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Detection of *B. anthracis* from Environmental Samples during Outbreak in Tamilnadu by Molecular Methods

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ABSTRACT

The study was undertaken during 2013–2017 at Zoonoses Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai. Blood smears, soil and environmental samples were collected from 23 sporadic incidences of anthrax reported in different parts of Tamil Nadu from 2013–2017. 11 incidences were presumptively diagnosed as anthrax by detecting capsulated bacilli on polychrome methylene blue staining and microscopy. The spores in soil samples (62) were extracted, cultured and DNA was extracted for molecular method of diagnosis. The molecular method detected twelve incidences as anthrax by amplifying the virulence factors like *lef* gene (385bp) and capsular gene (264bp) encoded in pXO1 and pXO2 plasmids respectively and chromosomal marker (Ba813) gene (152bp) using multiplex PCR. The partial sequence of *cap* gene of isolates (Vellore and Nolambur strain) revealed a close relationship with *B. anthracis* species. The relatedness of the isolated strains to virulent strains such as Ames and Vollum strain on phylogenetic analysis showed the nature of the outbreak. Since the multiplex PCR detects the outbreak with short turn-around time by detecting both chromosomal and Plasmid DNA, considered as a confirmative diagnosis. Further the molecular method of diagnosis precludes the need for culturing thereby avoiding unnecessary occupational risks and environmental contamination.

KEYWORDS: Anthrax, biosafety, cattle, environment, PCR, phylogenesis, soil, zoonoses

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1. INTRODUCTION

Anthrax is a bacterial zoonotic disease, caused by *Bacillus anthracis*, rod-shaped, capsulated, spore-forming bacterium (Anonymous, 2018). It is worldwide in distribution (Fasanella et al., 2010, Liskova et al., 2021). It is known since ancient times, however increased awareness of *B. anthracis* as a cause of anthrax and bio-warfare agent arouses after the anthrax terror attack in United State in 2001 (Jernigan et al., 2001, Goel, 2015). Although it affects wide range of animals including humans, it is primarily a disease of herbivores (Anonymous, 2008, Liskova et al., 2021). The disease is mediated by exotoxins encoded by plasmid pXO1 and pXO2 (Moayeri et al., 2015, Liang et al., 2016). In animals, it causes acute fatal disease, which causes economic loss to livestock farmers. The animals become infected mostly by ingestion of spores from the soil (Quinn and Turnbull, 1998, Finke et al., 2020). The organisms discharged from dead animals become spore under unfavourable conditions and persist for decades (Anonymous, 2005, Carlson et al., 2018, Gomez et al., 2018). In Tamil Nadu, several anthrax outbreaks have been reported sporadically from several parts which have alkaline soil are termed as "Anthrax belts". A total of 619 anthrax outbreaks in cattle were documented from 1991–2006 and observed more than 25 outbreaks year^{-1}. Recent countrywide surveillance has confirmed several anthrax outbreaks in the region of Odisha and Karnataka (Govindaraj et al., 2019, Roonie et al., 2020). Apart from disease in animals, several human outbreaks have been reported in India (Reddy et al., 2012, Nayak et al., 2019, Kumar et al., 2020). In humans, the infection is acquired from a contaminated environment or in contact with infected animals. The implementation of the 'One Health' intervention model is required to eliminate human anthrax (Bhattacharya et al., 2021).

The disease is diagnosed by observing the carcass characteristics or demonstration of encapsulated *B. anthracis* in smears stained with Polychrome methylene blue staining. In some time, capsules may not be detected in the blood smear from the animal that had died >24 h (Anonymous, 2018). The culture and isolation of *B. anthracis* or detection of virulence factors and chromosomal genes by molecular methods are the definite diagnosis of anthrax (Hornitzky and Muller, 2010). The culture and isolation of *B. anthracis* are restricted to Biosafety Level III facility laboratories, further the capsule is not present on *B. anthracis* grown aerobically on nutrient agar. The isolation of *B. anthracis* from decomposing carcasses, or soil with sporostatic chemicals that may outgrow and obscure *B. anthracis*. (Zasada, 2020). These limitations highlighted the need for the rapid diagnostic tests.

