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Madhanmohan M
Vaccine Research Centre-Viral Vaccines, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Karthik K
Central University Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Ramya R
Central University Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Meenambigai TV
Vaccine Research Centre – Viral Vaccines, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Sivaseelan S
Veterinary University training and Diagnostic Centre, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Thirupparankundram, Madurai, Tamil Nadu, India

Corresponding Author:
Madhanmohan M
Vaccine Research Centre-Viral Vaccines, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Investigation of sheep anthrax outbreak in Madurai district of Tamil Nadu, India

Madhanmohan M, Karthik K, Ramya R, Meenambigai TV and Sivaseelan S

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Abstract
Anthrax is an acute bacterial disease primarily affecting the herbivores and is transmissible to humans. There was a history of sudden death of five adult sheep at T. Kallupatti, Madurai district of Tamil Nadu during the month of January 2020. The disease investigation was carried out. External examination of the carcasses revealed that there was an unclotted blood near the nostrils, at anal orifice and on the floor. Blood smears were prepared from blood from ear vein of all the five dead animals for staining and one blood sample was collected in an EDTA vial from the fresh carcass for molecular diagnosis. Clinical examination of carcasses, positive M'Fadyean reaction and positive polymerase chain reaction amplification of *B. anthracis* protective antigen (596 bp) and capsule (846 bp) confirmed the anthrax outbreak in sheep flock. The outbreak was successfully controlled by using bio security measures and anthrax vaccination of the animal population at risk. Regular surveillance and periodic anthrax vaccination of domestic animals will be useful to control the disease in animals and humans.

Keywords: Sheep, Anthrax, *B. anthracis*, M'Fadyean reaction, Molecular diagnosis, Tamil Nadu

1. Introduction
Anthrax is an acute bacterial disease primarily affecting the herbivores and is transmissible to humans. Anthrax is enzootic in eastern and southern India but is less frequent to absent in the northern Indian states where the soil is more acid (WHO, 2008) [12]. The causative agent *Bacillus anthracis*, is a Gram-positive, spore-forming rod shaped bacterium (WOAH, 2023) [11]. The organism survives in the soil under suitable conditions for long periods of time. The spore forms are markedly resistant to biological extremes of heat, cold, pH, desiccation, chemicals, irradiation and other such adverse conditions (WHO, 2008) [12]. Therefore, the spore forms are the predominant phase in the environment and animals possibly get the infection by the uptake of spores. Climate probably acts directly or indirectly by influencing the way in which an animal comes into contact with the spores. For example, during the summer months the availability of grass is limited and animals grazing closer to the soil, or movement of herds to contaminated sites when water becomes scarce, or by affecting the general health status of the animals and thereby affecting their level of resistance to infection. Anthrax outbreaks in cattle (Gunaseelan *et al.*, 2011; Rajasokkapan *et al.*, 2016) [3, 7] were reported earlier in Tamil Nadu. However, very limited number of anthrax outbreaks in small ruminants particularly in sheep was reported from Tamil Nadu. In this study, we report the anthrax outbreak in sheep flock at T. Kallupatti, Madurai district, Tamil Nadu.

2. Materials and Methods
2.1 History, examination and sample collection
There was a history of sudden death of five adult sheep at T. Kallupatti (Latitude and longitude coordinates**;** 9.7214° N, 77.8553° E), Madurai district of Tamil Nadu (Fig.1) during the month of January 2020. The sheep flock consisted of 113 animals (Ram-03, ewe -90 and lamb -20). The sheep flock was maintained as migratory system of management or rearing. In this practice, the animals are regularly migrated to other places for search of food on natural

vegetation on common grazing lands, wastelands and uncultivated lands, stubbles of cultivated crops. The disease investigation was carried out. External examination of the carcasses revealed that there was an unclotted blood near the nostrils, at anal orifice and on the floor. Blood smears were prepared from blood from ear vein of all the five dead animals for staining. Further, one blood sample (1 ml) was collected in an EDTA vial from the fresh carcass for molecular diagnosis. The suspected blood sample in dry ice was transported to the Central University Laboratory, Chennai through a special messenger.

