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Epidemiological and laboratory investigation of a zoonotic anthrax outbreak in West Bengal, India

Premanshu Dandapat[1*], Arijit Chakrabarty[1], Sonjoy Dey[2], Pramod Kumar Nanda[1], Suresh Chandra Das[1], Suman Dey[3], Arun Kurien[4], Apurba Chakraborty[5], Samiran Bandyopadhyay[1], Subhasish Bandyopadhyay[1], Raj Kumar Singh[6]

[1]*Eastern Regional Station, ICAR-Indian Veterinary Research Institute, 37, Belgachia Road, Kolkata, PIN 700 037, West Bengal, India*

[2]*Department of Paediatrics, Bankura Sammilani Medical College & Hospital, P.O.: Kenduadihi, Dist.: Bankura, PIN – 722 102, West Bengal, India*

[3]*Animal Resources Development (Veterinary Research & Investigation), Toxicology Laboratory, Jhargram, Dist: West Midnapur, PIN 721 507, West Bengal, India*

[4]*Department of Animal Husbandry, Mattukatta, Idukki, PIN 685 584, Kerala, India*

[5]*Midnapur Sadar Block, Village Satgerya, P.O. Pachkhuri, Dist: West Medinipur, Pin: 721 150, West Bengal, India*

[6]*ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, PIN 243 122, Uttar Pradesh, India*

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ABSTRACT

Objective: To investigate an anthrax outbreak affecting multi-species of animals including human in five remote tribal villages in West Midnapur district, India, with no previous history of anthrax cases for decades.

Methods: A systematic epidemiological investigation, along with bacteriological examination, cultural isolation, biochemical and molecular characterization of the samples (blood, dried meat, pieces of bones) was carried out to confirm diagnosis, identify transmission routes and risk factors to recommend suitable control measures.

Results: Samples from nine animals were confirmed for presence of *Bacillus anthracis* by characteristic morphology, biochemical profile, McFadyean reaction in blood smear, 'medusa head' appearance of colonies and PCR based detection of pXO1 plasmid. Epidemiological investigation revealed that the human patients contracted the infection during butchering or while handling contaminated animal products. In total, 14 animals died and 11 people got infected with cutaneous anthrax during the outbreak period.

Conclusions: Anthrax continues to be a persisting problem in Indian subcontinent causing considerable morbidity and mortality in animals and human. Collaborative efforts of human and animal health officials through various controlled measures *viz.*, ring vaccination, early treatment of human cases, quarantine of affected animals, safe disposal of carcass and public health campaign effectively controlled the zoonotic anthrax outbreak.

1. Introduction

Amongst important zoonotic diseases reported till date, anthrax is a fatal infectious disease and has a nearly worldwide distribution. It is caused by *Bacillus anthracis* (*B. anthracis*), a capsulated non-motile, aerobic, Gram positive and spore forming organism. Although almost all mammals including domestic and wild herbivores (such as cattle, sheep, goats, bison, deer, antelope and hippos) are affected by anthrax, higher mortality is recorded in ruminants[1-3]. Outbreaks of anthrax in animals mostly occur due to exposure to contaminated soil or ingestion of bacillus spores adhering to vegetation during grazing[4]. Because of the disease, infected animals not only loose body weight with reduced milk yield, but also collapse and die induced by high fever, breathing problems and presence of blood in the urine, faeces or milk[5]. A study conducted in India (1991–2005) estimates the average annual economic loss in cattle due to anthrax at 1.3%[6], which is

*Corresponding author: Premanshu Dandapat, Senior Scientist, Eastern Regional Station, ICAR-Indian Veterinary Research Institute, 37, Belgachia Road, Kolkata, PIN 700 037, India.
Tel: +91-33-25565725
E-mail: pdandapat@gmail.com

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ranked as third important zoonotic disease in India[7].

