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Detection of *Mycobacterium tuberculosis* in Captive Sloth Bears in Karnataka

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A B S T R A C T

Tuberculosis (TB) is contagious disease of zoonotic importance. The disease is caused by organisms belonging to the *Mycobacterium* spp. The important species responsible for TB are *M. bovis*, *M. tuberculosis* and *M. avium*. Mycobacteria infect a broad range of species including humans, wild mammals and birds in captivity and free-living state. Diagnosis of mycobacterial infection is very important from the public health perspective, particularlyin captive animals. In the present study a total of 25 sloth bears kept in bear rescue center, Bannerghatta National Park, Bengaluru, Karnataka, India were screened for tuberculosis. The Bronchial wash, direct tracheal tube smear and fecal samples from all the sloth bears were subjected for Ziehl-Neelsen staining technique. Among these samples, only one fecal sample was found positive for *M. tuberculosis*. None of the bronchial wash samples were positive by culturing in Lowenstein Jensen (LJ) media. Six samples yielded *M. tuberculosis* by culturing in Mycobacteria Growth Indicator Tube (MGIT) 960 media. Three out of 25 bronchial wash samples were positive for *M. tuberculosis* by RT micro PCR. None of the blood samples of sloth bears were positive for *M. tuberculosis* species by PCR. This study demonstrated that culturing in MGIT960 media and RT micro PCR are the useful diagnostic tests for detection and identification *M. tuberculosis* in captive sloth bears. This study will be helpful for the screening of the tuberculosis in animals and prevent the spreading of the infection to other wild animals and humans visiting to the Biological National Park.

Introduction

Tuberculosis is a chronic infectious disease of public health significance. It is primarily a pulmonary disease; however, any organ system may become infected and extra pulmonary infection may occur alone or concurrently. The disease is caused by organisms belonging to the *Mycobacterium*

spp. They are distinguished from other bacteria by their ability to synthesize mycolic acids and the presence of a thick, waxy cell envelope. On the basis of growth rate, catalase and niacin production, and pigmentation in light or dark, mycobacteria are classified in to members of the *Mycobacterium* tuberculosis complex (*M. tuberculosis, M. bovis, M. africanum, M. canetti, M. caprae, M. pinnipedii and M. microti*). Three important species of genus *Mycobacterium* responsible for TB are *M. bovis, M. tuberculosis* and *M. avium*.

Mycobacteria infect a broad range of species including humans, wild mammals and birds in captivity and free-living state (Rathore and Khera, 1982), non-human primates, sloth bears (Mehrotra *et al.,* 1999), free ranging carnivores (Brunning-Fann *et al.,* 2001), marine mammals, reptiles, fish, pachyderms, domestic and non-domestic ungulates. Mammals are found to be more susceptible to bovine and human type of TB than to avian type.

For more than a century, tuberculosis has been recognized as a serious clinical entity in wild animals maintained in captivity. Tuberculosis is more prevalent in sloth bears which are found in India and Sri Lanka. The disease is probably non-existent in wild sloth bears remote from human habitationor association. Tuberculosis caused by *M. tuberculosis* has become a major killer of bears, especially of those which have been rescued from nomadic tribes/Kalandars (bear charmers). They are exposed to severe stresses like trauma, various physical and psychological illness, malnourishment etc., during their stay with Kalandars and hence making them easily susceptible for various diseases like tuberculosis (Renukaprasad *et al.,* 2013).

Diagnosis of disease during early stages in bear population is challenging. Due to its importance as a zoonotic and regulatory disease, accurate diagnostic testing is crucial for detection and control of the disease. Lack of diagnostic tools for most species and the absence of an effective vaccine make it currently impossible to contain and control this disease within an infected free-ranging ecosystem. In view of the significance of tuberculosis infection among captive sloth bears, detailed study was undertaken for detection and identification *M. tuberculosis* in captive sloth bears.

Materials and Methods

Collection of Samples

Twenty five sloth bears were selected for this study from bear rescue center, Bannerghatta Biological Park, Bengaluru, Karnataka, India. The bronchial wash, blood, direct tracheal tube smear and fecal swab were collected from each animal after anaesthetizing with Xylazine and Ketamine at the dose rate of 1mg and 3mg per kg body weight respectively.

