Full Length Article

DETECTION OF *MYCOBACTERIUM* SPP. IN DUNG PELLETS OF SPOTTED DEER (*AXIS AXIS*) BY PCR

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ABSTRACT

The study was conducted to investigate the presence of Mycobacterium spp. in dung of spotted deer (Axis axis). Non-invasive sample collection methods were followed to avoid stress. Genomic DNA was extracted from stool samples of spotted deer from Indian Institute of Technology-Madras (IIT-M) and Arignar Anna Zoological Park (AAZP), Vandalur. Samples were collected from five and four different sites of IIT-M and AAZP, Vandalur respectively. The pooled samples from each site were utilized for genomic DNA extraction. Duplex PCR with specific primers, were used to differentiate MTB (M. tuberculosis, M. bovis) and NTB (M. avium). The five samples from IIT-M amplified fragments specific to M. tuberculosis and M. bovis. All the samples collected from AAZP, Vandalur were negative. As the PCR results are presumptive, the PCR positivity of IIT-M samples should be further investigated and confirmed individually with other molecular techniques like sequencing to understand the exact species/genotype circulating in urban wildlife.

Keywords: Mycobacterium tuberculosis complex, faecal pellets, spotted deer, duplex PCR.

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INTRODUCTION

Mycobacterium tuberculosis complex (MTBC) includes *Mycobacterium bovis* and *Mycobacterium tuberculosis* which causes tuberculosis. Non tuberculous Mycobacteria (NTB) *Mycobacterium avium* causes paratuberculosis in wild mammals. The term 'tuberculosis' is derived from the nodules, or 'tubercles,' found in the lymph nodes and tissues of affected animals and humans. While bovine tuberculosis is widespread globally, some countries have successfully eliminated it from their cattle populations, keeping the disease under control (Borham *et al.*, 2022). However, wildlife remains a reservoir for significant infections. Africa and Asia exhibit the highest occurrence of bovine tuberculosis, with documented instances of diverse deer species in distinct locations. Examples include *Odocoileus virginianus* (white-tailed

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deer) in America, *Axis axis* (axis deer) in Hawaii, *Dama dama* (fallow deer) in Ireland, *Cervus nippon* (sika deer) in Ireland, and *Capreolus capreolus* (roe deer) in England. Tuberculosis, attributed to *M. tuberculosis*, has been detected in deer in zoological parks in India and within Indian zoos. The vulnerability of deer to mycobacteria is apparent in the elevated rates of *M. avium* recovery observed across various cervidae families. The objective of the study is to detect the presence of *Mycobacterium* spp. DNA in spotted deer dung pellet samples by PCR. As detection is aiming for screening of common *Mycobacterium* spp. an already reported differential PCR for detection of both MTBC and NTB is utilized in this study.

MATERIALS AND METHODS

Collection of faecal pellet samples

Permission was obtained for collecting the fecal samples of spotted deer (*Axis axis*) from IIT Madras and AAZP, Vandalur campuses in the period of December 2021 to May 2022. Non-invasive sampling method (Fig.1) was followed in the study. Faecal pellets of deer from in and around IIT Madras and AAZP, Vandalur were collected in March 2022 from five different hotspots ranging across a distance of 1600 m (Table 1 and 2, Fig.2 and 3). Five fresh pellets were collected from each location in separate falcon tubes and the GPS coordinates were noted. The pellets were stored at -20ºC until further processing.

Sample preparation

The faecal pellets preserved at -20ºC were thawed down to room temperature, the pellets were scrapped using sterile scalpel and pooled in the falcon tube. 220 mg of pooled pellet sample was weighed from each tube and aliquoted into new 2 mL centrifuge tubes.

Genomic DNA extraction from faecal pellet samples

Nucleic acid extraction from fecal pellets of spotted deer was done using QIAamp Fast DNA Stool Mini Kit (USA) following manufacturer's instructions. After extraction, the eluted DNA was quantified in Nanodrop to evaluate the purity. The elutes were then stored at -20ºC. All centrifugation steps were carried out at 14000 rpm at room temperature.