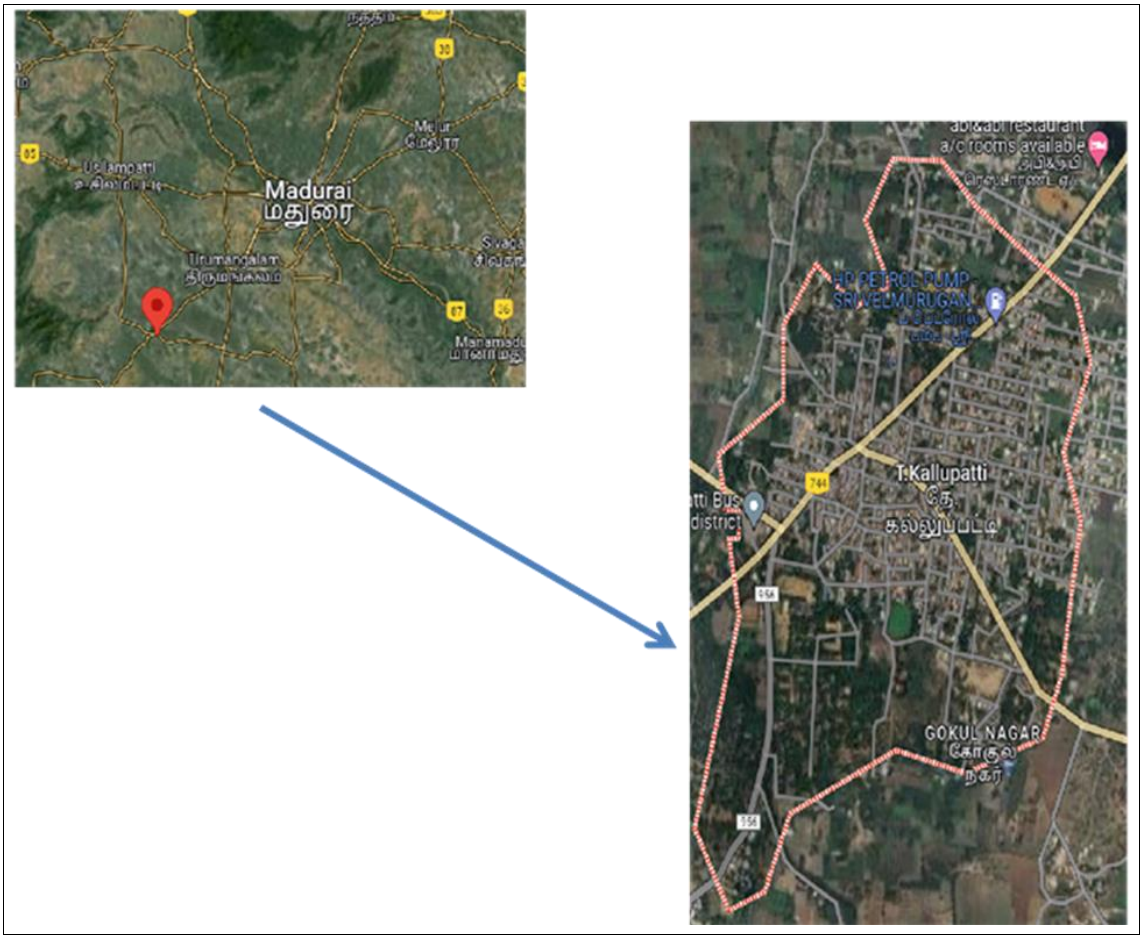


Fig 1: Google map of T. Kallupatti, Madurai district, Tamil Nadu

2.2 Polychrome methylene blue stain (M'Fadyean reaction)

The collected blood smears were dried, fixed using methanol for about 1 minute and air dried and then stained with polychrome methylene blue (M'Fadyean, 1903; WOAH, 2023) [6, 11].