Anthrax occurs in three forms *viz*. cutaneous, gastrointestinal and inhalational. Cutaneous infection usually occurs in herdsmen and butchers in India and also in other developing countries, while other two forms (inhalational and gastrointestinal) are uncommon[8]. Under natural conditions, humans usually acquire cutaneous infection by direct or indirect contact with infected dead animal and animal products[1,9,10]. Human anthrax cases range between 2 000 and 20 000 annually over the world with majority in cutaneous form[8]. Despite this, the ecology and transmission of anthrax are less understood because of the intermittent and variable nature of outbreaks, species affected, and associated environmental and climatic conditions[11]. As far as reports of anthrax cases from India are concerned, the actual incidence of this disease is not known exactly due to underreporting. But in reality, many regions in India are enzootic for animal anthrax[12,13] and therefore, early detection and epidemiological investigation of the disease through one health approach are very important to limit the transmission in human being. In this study, outbreak of anthrax in human beings involving death of cattle in West Midnapur district of West Bengal, India, during June–July 2014 is reported. The investigation carried out by a team of human and animal health personnel to isolate, characterize the pathogen employing biochemical, microbial and molecular means, and their collaborative effort in prevention and control of anthrax are discussed in this paper.

2. Materials and methods

2.1. Anthrax case definition and investigation report/data collection

A case of anthrax was suspected, if an animal had signs or symptoms of infection like sudden death, bloated carcass, bleeding of unclotted blood from natural orifices and it was confirmed, if rod-shaped bacilli were found by blood smear examination. In humans, symptoms of fever, dizziness, headache and skin lesions involving vesicular eruption and/or black eschar were suspected for anthrax infection. Besides, socio-demographic data including name, age, occupation, work practices, as well as clinical data such as disease onset, symptoms and treatments provided from the primary health centres were collected using questionnaires and face-to-face interviews. Further, their history of recent contact with dead animal(s) during the process of skinning, butchering, chopping or carrying of animal meat was also noted.

2.2. Area of disease outbreak/epidemiological investigation of disease outbreak

An outbreak of anthrax was reported with death of a cow (an index animal case) within 4 h of showing signs of respiratory distress on 19 June, 2014 evening in Birsinghpur, a remote village in Midnapur Sadar block (West Midnapur), India (latitude: 22.42524; longitude: 87.35579). A multisectoral team involving both animal and public health officials visited the area to investigate the outbreak and establish control programme as well. Upon survey and inquiring, it was found that the carcass was butchered by the tribals besides the bank of a canal and the long bones, excreta, head and offal were disposed off around 200 m away from the village perimeter in a canal. Oozing of unclotted blood was noticed from anal orifice of the dead animal. Four days after the first case report, enhanced surveillance within 2 km radius of the outbreak epicenter revealed death of more cows in nearby villages (Ayma, Jamkunda, Boxakura and Maharajpur). On compilation of information, it was concluded that a total of 14 animals died during the outbreak period (19th June–7th July, 2014).

The first case of anthrax in human was reported three days after the death of first animal in village situated 1 km away from disposal site of the carcass. On investigation, it was found that a total of 17 tribal people came in direct contact with the carcass while skinning, butchering, chopping, dressing or carrying the meat without using any protective clothing or equipment and got infected.

2.3. Sample collection

Samples from eight (seven cattle and one goat) out of 14 dead animals (13 cattle and one goat) were collected from intact carcasses. Blood from tip of the ear of dead carcasses along with other available samples like dried meat, pieces of bones and cloth having contact history with hide of carcass (first animal case) were collected and packaged aseptically. The bone samples were stored at room temperature while other collected samples were brought under ice pack and kept at –70 °C for laboratory investigation. All isolations were performed under biosafety cabinet level 3 conditions.

