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Short Paper

A study on clinical diagnosis of tuberculosis in free ranging and captive wild animals of India

Sharma, M.[1]; Karikalan, M.[2*]; Asok Kumar, M.[3]; Sree Lakshmi, P.[1]; Sharma, K.[2]; Ilayaraja, S.[4]; Mathur, A.[5] and Pawde, A. M.[2]

[1]Ph.D. Student in Pathology, Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India; [2]Centre for Wildlife Conservation, Management and Disease Surveillance, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India; [3]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India; [4]MSc in Microbiology, Agra Bear Rescue Centre, Wildlife SOS, Keetham-281122, Agra, Uttar Pradesh, India; [5]MVSc in Surgery and Radiology, Nahargarh Biological Park, Kukas-302028, Rajasthan, India

[*]***Correspondence:*** *M. Karikalan, Centre for Wildlife Conservation, Management and Disease Surveillance, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India. E-mail: karyvet11@gmail.com*

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Abstract

Background: Tuberculosis (TB) is a disease of paramount importance at the wildlife-livestock-human interface. **Aims:** To study the occurrence and *Mycobacterium* (M) species involved in the TB of free-ranging and captive wild animals in various Indian states. **Methods:** A total of 396 clinical samples from 207 different wild animal species from various Indian national parks, zoological gardens, etc., were analyzed by lateral flow assay (LFA), Ziehl-Neelsen (ZN) staining, and PCR. Clinical samples include blood (n=156), faecal swabs (n=103), serum (n=73), and nasal swabs or trunk wash fluids (n=64). **Results:** Clinical signs of TB were absent in 202 animals, although 21 wild animals were seropositive for pathogenic *Mycobacterium* antigens by LFA. Clinical signs like progressive weight loss, and respiratory distress were exhibited by 4 sloth bears (*Melursus ursinus*) and an elephant (*Elephas maximus*), which were also found positive for LFA, PCR, and ZN staining. ZN staining showed positivity for acid-fast bacilli (AFB) in 9 (8.74%) faecal and 9 (14.06%) nasal swabs or trunk wash fluids of sloth bears (7 samples) and elephants (2 samples). *M. tuberculosis* was detected in 7 sloth bears and 2 elephants, whereas *M. bovis* was found in a spotted deer (*Axis axis*) by species-specific PCR. **Conclusion:** The circulation of TB organisms in wild animals warrants a strict surveillance programme to identify the carrier status of these animals so that effective TB control strategies can be formulated.

Key words: Elephant, Lateral flow assay, *Mycobacterium*, Sloth bear

Introduction

The complex and dynamic interactions between humans, domestic animals, and wildlife lead to an increased chance of the emergence of diseases in new host species (Hassell *et al*., 2017). Tuberculosis (TB), a deadly air-borne disease, has proved to be a worldwide problem at the wildlife-livestock-human interface. It is caused by a group of intimately related bacterial species termed the *Mycobacterium tuberculosis* complex (MTBC) (Brites and Gagneux, 2017). According to WHO's global TB report 2019, under-developed and developing countries record nearly 1.4 million human deaths every year due to TB. India has the world's highest bovine population with 21.8 million TB affected cows (Srinivasan *et al*., 2018), thus creating a major wellspring for spill-over to various hosts like humans and wild animals. Amongst the members of MTBC, *Mycobacterium bovis* is having the widest host range thus infecting many species of domestic animals, wildlife, and also humans (Bernitz *et al*., 2021; Ncube *et al*., 2022). *M. tuberculosis* is majorly a human pathogen, however, causes TB in domestic and wild animals living in close and prolonged contact with humans (Teppawar *et al*., 2018). Ante-mortem diagnosis of TB in wild animals is difficult due to the sub-clinical nature of the disease and the limited application of the standard diagnostic tests employed for domestic animals and humans (Thomas *et al*., 2021). Culture stands as the gold standard, but culture-based approaches to wild animals have several shortcomings, like trouble with sample collection, problems related to sample contamination, and transport (Lekko *et al*., 2021; Thomas *et al*., 2021). The tuberculin skin test (TST) is the most commonly used test in domestic animals, but difficulties in capturing wild animals after 72 h and optimizing tuberculin doses make it unsuitable for free-roaming wild animals (Che-Amat *et al*., 2016). Serological assays, being simple, rapid, and relatively inexpensive, offer a useful alternative. The immunochromatographic test