2.3 Molecular diagnosis

The DNA was extracted from blood sample using QIAamp Blood MiniKit (Qiagen, Germany) following the manufactures protocol, quantified and stored at -80°C until further use. PCR was performed to confirm the presence of *B.anthracis* genome in the sheep blood (Beyer *et al.*, 1996; Hutson *et al.*, 1993; WOAH, 2023) [1, 4, 11]. PCR was carried out using the primers PA5 and PA8 specific to protective antigen and primers 1234 and 1301 specific to capsule of *B.anthracis.* The oligonucleotide primer details are presented in Table 1. A 20 µl PCR reaction containing 10 µl PCR master mix (Taq 2X Master mix Red, 1.5 mM $MgCl_2$, Ampliqon), was carried out including specific forward and reverse primers 1 µl (10 pmol) each, 2 µl of genomic DNA (100 to 150 ng) and 4 µl of nuclease free water. PCR amplification was performed in a thermal cycler-T100 (Bio-Rad, USA) with the cycle parameters for PCR were, initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 67°C for 1 min and 72°C for 1 min, and final extension at 72°C for 5 min. Amplified PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized under UV illumination of Gel Documentation system (Bio-Rad, USA).

Table 1: Oligonucleotide primer details

Target	Primer ID	Sequence (5'-3')	Product size
Protective antigen	PA5 (F)	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp
	PA8 (R)	GAG-GTA-GAA-GGA-TAT-ACG-GT	
Capsule	1234 (F)	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bp
	1301(R)	TCC-CAC-TTA-CGT-AAT-CTG-AG	

3. Results and Discussion

3.1 Clinical examination

External examination of the carcasses (n=5) revealed that there was an unclotted blood near the nostrils, at anal orifice and on the floor (Fig.2). There were three ailing lamb showed clinical signs like pyrexia, anorexia, dyspnoea, highly congested mucous membrane and presence of unclotted blood near the nostrils. These clinical signs suggested that the sheep flock was affected with anthrax. Furthermore, other causes of sudden death like botulism, blackleg, chemical poisoning, ingestion of toxic plants, snake bite, lightning strike or metabolic disorders such as lactic acidosis, magnesium deficiency and bloat were ruled out. Anthrax outbreaks in sheep were reported in Andhra Pradesh and Karnataka (Dabbir, 2017; Suresh *et al.*, 2023) [2, 9].

Fig.2: Unclotted blood near the nostrils, at anal orifice and on the floor

3.2 Capsule visualization

The collected blood smear samples were stained with Polychrome methylene blue stain (M'Fadyean reaction) and found the all samples were positive for *Bacillus anthracis* organism. The capsule stains pink, whereas the bacillus cells stain dark blue. The cells were found in pairs or short chains and are often square-ended ('box-car' or 'jointed bamboo-rod' appearance) (Fig. 3) (WOAH, 2023) [11].