2.4. Testing of samples

2.4.1. Microscopic examination, cultural isolation and biochemical characterization

Each of the samples collected was processed and investigated through microscopic examination followed by cultural isolation before confirming them positive for anthrax by PCR. The smears of blood and impressions from smear of dried meat were air dried, fixed and stained with polychrome methylene blue (0.5%) and examined under microscope for bacterial morphology and presence of capsule. Besides, to demonstrate growth of *B. anthracis*, routine culture of the sample was spread on agar plates supplemented with 5% sheep blood and incubated aerobically at 37 °C for 24 h. The isolates were characterized following standard microbiological methods[14,15]. Motility of the bacilli

was examined by microscopic observation of wet mount of cells grown in nutrient broth.

2.4.2. DNA extraction and confirmation by PCR

Fresh culture of *B. anthracis* grown on nutrient agar was used for extraction of genomic and plasmid DNA using commercially available QIAamp DNA Mini Kit (QIAGEN, Germany) following protocol provided by the manufacturer. PCR for amplification of pX01 plasmid targeting protective antigen was carried out using the oligonucleotide and PCR conditions as previously described[15] in a Veriti 96-Well Thermal Cycler (Applied Biosystem, USA). The primers used for amplification of 596 bp sequences were: Forward primer 5'TCCTAACACTAACGAAGTCG3'; Reverse primer 5'GAGGTAGAAGGATATACGGT3'.

The PCR mixture (100 μL total reaction volume) contained 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTPs, 20 pmol of each primer, 2.5 units of *Taq* DNA polymerase, and 5 μL template DNA, all in NH_4 buffer. Template DNA was initially denatured by heating at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and primer extension at 72 ℃ for 30 s. Finally, the cycle was completed after incubation for 7 min at 72 ℃ to complete the extension followed by cooling at 4 ℃. The amplified PCR products obtained were visualized in a gel documentation system [Ultraviolet Transilluminator (DNR Bioimaging System)] after electrophoresis in 2% (w/v) agarose gel containing ethidium bromide (0.5 mg/mL; Sigma-Aldrich).

2.5. Cloning and sequencing

The amplified product was purified using the gel purification kit (Fermentas, USA) and ligated with a pTZ57R vector (Fermentas, USA) following the prescribed protocol. The plasmid containing the expected insert was sequenced using commercially available facility (Xcelris, India). After obtaining the sequencing results, homology searches were conducted using the BLAST algorithm available at http://blast.ncbi.nlm.nih.gov/Blast.cgi to find out the similarity of the sequence with those available in database.

3. Results

3.1. Outbreak of anthrax in animals

Upon compilation of information after field investigation and face-to-face interviews, it was concluded that a total of 14 animals (13 cattle and one goat) died during the outbreak period (19th June, 2014 to 7th July, 2014) from five villages (Birsinghapur, Ayma, Jamkunda, Boxakura and Maharajpur) of West Midnapur district, West Bengal (Figure 1).

3.2. Outbreak of anthrax in humans

After three days of the animal anthrax outbreak, the first case of disease outbreak in human was detected on 21st June, 2014 which lasted for 10 days. Upon investigation, it was found that a total of 17 persons were involved in handling the hide of the dead animal, skinning or disposing off either the dead carcasses. A total of 11 persons (8 male and 3 female) developed painless vesicular cutaneous lesions on arm, finger, leg and back with itching sensation with an attack rate of 64.7% (Figure 2, Table 1). The affected people detected with cutaneous anthrax were treated with doxycycline for a period of 60 days. The epidemiologic

Figure 1. Map showing anthrax affected areas in West Bengal state.

