Beldanga-II and Burwan block in the district of Murshidabad (2016-17, 2017-18 and 2018-19), from Kaliganj and Tehatta-II block of Nadia (2016-17 and 2017-18) , from Pandua Block of Hooghly (2018-19) (Table 1). Repeated outbreaks in adjacent area of Murshidabad and Nadia districts confirmed the circulation of anthrax bacteria or spores within this belt. After thorough investigation in the outbreak area, whole blood, smear and soil were collected either by the scientists of IAH&VB or local Veterinary officers and samples were brought to the laboratory for investigation. Sample of each outbreak was subjected to investigation by microscopic, mice inoculation test, cultural and PCR methods. Microscopic observation of blood smear stained with polychrome methylene blue showed the presence of large blue rod with truncated ends in short chains with purple/pink stained capsule (Fig. 1). The colonies of *B. anthracis* isolates were non-motile, white to grey, often looking like ground glass and non-haemolytic on sheep blood agar. Colonies grew rapidly at a temperature of 37°C and formed large colonies with irregularity tapered outgrowths as seen in a low power microscope. PCR analysis of genomic and plasmid DNA extracted from whole blood of each outbreak gave positive result, as evidenced by specific amplicons of 596 bp seen in agarose gel electrophoresis (Fig. 2). Prompt outbreak investigation, rapid diagnosis, strict bio-security measures and use of anthrax spore vaccine (Sterne strain, manufactured by IAH&VB, @ 5 ml to cattle and 2 ml to sheep and goat S/C

ly) not only controlled the outbreak among the domestic animals but also prevented the spreading of infection to the animal owners.

Epidemiological and laboratory investigation of a zoonotic anthrax outbreak at Paschim Medinipur district of West Bengal was confirmed by microscopic, cultural and molecular techniques (Dandapat *et al*. 2017). An incidence of 21.17% from May- June was reported from Sirajganj, Bangladesh due to weak acidic soil (pH 6.38 ± 0.15), temperature (32°C) and average rainfall 158 mm (Ahsan *et al*. 2013). Suspected anthrax outbreak was reported in 14.28% humans with the symptoms of haematuria, convulsion and respiratory distress from Oregram and Kathaldanga villages in Burdwan from May-June-2013 (Mondal *et al.* 2015). Molecular confirmation of anthrax outbreak was done in different villages of Simdenga district, Jharkhand due to transmission through direct contact with hide and meat of anthrax-affected animals (Kumar *et al*. 2019). Anthrax outbreak in cattle, sheep and goat was recorded from district of Ramananathapuram, Tamil Nadu from 2010-2015 (Rajasokkappan *et al.* 2016). PCR offered reliable diagnosis from blood even after 15-17 days after collection of samples, microscopy from 2-6 days and cultural isolation of organism from 2-17 days (Berg et al. 2006). Anthrax outbreak among domestic cattle in Gundlupet near Bandipur National Park area was examined by EA-1 sandwitch ELISA and immunocapture- PCR (Kingston *et al*. 2015). Examination of smear as a diagnostic tool may become unreliable about 6 hrs after death (Hornitzky 2004). Demonstration of encapsulated *B. anthracis* in blood or fluid is a straightforward and reliable means of diagnosis of anthrax. Microscopic examination of smears usually remains the quickest means of obtaining a reliable result in fresh sample; however, stain failure has been reported (Forshaw *et al*. 1996).

The rapid and accurate diagnosis of anthrax is vital to implement appropriate control measures for minimizing the zoonotic transmission, reducing the chances of spore formation and preventing the infected animal from entering the food chain. Molecular methods have been the greatest advantage that they are able to detect the presence of even one live or dead cell which significantly reduces the time required for confirmation of final result.

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