Duplex PCR

The duplex PCR reported for differentiation of MTB (*M. bovis*, *M. tuberculosis*) and NTB (*M. avium*) were followed in this study.

The reaction mixtures were prepared in 25.0 µL volume as given : Red dye master mix(2X) (Takara)-12.5 µL, Tbc1 Forward primer (10 pmol/µL)-1.0 µL, TbcR5 Reverse primer (10 pmol/µL)-1.0 µL, M5 Forward primer (10 pmol/µL)- 1.0 µL, Rm3 Reverse primer (10 pmol/µL)- 1.0 µL, DNA template-2.0 µL, Nuclease free water-6.5 µL. *M. tuberculosis* positive DNA was received from Translational Research Platform

for Veterinary Biologicals (TRPVB), TANUVAS, Madhavaram, Chennai-51 and treated as positive control. Normal healthy goat faecal pellet sample DNA was used as negative control. The reaction conditions were as follows: Initial denaturation of 94°C for 5 min, 35 cycles of denaturation 94°C for 30 sec, annealing 65°C for 30 sec, extension 72°C for 1 min and final extension of 72°C for 1 min.Ten µL of the amplified product was electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) at constant 80V for 30 min in 1X TAE buffer along with DNA molecular weight marker 100 bp based on PCR product size.

Organisms	Primer	Size	Target
MTB (M. bovis and M. tuberculosis)	Tbc1:5' CGTACGGTCGGCGAGCTGATCCAA 3' TbcR5:5' CCACCAGTCGGCGCTTGTGGGTCAA 3'	235 bp	gene rpoB encoding RNA polymerase b-subunit
NTB (M. avium)	M5: 5' GGAGCGGATGACCACCCAGGACGTC 3' Rm3: : 5' CAGCGGGTTGTTCTGGTCCATGAAC3'	136 bp	

RESULTS AND DISCUSSION

The number and positive results of samples screened by duplex PCR primers are listed separately in Table 3 and shown in figures 4 and 5. Four numbers of pooled samples collected from AAZP, Vandalur showed no positive amplification whereas five numbers of pooled samples collected from IIT-M showed positive amplification for *M. bovis* and MTb.

Tuberculosis in deer is primarily caused by *M. bovis* and only rarely by *M. tuberculosis*. Reports of avian tuberculosis associated with *M. avium* infection in deer are scarce in India and are therefore considered to pose a comparatively lower risk than infections caused by *M. bovis* and *M. tuberculosis*. However, *M. avium* infection in deer has been reported more frequently in other countries.

The purpose of this study was to investigate the presence of Mycobacterium DNA in dung pellet DNA samples of free-ranging spotted deer in two campuses of Chennai by PCR. Dung pellet is the most favorable clinical sample for disease diagnosis in deer, as the animals are prone to capture myopathy while restraining for other sample collection methods and diagnostic tests. Attempts were made to detect the presence of DNA of *Mycobacterium* spp. from dung pellet samples collected from free-ranging spotted deer. All the samples collected from AAZP, Vandalur were negative by PCR.

Pooled sample methodology is based on the Wassermann test and involves combining small portions of several samples to create a pooled sample equal to the amount needed for a single test. A single test is then performed on the pooled sample, enabling the detection of one or more

infected individuals within a larger group without testing each sample separately. If the pooled sample tests positive, the individual samples are subsequently tested to identify the true positive cases. This approach facilitates rapid large-scale screening while conserving resources and reducing testing time (Leonardi-Cattolica *et al*. 2024).