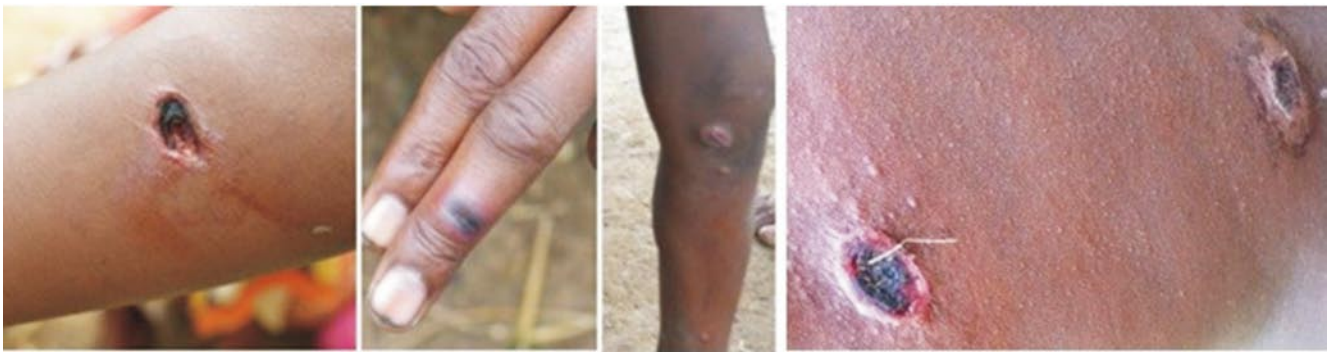

Figure 2. Lesions on the arm, finger, leg and back of patients affected by anthrax.

curve for both animal and human infection is shown in Figure 3.

Table 1
Incidence of cutaneous anthrax in human by age and sex in West Midnapur, West Bengal, India.

Characteristics		Cases	Exposed population	Attack rate per 100
Age	0–15	3	7	43
	15–40	5	6	83
	41–60	3	4	75
Sex	Male	8	12	67
	Female	3	5	60

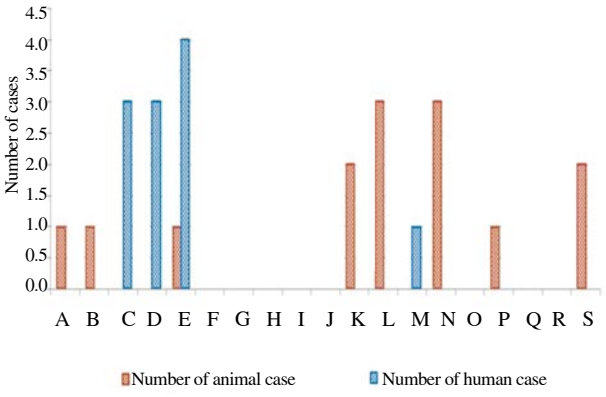

Figure 3. Epidemiologic curve of anthrax outbreak (June–July 2014) in animal and human population in West Midnapur, West Bengal, India.
A: 19-Jun; B: 20-Jun; C: 21-Jun; D: 22-Jun; E: 23-Jun; F: 24-Jun; G: 25-Jun; H: 26-Jun; I: 27-Jun; J: 28-Jun; K: 29-Jun; L: 30-Jun; M: 01-Jul; N: 02-Jul; O: 03-Jul; P: 04-Jul; Q: 05-Jul; R: 06-Jul; S: 07-Jul.

3.3. Confirmation of anthrax in animals

Microscopic examination of the blood smears from nine animal cases (eight cattle and one goat) and impression smear from dried meat on polychrome methylene blue staining showed spore bearing, capsular bacillus with presence of characteristic pink capsule surrounding the blue bacilli of *B. anthracis* (Figure 4). The greyish-white colonies in blood agar were found to be non-haemolytic with a ground glass surface with 'medusa head' like appearance (Figure 5). The bacillus was further found to be non-motile and negative for salicin fermentation distinguishing them from other bacillus species including *Bacillus cereus*. The virulence of *B. anthracis* isolate was also confirmed by PCR establishing the presence of pXO1 plasmid. The sequence was deposited in NCBI database and accession number (KT831967) was obtained. Although, on microscopic examination, none of the skin smear of 11 human cases was found positive for Gram-positive, spore-forming bacilli resembling *B. anthracis*, the pathognomic lesions with epidemiological analysis revealed that the affected humans contracted the diseases during butchering or while handling infected animal products.