Ziehl-Neelsen acid-fast staining

The bronchial wash and fecal swab contents were centrifuged at 10,000 rpm for 15 min. and smear was prepared from a drop of sediment, air dried and heat fixed. After collecting bronchial wash, the tracheal tube was removed and the smear was taken immediately by placing a clean glass slide on to the tracheal tube air dried and heat fixed. Smears prepared from bronchial wash, direct tracheal tube and fecal swab were subjected for Zeil-Neelsen staining as described by Coles (1986) for the demonstration of acid fast bacilli.

Culture and isolation by LJ media

The bronchial wash was decontaminated with

double the volume of 4% Sodium hydroxide by Petroff's method adapted by Srivastava *et al.,* (2008). The decontaminated bronchial wash was centrifuged at 10,000 rpm for 15-20 min. The supernatant was discarded. The sediment was washed with normal saline and centrifuged at 10,000 rpm for 5-10 min. A loopful of sediment was inoculated onto the Lowenstein Jensen (LJ) media slants. The slants were incubated at 37°C for 6 to 8 weeks and observed at weekly intervals.

Culture and isolation by MGIT960 Liquid media

About 10 ml of bronchial wash from sloth bears was mixed with 50 ml of 4% sodium hydroxide, 50 ml of 2.9% sodium citrate and 0.5% N -acetyl cysteine for digestion and decontamination. Centrifuged for 20 min at 3,000rpm and supernatant was discarded. The pellet was reconstituted with 1 ml of sterile phosphate buffer, 0.5 ml of this was inoculated to the BACTEC MGIT (Mycobacteria Growth Indicator Tube) 960 liquid culture media. Immediately the tube was recapped tightly, mixed by inverting the tube several times and the inoculated tubes were left at room temperature for 30 minutes. BACTEC MGIT960 tubes were loaded into the BACTEC MGIT960 instrument within 2 hrs of inoculation at National Tuberculosis Institute, Bangalore. The instrument was maintained 37ºC + 1ºC temp. and growth in the BACTEC MGIT960 medium tubes was automatically detected by the BACTEC MGIT960 instrument.

Polymerase Chain Reaction (PCR)

DNA extraction from blood samples was carried out as described by Sambrook and Russel (1989). PCR amplification was done by using primers TB1F (5'-CCTGCGAGC GTAGGCGTCGG- 3') and TB2R (5'-CTCG TCCAGCGCCGCTTCGG- 3'). The PCR reactions were performed by adding 1 µl of extracted DNA as a template to 50 µl of a reaction mixture containing 10x PCR buffer, 25 pMol TB1F, 25 pMol TB2R, 10 mM of each deoxynucleoside triphosphate (dNTP) and 1U of Taq polymerase. The amplification was carried out with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58° C for 1 min and extension at 72° C for 1 min. The final extension was carried out at 72°C for 10 min to obtain final PCR products. The amplified PCR products were run on 2% agarose gel electrophoresis at 60 volts for 45 min. and visualized for bands under UV trans illuminator.

Real time Micro PCR

The bronchial wash from sloth bears were also subjected for RT (Reverse Transcriptase) micro PCR which is a patented technology and the assay was done using micro PCR kits (Bigtec Labs, Bangalore) and procedure was followed according to manufacturer's protocol.

Results and Discussion

In the present study bronchial wash, direct tracheal tube smear, fecal swab and blood samples were collected from sloth bears of Bannerghatta National Park, Bengaluru, Karnataka, India for the screening of tuberculosis by Ziehl-Neelsen staining technique, Culture by LJ media, Culture by MGIT960, PCR and RT micro PCR molecular techniques (Table 1).

None of the 25 bronchial wash smears and direct tracheal tube smears from sloth bears revealed positive for the presence of mycobacterial organisms by Ziehl-Neelsen staining technique. This may be attributed to fact that shedding of small quantity of bacilli during the sample collection and minimum of

10^4-10^6 bacilli/ml of tissue or fluid specimens are required to give a positive result. In contrast, Rishikesavan *et al.,* (2008) reported eight positives out of 25 nasal discharges from the sloth bears. This may be due to variation in the stage of the disease and extent of lesion in the lungs as shedding of organisms will be minimal once the lesion is encapsulated.

One out of 25 fecal smear samples revealed presence of mycobacterial organism by Ziehl-Neelsen staining technique with 4% occurrence of the disease. However, perusal of literature did not reveal any information on diagnosis of TB by acid-fast staining in fecal sample of sloth bears. Veeraselvam *et al.,* (2008) reported five out of 42 fresh fecal samples positive for *M. bovis* by PCR method. This may be attributed to swallowing of tuberculous bacilli in the nasal discharges and excretion in the fecesas opined by Good (1984). However, the tuberculous bacilli can be found in the fecal samples of animals with disseminated and intestinal form of tuberculosis.