(ICT), viz., lateral-flow assay (LFA), an animal-side test that uses a cocktail of a limited number of antigens, has several advantages for application in wild animals (Bruns *et al*., 2017; Fresco-Taboada *et al*., 2019). In addition, PCR primers designed to target *16S rRNA*, genes coding for MTBC specific proteins and insertion sequences (IS6110) can be used as a rapid identification tool of mycobacterial isolates (Verma *et al*., 2022). The majority of the work on TB in wild animals in India is based on morbid data and there are very few reports on ante-mortem diagnosis of TB in wild animal species that are too limited in terms of identification of the MTBC species involved (Sharma *et al*., 2014). This study documents *M. tuberculosis* infection in seven sloth bears (*Melursus ursinus*) and two elephants (*Elephas maximus*) as well as *M. bovis* in a spotted deer (*Axis axis*), based on LFA, ZN staining, and PCR.

Materials and Methods

Clinical samples and DNA isolation

A total of 207 wild animals belonging to various species of bovids, cervids, carnivores, non-human primates, and pachyderms from various national and zoological parks, rescue centers, etc. across 10 Indian states (Fig. 1; Supplementary Table 1 (ST1)) with or without any apparent disease symptoms were examined clinically from August 2017 to November 2020. Samples included 156 blood samples, 103 faecal swabs, 73 serums, and 64 nasal swabs/trunk wash fluids were collected (Table 1). Total DNA was isolated using the

Fig. 1: The geographical distribution of different zoological and national parks, safaris, and state forest departments from where the clinical samples were collected/received in India. The stars (★) show the distribution in 10 states of India

Table 1: Species-wise details of different clinical samples

S. No.	Species	Total clinical cases screened	Samples screened				
			Nasal swab/trunk wash fluid[a]	Faecal swab	Blood	Serum	Total
A	**BOVIDS**						
1	Chinkara (*Gazella bennettii*)	6	6	6	3	6	21
2	Wildebeest (*Connochaetes taurinus*)	6	6	-	6	6	18
3	Oryx (*Oryx gazella*)	4	4	4	4	4	16
4	Blackbuck (*Antilope cervicapra*)	3	-	2	2	3	7
5	Giraffe (*Giraffa camelopardalis*)	2	2	2	2	2	8
6	Nilgai (*Boselaphus tragocamelus*)	2	-	2	-	-	2
7	Impala (*Aepyceros melampus*)	1	1	1	1	1	4
8	Indian gaur (*Bos gaurus*)	1	-	1	-	-	1
Total		25	19	18	18	22	77
B	**CERVIDS**						
1	Spotted deer (*Axis axis*)	13	5	7	3	3	18
2	Sambar deer (*Rusa unicolor*)	5	-	3	2	2	7
3	Thamin deer (*Panolia eldii*)	2	2	2	2	2	8
Total		20	7	12	7	7	33
C	**CARNIVORES**						
1	Lion (*Panthera leo*)	78	-	9	69	1	79
2	Sloth bear (*Melursus ursinus*)	11	11	10	-	7	28
3	Leopard (*Panthera pardus*)	5	-	5	2	2	9
4	Tiger (*Panthera tigris*)	3	-	3	-	1	4
5	Jackal (*Canis aureus*)	1	-	1	-	-	1
Total		98	11	28	71	11	121
D	**NON-HUMAN PRIMATES**						
1	Bonnet macaque (*Macaca radiata*)	1	-	1	-	-	1
2	Hoolock gibbon (*Hylobates hoolock*)	10	5	10	10	5	30
3	Rhesus macaque (*Macaca mulatta*)	2	-	2	-	-	2
Total		13	5	13	10	5	33
E	**PACHYDERMS**						
1	Elephant (*Elephas maximus*)	50	22	31	50	28	131
2	Rhinoceros (*Rhinoceros unicornis*)	1	-	1	-	-	1
Total		51	22	32	50	28	132
	Grand total A+B+C+D+E	207	64	103	156	73	396

[a] In the case of elephants

DNeasy blood and tissue kit and the QIAamp DNA Stool Mini kit (Qiagen, Germany) as per the recommendations of the manufacturers along with suitable modifications. All samples were properly labeled and stored at -20°C until further use.