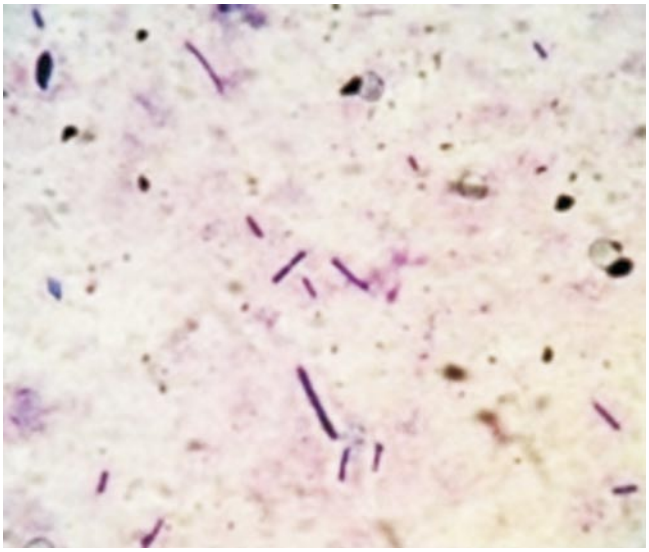

Figure 4. *B. anthracis* in blood of dead cattle (methylene blue staining, 1 000×).

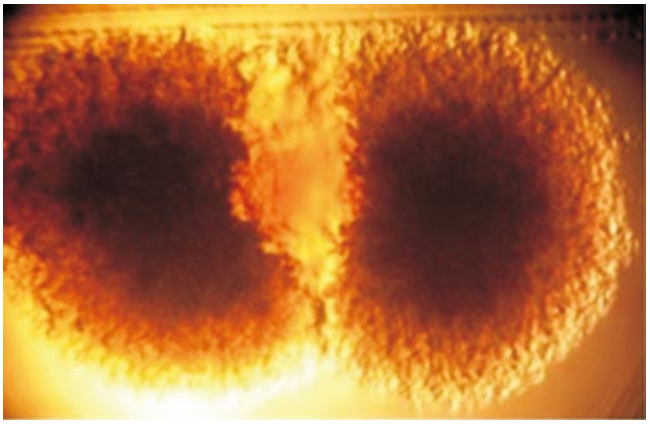

Figure 5. Characteristic medusa head colony of *B. anthracis* isolate in nutrient agar (10×).

4. Discussion

Out of 1 465 infectious diseases reported globally, about 75% are zoonotic and almost 61% are communicable from animals to man[16]. Amongst these, *B. anthracis* is a highly fatal infectious agent in animals and humans and therefore, its early and unambiguous diagnostic detection is essential for successful treatment and disease prevention.

In this study, an outbreak of anthrax in human epidemiologically linked with sudden death of livestock (cattle and goat) was investigated which occurred during June–July, 2014. Cattle are the most susceptible species to *B. anthracis* and the disease appears in acute or peracute form with higher mortality rate[3]. In fact, anthrax outbreaks are very common in animals than humans in many parts of India[12]. A total of 612 anthrax outbreaks were reported in India over a period of 5 years during 1991–1996[16]. These spores can remain in avirulent form in the soil for several decades[8,11]. Under favourable soil conditions, they become viable and the herbivore animals like cattle may have a higher chance of contracting infection while grazing in the contaminated pasture land[17], as also found in this study. Diagnosis of anthrax was done by direct examination of blood and tissue smears, bacterial isolation and PCR. *B. anthracis* colonies were non-hemolytic and grew well on 5% sheep blood agar. The colonies were medium to large, gray, flat, and irregular with swirling projections, having a "medusa head" appearance. The absence of hemolysis and lack of motility of the isolates in this study are the characteristics that differentiate *B. anthracis* from other *Bacillus* sp., as also recorded by Ibrahim *et al.*[18].