None of the 25 bronchial washes from sloth bears on LJ media yielded growth of mycobacteria. Perusal of literature did not reveal any studies on culturing of mycobacteria in bronchial wash. However, LJ media has been used for diagnosis of *Mycobacterium* using tracheal/bronchial wash culturing in other species of animals by various workers (Witmer *et al.,* 2010, Traversa *et al.,* 2009, Corner *et al.,* 2008). Number of organisms shed in the bronchial washes of sloth bear may not be sufficient for culturing in LJ media. Chawla *et al.,* (2009) was of the opinion that culture for *Mycobacterium* needs 10^1-10^2 bacilli/ml of sample for the diagnostic yield. In the present study less number of organisms could be the reason for negative results.

In the present study, six out of 25 bronchial wash samples from sloth bears were positive for *M. tuberculosis* by MGIT 960 culture media with the occurrence rate of 24% (Fig.1). However, no work has been carried out in diagnosis of TB in sloth bears by MGIT culturing and this seems to be the first report. Further, when compared to LJ media, even small numbers of organism were detected by MGIT 960 instrument. This may be the reason for getting higher positive result in MGIT 960 media. Further, all the isolates were confirmed as *M. tuberculosis* based on biochemical tests carried out at National Tuberculosis Institute (NTI), Bangalore. The sloth bears in the study had been rescued from kalandars and they could have picked the infection from other domestic animals and humans.

All the 25 blood samples in the present study revealed negative results for *M. tuberculosis* by PCR. This maybe due to low number of organisms or no organisms in the blood of affected bears. This is in agreement with Alfonsoa *et al.,* (2004) who opined that mycobacterial presence in blood is quite rare; however, it is a consequence of hematogenous bacilli dissemination.

In the present study, three out of the 25 bronchial wash from sloth bears were positive for *M. tuberculosis* by RT micro PCR with occurrence rate of 12% (Fig.2). Scanning of literature did not reveal any information on application of RT micro PCR for diagnosis of tuberculosis in bears and this seems to be the first work on diagnosis of tuberculosis by RT micro PCR. Chaitali *et al.,* (2013) applied RT micro PCR for detection of *M. tuberculosis* in clinical sputum specimens of human beings by using the same RT micro PCR and Truenat MTB test. They opined that, the load of bacilli required to obtain a positive culture was 100 viable bacilli, the lower detection limit of conventional PCR was 10 copies and for RT micro PCR was six copies.

Table.1 Results of the screening of various samples by different tests from sloth bears for tuberculosis

Sl No.	Samples	Tests conducted	No. tested	No. positive	Per cent positive
1	Bronchial wash	Ziehl-Neelsen staining technique	25	0	0
2	Direct tracheal tube smear	Ziehl-Neelsen staining technique	25	0	0
3	Fecal swab	Ziehl-Neelsen staining technique	25	1	4
4	Bronchial wash	Culture by LJ media	25	0	0
5	Bronchial wash	Culture by MGIT960	25	6	24
6	Blood	PCR	25	0	0
7	Bronchial wash	RT-PCR	25	3	12

Fig.1 Mycobacterial growth in the BACTEC MGIT960 medium tubes.