As the PCR results are presumptive, the PCR positivity of *Mycobacterium* spp. from fecal pellet samples of IIT-M should be confirmed individually with other molecular techniques i.e. sequencing for further confirmation. The PCR performs well as a confirmatory test on cultures, being its sensitivity close to 100% (Manning and Collins, 2001), but its application to clinical samples especially faecal pellet samples has been problematic, mainly due to the problems associated with DNA extraction from complex matrices such as milk, feces and blood, and the presence of PCR inhibitors (Stevenson and Sharp, 1997; Grant *et al*., 1998; Aly *et al*., 2010; Stevenson, 2010), decreasing its sensitivity. The limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, the matrix tested, and the PCR format (conventional gel- based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, or multiplex PCR) (Mobius *et al*., 2008; National Advisory Committee on Microbiological Criteria for Foods, 2010; Stevenson, 2010). Ideally, environmental sampling, serial testing, and the use of two to three diagnostic tests would be the recommendation for herd screening, to increase the accuracy of Mycobacterium spp. diagnosis (Collins *et al*., 2006; Stevenson, 2010).

Tuberculosis in deer may present either as a subacute form, in which animals become severely affected within six months of infection, or as a chronic form, where no obvious clinical signs are evident for several years. Clinical manifestations are variable depending on lesion distribution, but progressive emaciation and enlargement of lymph nodes are commonly observed. Chronically infected animals without overt clinical signs may act as carriers, harboring significant numbers of bacilli that can subsequently be disseminated into the environment. Recently, Sharma *et al.* (2022) confirmed the presence of *M. bovis* in samples collected from both free-ranging and captive spotted deer across India.

The organism is relatively resistant and can survive in the environment for prolonged periods under favorable conditions. *Mycobacterium* spp. may persist on pasture for up to eight weeks when protected within fecal matter. Since these organisms act as obligate pathogens upon entering the host, ingestion of contaminated feed or water, as well as inhalation of infectious aerosols, can lead to infection in susceptible animals.

A one health approach should be adopted to assess the risk of tuberculosis in animals, humans, and the environment. Further investigations, including species-level identification and sequencing, are necessary to confirm the presumptive PCR-

positive findings in animals. In addition, random screening of human samples from IITM may help evaluate the potential zoonotic risk and possible transmission of the organism.

CONCLUSION

Zoonotic infections originating from Mycobacterium species pose a potential threat to both humans and animals. In this study, faecal pellet sampling of cervids is used as noninvasive technique for identification of organism by molecular technique like PCR However, the initial screening was performed using pooled samples, and the PCR results obtained are therefore considered presumptive. Further epidemiological herd-level screening is urgently required to confirm and assess the extent of infection.

Table.1. Sampling locations of IIT-M

Sampling site number	Location name	Google map coordinates
SITE - 1	Hostel office	12.987377981833404N, 80.23745398590795E
SITE - 2	Sangam ground	12.985575838509849N, 80.23765956939083E
SITE - 3	Watsa stadium	12.992265966027755N, 80.23668605982596E
SITE - 4	NAC lawn	12.992811659364024N, 80.22979841631648E
SITE - 5	Biotech lawn	12.989609864538652N, 80.22785714959663E

Table.2. Sampling locations of AAZP, Vandalur

Sampling site number	Location name	Google map coordinates
SITE - 1	Deer safari	12.879822116938268N, 80.08285117382238E
SITE - 2	Animal quarantine area	12.875120145435702N, 80.08988836813867E
SITE - 3	Hospital percolation pond	12.874586731178878N, 80.09305337466735E
SITE - 4	Veterinary hospital	12.874584732031742N, 80.09062012899041E
SITE - 5	Biotech lawn	12.989609864538652N, 80.22785714959663E

Table.3: Number of faecal DNA samples screened by duplex PCR primers for *Mycobacterium* spp.

Place of sample collection	Number of pooled faecal samples screened by duplex PCR primers	Mycobacterium tuberculosis complex(MTBC)	Non tuberculous Mycobacteria (NTB)
AAZP, Vandalur (4 places)	4 X 5 = 20	-	-
IIT-M (5 places)	5 X 5 = 25	5	-

Fig. 1. Collection of dung pellet samples from IIT Madras and AAZP, Vandalur campuses (a) Physical consistency, (b) of dung pellet samples during collection