The blood smears, stained with polychrome methylene blue, showed characteristic McFadyean reaction characterized by the presence of large square ended blue rods in short chains with purple/pink stained capsules (Figure 4) as also reported by Radostits *et al.*[19]. Presence of capsular material is a positive test for *B. anthracis*, as none of its close relatives like *Bacillus cereus* nor *Bacillus thuringiensis* synthesize this capsular polymer[20]. However, Dragon and Rennie[21] did not recommend diagnosis of anthrax based on this test because of higher rate of false positivity (20%). Furthermore, biochemical and culture-based diagnostic tests take more time (24–48 h) and give presumptive diagnosis[22].

To overcome this problem, other rapid tests like PCR detection systems have been developed for *B. anthracis* and a positive result by this method is accepted as a confirmatory diagnosis of suspected anthrax cases for clinical samples[15]. *B. anthracis* contains two virulence determinant plasmids, pXO1 and pXO2. These virulence genes are restricted to *B. anthracis*, thereby giving the plasmid-based assays a high degree of specificity[23]. In our PCR-based test of clinical samples, presence of pXO1 plasmid was confirmed thereby establishing virulence of *B. anthracis* isolate(s) and distinguishing them from other soil-borne bacteria as well.

In India, a number of states including West Bengal are enzootic for animal anthrax with human anthrax cases[24] and since 1953 till 2000, about 182 human anthrax cases were reported[3]. According to the World Health Organization, the incidence of human anthrax depends on exposure to affected animals[25]. Depending upon the exposure of human, incubation period for anthrax varies from a few hours to seven days, although in most of the cases, it occurs within 48 h of exposure to animals. Based on the investigation reports and data collected from the villagers, first case of anthrax was detected in human upon exposure to or in contact with animal or its product after three days of anthrax outbreak in animals. Out of 17 people involved in handling the hide of the dead animal, skinning or disposing off the dead carcasses, 11 (64.7%) were diagnosed with cutaneous anthrax. The vesicular cutaneous lesions were like black eschars, typical of anthrax, and occurred on the arm, finger, leg and back of the patients (Figure 2). Compared to female, 67% of the male population were found to be exposed to cutaneous anthrax. Such higher incidences of cutaneous anthrax in male population could be due to their increased exposure to infected animals during handling, skinning or butchering and direct contact with uncooked meat as also reported by other workers[26-28].

According to available reports, up to 20% of persons with cutaneous anthrax may progress to sepsis leading to death, if not treated at the earliest[29,30]. On contrary to this, World Health Organization[31] report indicates that almost 95% of such cases of human acquired through contact with infected carcasses or animal products are non-fatal in nature. In this study, none of the human anthrax cases were fatal, as probably all patients received early and proper medical treatment.

On confirmation of outbreak and to stop further spread of the disease, the Animal Resources Development Department, Government of West Bengal swung into action and immediately carried out various prevention and control measures including vaccination of animals in surrounding villages of the epicentre. Control was further effected by recalling all the animal and animal products from confirmed and suspected animal cases and disinfected and destroyed as per scientific protocols to combat the spread of the disease. The villagers were made aware of associated risk factors and public health implications of anthrax and advised to adopt safety and precautionary measures (simple interventions like wearing gloves and mask) during disinfection, decontamination of materials and disposal of carcasses.

Based on the epidemiological investigations, observations of the symptoms and lesions, a systematic bacteriological examination and molecular characterization carried out by a team of researchers confirmed that the death of animals were due to anthrax outbreak caused by *B. anthracis*. As anthrax is zoonotic, a better surveillance and reporting system, in collaboration between human and animal health department, for early identification of anthrax cases is very important and crucial in prevention and control of this disease. Educating meat handlers and creating awareness among human and animal healthcare workers at grass root level would also help in prompt recognition and reporting of cases for effective control of anthrax. Furthermore, a test kit, if available commercially, would help in risk assessment of disease in initial stages and prevent its further spread or exposure, thereby reducing the socio-economic impact of the disease. The findings of this study may help in

implementing appropriate control and prevention measures so as to manage future anthrax outbreaks, particularly in enzootic areas.

Conflict of interest statement

We declare that we have no conflict of interest.

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