Clinical examination

Clinical examination of wild animals under study was done following physical and chemical immobilization. The physical parameters like body condition, color of the mucous membranes, palpation of peripheral lymph nodes, and absence or presence of nasal discharge were recorded.

Lateral flow assay

Serum samples collected during clinical examination were screened for antibodies against MTBC organisms using the MycoPac Dual kit (Cisgen Biotech, Chennai, Tamil Nadu, India). The LFA kits and sera samples were first brought to room temperature, after which about 10 µL of the test serum was added to the two sample wells of the kit, and after a few minutes, 2 drops of sample buffer were added into both the wells. The results were read within 5-10 min. The appearance of the colored line in the control area is a must to validate the results of the kit. The appearance of a colored reaction in either of the test lines (1 and 2) or in both of them was considered as seropositive against TB. The presence of a colored reaction in test lines 2 and/or 4 was an indication of seropositivity against pathogenic MTBC organisms. The development of a colored reaction only in test line 3 was interpreted as sero-reactive against NTM organisms.

Ziehl-Neelsen staining

Ziehl-Neelsen (ZN) staining method was used to detect acid-fast mycobacteria in heat fixed smears prepared from nasal and faecal swabs as described earlier (Sharma *et al*., 2022).

Molecular diagnosis

DNA samples were examined by conventional PCRs using primers IS6110 F: CTC GTC CAG CGC CGC TTC GG, IS6110 R: CCT GCG AGC GTA GGC GTC GG for identification of MTBC organisms (Miller *et al*., 1997) and 12.7 kb F: TTC CGA ATC CCT TGT GA, 12.7 kb R1: GGA GAG CGC CGT TGT A, 12.7 kb R2: AGT CGC CGT GGC TTC TCT TTT A for differentiation of *M. bovis* and *M. tuberculosis* (Bakshi *et al*., 2005). The amplified products obtained were confirmed to be of expected size in 1.5% (w/v) agarose gel (Thermo Scientific, USA) in 1X Tris Acetate EDTA buffer.

Results

The clinical signs of TB were found to be absent in most (n=202) of the examined wild animals. There was no manifestation of any clinical signs, even in 16 sero-positive (LFA) animals. However, four sloth bears exhibited clinical signs like variable degrees of emaciation, dullness, pale mucous membranes, intermittent anorexia, and respiratory distress. Progressive weight loss was also observed in an elephant (Fig. 2). Out of the total 73 serum samples screened by LFA, 21 (28.76%) serum samples, comprising of eight (38.09%) bovids, i.e., three wildebeest (*Connochaetes taurinus*), two blackbuck (*Antilope cervicapra*), two giraffe (*Giraffa camelopardalis*), and one chinkara (*Gazella bennettii*), seven (33.34%) sloth bears, two (9.52%) cervids, i.e., sambar deer (*Rusa unicolor*) and spotted deer (*Axis axis*), and four (19.04%) elephants (*Elephas maximus*) were tested positive for antibodies against MTBC organisms (Fig. 3; Supplementary Tables 2 and 3 (ST2 and ST3)).