Because of rapidity, specificity and ease of use for various targets and sample types, PCR and real-time PCR based methods are used for detection of *B. anthracis* (Ramisse et al., 1996, Makino et al., 2001, Rantakokko-Jalava and Viljanenm, 2003). The molecular methods overcome the biosafety concern of individuals and the hazardous nature of the environment. (Ko et al., 2003). In some cases, *B. anthracis*, fails to produce capsules or induce anthrax in test animals, requiring DNA probe or PCR to reveal such a strain as *B. anthracis*. (Turnbull et al., 1992). Emerging strains of *Bacillus cereus*, associated with anthrax-like disease in mammals as exchange of genetic material in the *Bacillus* sp have been reported (Baldwin, 2020). Hence in this study, multiplex PCR, for detecting virulence factors (*lef* and *cap* gene) and chromosomal gene (Ba813) of *B. anthracis* in clinical and environmental samples was carried out as the confirmative diagnosis of the anthrax, which may also be used for surveillance of disease and planning the prevention measures to control of the disease.

2. MATERIALS AND METHODS

2.1. Collection of samples

Depending upon the availability of materials, appropriate samples such as blood smears (27) from dead animals and soil (62) were collected from different outbreaks (total 22) with suspicious Anthrax noticed in Tamil Nadu during the period 2013–2017. The samples collected are detailed in Table 1.

After the blood smears were screened by staining and microscopy, the soil samples at the carcass disposal site, environmental samples composed of heterogeneous mixture of soil, animal faeces, vegetation, etc were collected with Biosafety measures to detect the spore contamination of the soil. Approximately 500 g of the top soil to a maximal depth of 20 cm were collected in double sealed plastic bags, labelled, and transferred to the laboratory.

2.2. Staining and microscopy

The blood smears were stained with Polychrome methylene blue (M/s Merck, India) for 30–60 seconds (M'Fadyean reaction) after fixing the smear by dipping in absolute or 95% methanol for 30–60s. The slides were viewed to demonstrate encapsulated bacilli.

2.3. Spore extraction and culture

The spores were extracted from soil and other environmental samples as per the spore extraction method described with some modifications (Moazeni Jula et al., 2004). Briefly, 50 g of the soil sample is blended in 100 ml of sterile distilled water, soaked overnight, and placed in a water bath at 62.5±0.5°C for 60 m, filtered through Whatman No 1 filter paper, and centrifuged at 6000 rpm for 15 m. The pellet was resuspended in 1 ml of PBS and used for culture. The spore

Table 1: Details on collection of samples from different sources and detection of *B. anthracis* genomic DNA

Sl. No.	Source	Incidence Positive / total	Type of sample	Number	Test			Result		
					Staining	Culture	PCR	Staining	Culture	PCR
1.	Villupuram Dt	3/4	Blood smear	17	17	-	-	11	-	-
			Soil	6	-	6	6	-	6	6
2.	Vellore Dt	3/4	Blood smear	4	4	-	-	4	-	-
			Soil and environmental samples	12	-	12	12	-	4	4
3.	Tiruvannamalai Dt	4/6	Blood smear	5	5	-	-	3	-	-
			Soil and environmental samples	14	-	14	14	-	3	3
4.	Theni	0/1	Soil	15	-	15	15	-	-	-
5.	Kancheepuram Dt	1/1	Blood smear	1	1	-	-	1	-	-
			Soil	5	-	5	5	-	-	-
6.	Dharumapuri Dt	1/1	Soil	4	-	4	4	-	1	1
7.	Krishnagiri Dt	0/1	Soil	2	-	2	2	-	-	-
8.	Tiruvallur Dt	0/4	Soil	4	-	4	4	-	-	-
	Total	12/22			27	62	62	19	14	14

extract was cultured on blood agar (M/s Himedia, Mumbai) and PLET (Polymyxin, Lysozyme, EDTA, Thallus acetate) agar (M/s Himedia, Mumbai) by streak plate method and incubated at 37˚C for 24–48 h.

2.4. Multiplex polymerase chain reaction (PCR)

DNA was extracted from bacterial colonies on culture as described (Hornitzky and Muller, 2010). The bacterial colonies from blood agar were resuspended in 200 µl of dH_2O in a centrifuge tube and the suspension was used for DNA extraction. Briefly, the suspension was incubated at 56°C for 20 minutes, vortexed, then incubated at 100°C for 20 m, centrifuged at 12,000 rpm for 3 m and the supernatant was collected as genomic DNA, stored at -30˚C. A multiplex PCR as approved by SCAHLS (Sub-committee on Animal Health Laboratory Standards, Australia.) to confirm the presence of virulence genes for lethal factor in pX01 and capsular antigen in pX02 plasmids and chromosomal marker (Ba813) specific for *B. anthracis* was carried out (Hornitzky and Muller, 2010). The targets, primers, sequences are described by are given in Table 2.