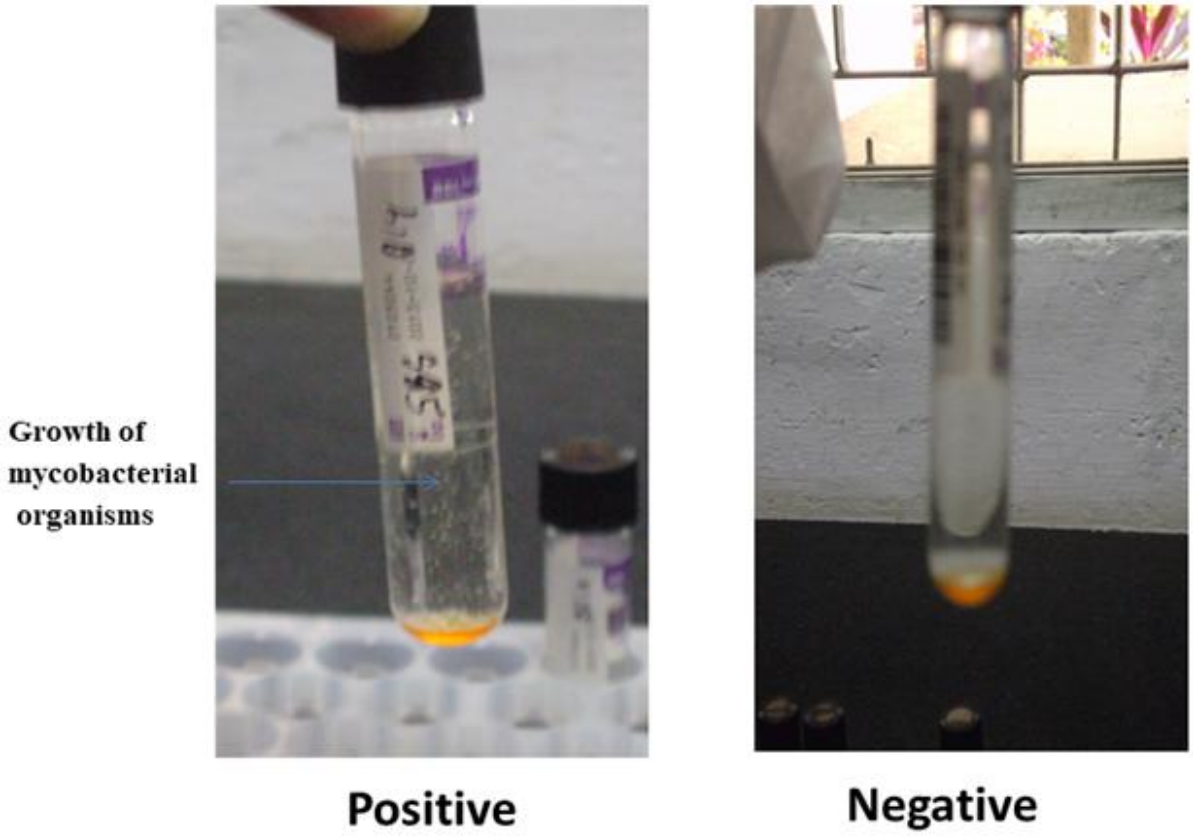

Fig.2 Screen capture images of micro RT PCR while running the test procedure: A. Graphical representation showing positive result of animal 1 indicated by elevated green curve in correspondence with red internal control. B. Screen capture image showing animal details and results positive for *M. tuberculosis*.

A B

In the present study, MGIT 960 was found to be more beneficial as it detected six animals positive for tuberculosis as compared to RT micro PCR which detected only three animals. However, RT micro PCR although it is sensitive, requires more number of organisms than MGIT 960 method for DNA extraction and PCR detection. When compared to culture by MGIT 960 the RT micro PCR was found to be 33.33% sensitive. However, RT micro PCR was found to be quicker (45 min) as compared to MGIT 960 (12-15 days) and easy to perform. RT micro PCR is supposed to be more sensitive than culturing. However, RT micro PCR kit method is standardized for sputum in human beings. The samples collected in bears were bronchial washes and kit procedure may also require modification for DNA extraction. Always culturing is considered as confirmative and this MGIT 960 method is more sensitive and faster than LJ media. Even a few organisms can be detected as they develop onto individual colonies.

Subsequently, five out of seven cases detected positive by various tests have died of tuberculosis in Bannerghatta Biological Park, Bengaluru (Arun Sha, 2013, personal communication). This indicates the benefit of testing sloth bears in endemic groups which were hitherto detected mostly by post-mortem examination.

The screening for tuberculosis was carried out in 25 sloth bears kept in bear rescue centre, Bannerghatta National Park, Bengaluru, Karnataka, India. The Bronchial wash, direct tracheal tube smear and fecal samples from 25 sloth bears were subjected for Ziehl-Neelsen staining and only one fecal sample was found to be positive. None of the 25 bronchial wash were positive by culturing in LJ media, while

six samples yielded *M. tuberculosis* by culturing in MGIT 960 media. None of the blood samples of sloth bears were positive for mycobacteria species by PCR. Three out of 25 bronchial wash samples were positive for *M. tuberculosis* by RT micro PCR. This study is helpful for the screening and identification of the *M. tuberculosis* in the sloth bears which is necessary for control and prevention of zoonotic disease.

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