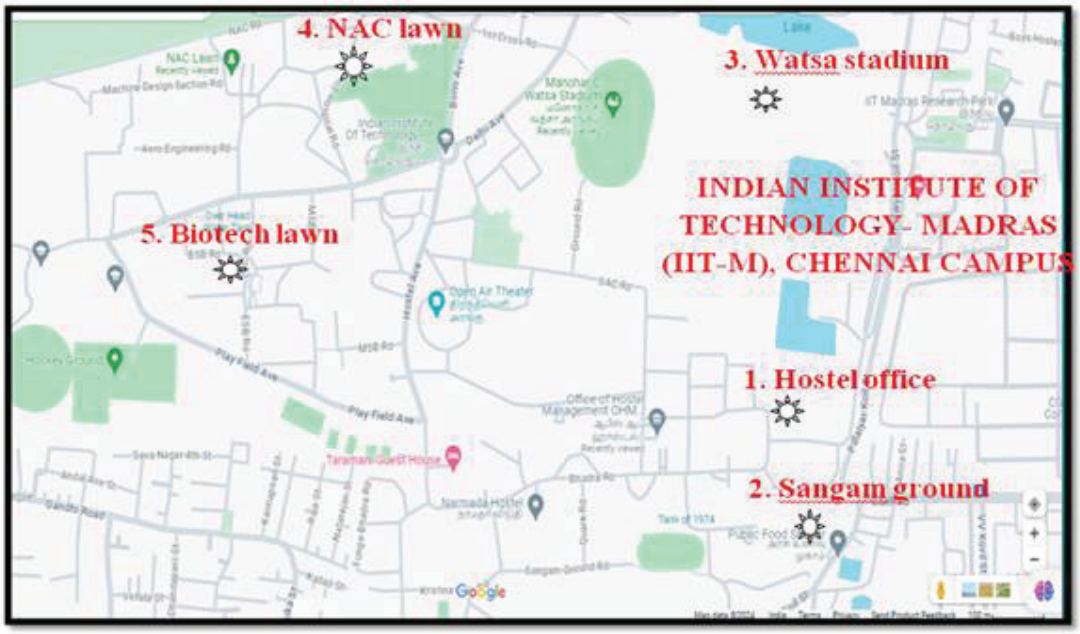

Fig. 2. Google map representation of sampling locations (1-5 represented by stars) at IIT-M, Chennai

Fig. 3. Google map representation of sampling locations at AAZP, Vandalur

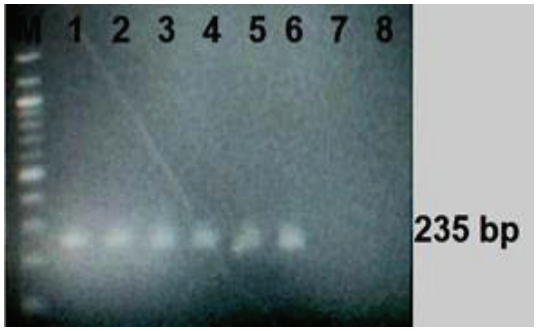

Fig.4. Agarose gel electrophoresis of PCR product on faecal pellet DNA samples collected from IIT-M by duplex PCR. Lane M: 100 bp ladder, Lane 1: *Mycobacterium tuberculosis* positive control, Lane 2 to 6: MTB (235 bp), Lane 7: negative control, Lane 8: non-template control (NTC)

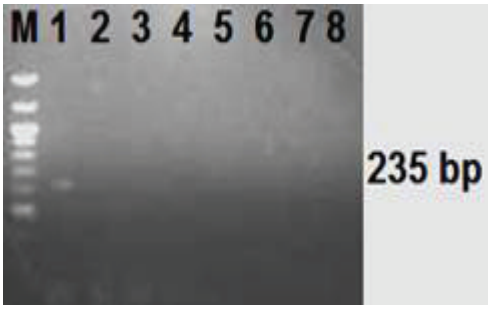

Fig.5. Agarose gel electrophoresis of PCR product on faecal pellet DNA samples collected from AAZP,Vandalur by duplex PCR. Lane M: 100 bp ladder, Lane 1: *Mycobacterium tuberculosis* positive control, Lane 2 to 6: negative samples with no amplification, Lane 7: negative control, Lane 8: non-template control (NTC)

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