The PCR reaction mixture (25 µl) containing 20 picomoles of each primers Ba813 R1, Ba813 R2, *cap* 57, and *cap* 58, 30 picomoles of *lef* 3 and *lef* 4, 12.5 µl of Exprime PCR reaction mixture (M/s GeNet Bio), 1.5 µl nuclease-free water and 20 µg of DNA was prepared. The reaction was performed in a thermal cycler (M/s BioRad) with initial denaturation at 94˚C for 4 minutes; 35 cycles with cycling conditions of 40 sec at 94˚C, 40 s at 57˚C, 40 s at 72˚C, followed by extension at 72˚C for 5 m. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide (50 µg ml^{-1}) (M/s Bio Basic, USA) at 90 V for 40 m, viewed under a gel documentation system. The amplification of 2 or 3 of the targets by multiplex PCR is considered to be positive.

Table 2: List of Oligonucleotide used for PCR assay

Target	Primer	Sequence	Product size
Chromosomal	Ba813R1 Ba813R2	TTAATTCACTTG-CAACTGATGGG CGATAGCTCCTA-CATTTGGAG	152 bp
pX01	lef 3 lef 4	CTTTTGCATAT-TATATCGAGC GAATCACGAATAT-CAATTTGTAGC	385 bp
pX02	cap 57 cap 58	ACTCGTTTTTAAT-CAGCCCG GGTAACCCTT-GTCTTTGAAT	264 bp

2.5. Cap gene sequencing and phylogenetic analysis

The cap gene of anthrax strain isolated from Vellore and Nolambur was partially sequenced commercially on ABI

3130 XL Genetic analyzer (Applied Biosystems, USA) through the Sanger sequencing technique. The nucleotide sequences were analyzed with the other anthrax cap gene sequences on blast analysis using clustal X2 (Larkin et al., 2007). The relatedness of the strain with other Bacillus sp was analyzed by phylogenetic analysis on MEGA-X software (www.megasoftware.net). The phylogenetic tree was constructed using the Neighbor-joining algorithm and the reliability of the branches was validated by the generation of 1000 'bootstrap' replicates.

3. RESULTS AND DISCUSSION

Out of 27 blood smears collected from dead animals from 15 incidences, 11 incidences were presumptively diagnosed as anthrax by demonstration of encapsulated bacilli in blood smears (19) by polychrome methylene blue staining. The capsule is stained in pink, whereas the bacillus is stained dark blue which is characteristic of *B. anthracis*. The capsulated bacilli were noticed in nineteen samples by polychrome methylene blue staining by M'Fadyean reaction (Quinn et al., 1994, Anonymous, 2018) but the organisms were isolated only from 14 samples, it could be due to invasion of the postmortem bacterial invaders into the blood from the GI tract producing similar morphology, undistinguished from anthrax bacilli (Davies, 2015). In some cases, the capsule may not be readily detected in a blood smear taken from animals that have died for ≥24 h due to disintegration of capsule (Hornitzky and Muller, 2010) or due to loss of plasmid pOX2 (Turnbull et al., 1992). Hence it is considered as a presumptive test. The culture on the blood agar, non-haemolytic grey-white to grey colonies, with a ground-glass appearance were noticed that were characteristic of *B. anthracis*. On PLET agar circular, creamy-white with ground glass texture colonies were noticed. *B. anthracis* was isolated in fourteen soil and environmental samples out of 62 samples that were collected from 22 incidences. The culture and isolation are considered as confirmatory diagnosis; however, it requires a high security, biosafety III laboratory to prevent environmental contamination. The overgrowth of contaminants, especially samples collected from the animal that had died a long time back, contaminated soil, and the environment hinder the isolation. Sometimes failure to culture the *B. anthracis* happened when the specimens from animals that have received antibiotic therapy or where environmental samples contain sporostatic chemicals. These constraints warrant the need for a molecular method of diagnosis (Hornitzky and Muller, 2010).

3.1. Detection of anthrax by multiplex PCR by amplifying targets on plasmid and chromosomal DNA

DNA was extracted from 62 samples that were collected from 22 incidences. Out of 62 samples, virulence factors such as *lef* gene, *cap* genes and chromosomal marker genes were amplified in 14 samples from 12 incidences by multiplex PCR. Multiplex PCR revealed the amplification of the expected 385 bp product for *lef* gene and 264 bp product for *cap* gene and 152 bp product for the chromosomal gene (Figure 1). The detection of plasmid and chromosomal genes of *B. anthracis* from the samples confirmed the disease outbreak as anthrax. The multiplex PCR, for detection of *B. anthracis* in blood, blood smear scrapping, culture, and tissue in this study, is an approved diagnostic test by SCAHLS (Sub-committee on Animal Health Laboratory Standards, Australia). Similarly, a multiplex PCR assay was used for the determination of anthrax foci in different regions of Iran (Moazeni Jula et al., 2004). The PCR assay (Sjostedt et al., 1997, Dragon et al., 2001) and real time PCR assay (Makino et al., 2001, Ryu et al., 2003) were most reliable, rapid, and minimizes the risks of transmission of anthrax in the laboratory The detection of both plasmid and chromosomal markers by multiplex PCR for confirming the virulence of *B. anthracis* (Hornitzy and Muller, 2010) is in accordance with the isolation of *B. anthracis* from the soil in all 12 incidences and indicated that molecular detection is an alternative to culture and isolation.