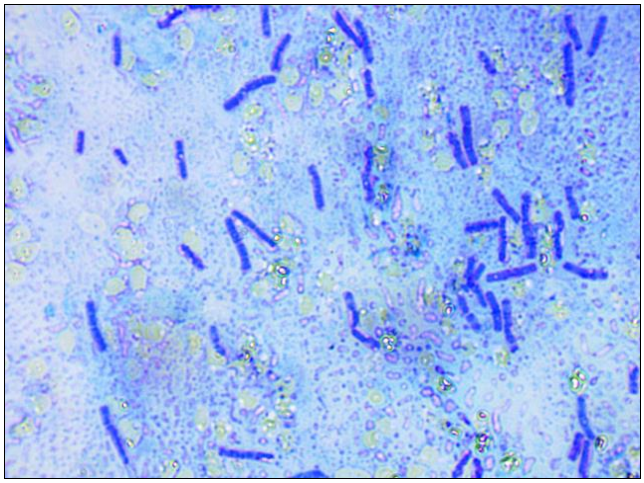
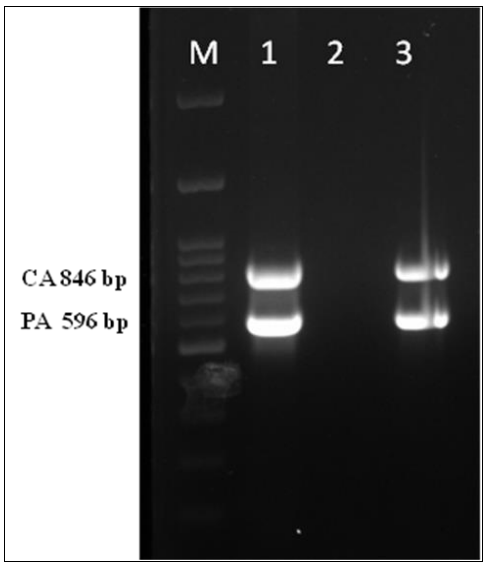


Fig 3: Capsule stains pink, whereas the bacillus cells stain dark blue

3.3 Confirmation of virulence

Confirmation of the virulence was carried out using the PCR for the detection of *B.anthracis* by targeting virulence genes for protective antigen (PA) and capsule (CAP) located on two plasmids, pXO1 and pXO2 respectively. In the current study, the blood sample was positive for both *B.anthracis* protective antigen (596 bp) and capsule (846 bp) (Fig.4) and thus confirmed the virulence of the *B.anthracis* involved in this outbreak. In earlier study (Shasikala, 2018) [8], PCR was successfully used to diagnose anthrax outbreaks in sheep in Karnataka.



M – 100 bp DNA marker; 1- Positive control; 2 – Negative control; 3 – Positive test sample

Fig 4: Agarose gel electrophoresis picture showed anthrax positive protective antigen (596 bp) and capsule (846 bp) amplicon

Once the anthrax outbreak was confirmed, the carcasses were buried following the WHO (2008) [12] protocol. The contaminated area was disinfected with 10% formaldehyde. The affected sheep flock was moved from the infected area. The apparently healthy animals in the flock were vaccinated with live anthrax spore vaccine (Institute of Veterinary Preventive Medicine (IVPM), Ranipet). Further, all the susceptible population at risk from 1-5 KM radius of the infected area was vaccinated with live anthrax spore vaccine (IVPM, Ranipet). Finally, the outbreak was successfully controlled.

Anthrax is a zoonotic disease. Human anthrax outbreaks were reported in Odisha, West Bengal, Assam, Jharkhand, Andhra Pradesh, Tamil Nadu, Puducherry and Karnataka states during 2000-2021 (Jayaprakasham *et al.*, 2023) [5]. The main mode of anthrax transmission to human is handling or consumption of sick or dead animals. Veterinarians, abbatoir workers, farmers, farm labourers, forest labourers and those dealing with animal products face greater occupational hazard of contracting anthrax disease. The interaction between wildlife and livestock may be another important contributor to inter-species transmission and subsequent spill-over to humans (Walsh *et al.*, 2018) [10]. Intensive and regular surveillance of anthrax and regular anthrax vaccination in domestic animals in the anthrax prone areas will be useful to control the disease in animals and humans.

4. Conclusion

Disease investigation was carried out to identify the cause of sudden death in adult sheep. Clinical examination of carcasses, laboratory and molecular diagnosis confirmed the anthrax outbreak in sheep flock. The outbreak was successfully controlled by using bio security measures and anthrax vaccination of the animal population at risk. Regular

surveillance and periodic anthrax vaccination of domestic animals will be useful to control the disease in animals and humans.

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Conflict of Interest
Not available

Financial Support
Not available

6. References

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