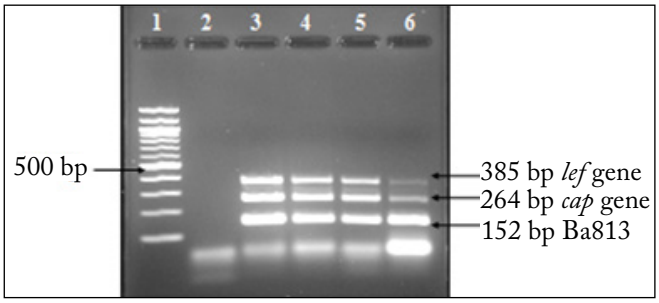

Figure 1: Agarose gel electrophoresis showing amplification of *lef*, cap and *chromosomal* (Ba813) gene of *B. anthracis* by multiplex PCR. Lane-1 100 bp DNA marker, Lane-2 Negative control, Lane-3 DNA - Blood in filter paper (*Tiruvannamalai* Dt), Lane-4 Soil DNA (Vellore), Lane-5 Soil DNA (Nolambur), Lane-6 Soil DNA (Villupuram)

3.2. Phylogenetic analysis of B. anthrax strain

The partial sequence of *cap* gene of isolates (Vellore and Nolambur strain) showed 100% identity with *cap* gene sequences of different strain of *B. anthracis* species on nucleotide BLAST analysis. The phylogenetic analysis of partial *cap* gene sequence of the strain isolated from soil collected from Vellore (accession number MT645311.1) and the strain isolated from Nolambur (accession number MT700999.1) revealed close relationship with *B. anthracis* species (Figure 2). Capsular gene is an important virulent factor on pathogenesis of anthrax; hence the partial sequence of cap gene of isolates and the phylogenetic analysis was carried out to assess the relatedness of the

circulating strain on its virulence. The phylogenetic analysis revealed that the isolates were related to other virulent strains such as Ames and Vollum strain and the nature of disease during outbreak.

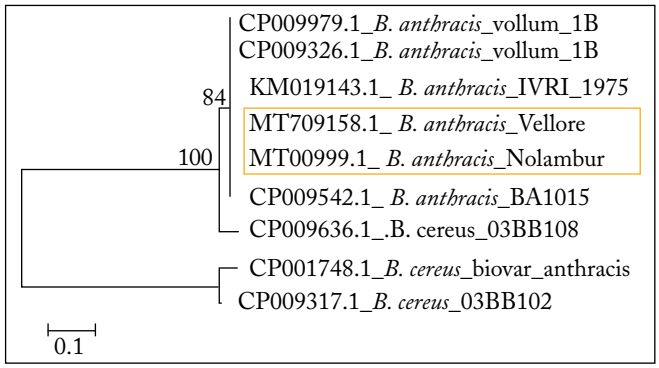

Figure 2: Phylogenetic analysis of isolates based on the partial sequence of cap gene. The dendrogram was built from a 264 bp-based alignment of nucleotide sequences by the Neighbor-joining method, using 1,000 bootstrap replications. Accession numbers are presented and they are followed by the species and strain designations respectively

The molecular method of diagnosis requires short turnaround time of 3–4 h when compared to culture and isolation which requires 2–3 days to confirm the disease. Since the molecular method of diagnosis preclude the need for culturing thereby avoiding unnecessary occupational risks and environmental contamination this molecular method can be used for confirmatory diagnosis of anthrax.

4. CONCLUSION

The anthrax outbreaks were diagnosed by multiplex PCR by amplifying the virulence factors like *lef* gene, capsular gene and chromosomal marker (Ba813) gene of *B. anthrax*. The short turn around time and preclusion of culture, make the molecular methods more advantages than the conventional method for confirmatory diagnosis of anthrax. In addition, this molecular method can be used for surveillance of potential sources of disease, which can be used for planning the prevention and control measures such as vaccination.

5. FURTHER RESEARCH

Further research on real time PCR detection of anthrax from the clinical samples is required since it is rapid, sensitive and avoidance of cross over contamination and post PCR procedures.

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