Fig. 2: Emaciated and debilitated TB-positive elephant

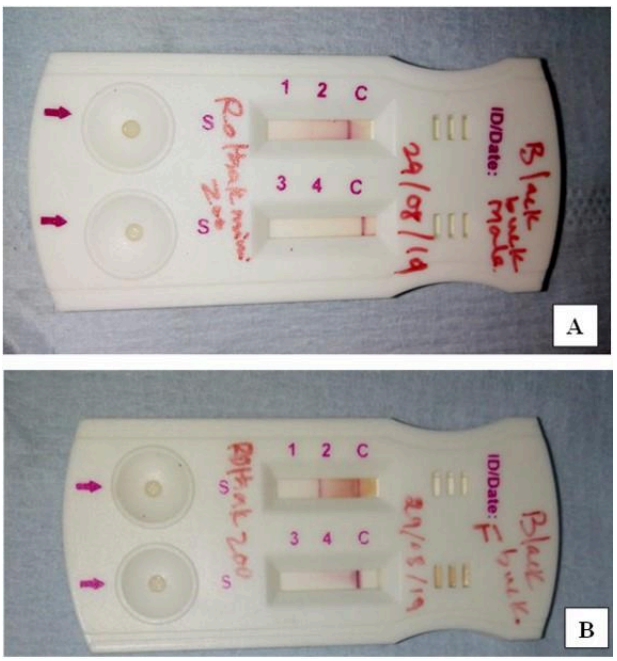

Fig. 3: Blackbuck. Negative result of lateral flow assay (LFA) shows the absence of a colored line in the test area and presence of a colored line in the control area (**A**), and positive result of LFA shows the presence of colored lines in both the test and control area (**B**)

A total of 103 faecal smears and 64 nasal or trunk

wash fluid smears were subjected to ZN staining. Only nine (8.74%) faecal and nine (14.06%) nasal or trunk wash fluid smears of seven sloth bears and two elephants were found positive for AFB (Fig. 4). Genomic DNA isolated from nasal swabs/trunk wash fluids, faecal swabs and blood samples was screened by the IS6110 insertion sequence and a 12.7 kb fragment-based PCR. In IS6110 insertion sequence-based PCR, out of the 156 blood samples, three samples (0.64%) of a spotted deer and two elephants were found TB positive with an amplicon size of 123 bp. Out of the 103 faecal swabs, 64 nasal swabs or trunk washes were screened of them nine (8.74%) faecal and nine (14.06%) nasal swabs or trunk wash fluids of seven sloth bears and two elephants were found positive (Supplementary Tables 2 and 3 (ST2 and ST3)) These positive samples were further subjected to duplex PCR using 12.7 kb fragment primers in which DNA isolated from nasal and faecal swabs of seven sloth bears and trunk wash fluids, faecal swabs and blood of two elephants revealed an amplicon size of 262 bp, i.e., specific for *M. tuberculosis*, and DNA isolated from the blood of spotted deer revealed an amplicon size of 168, i.e., specific for *M. bovis* (Fig. 5).

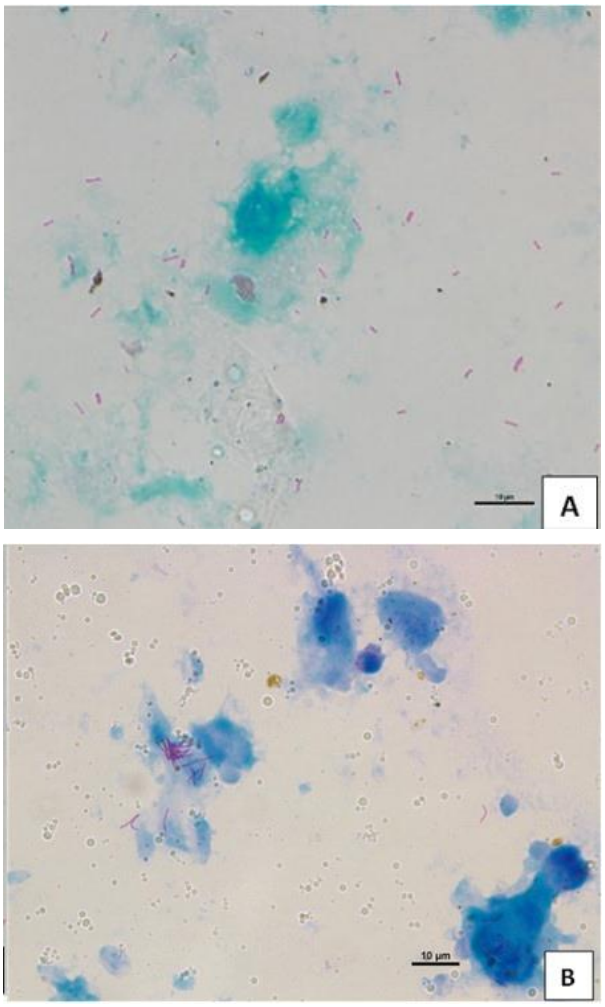

Fig. 4: Ziehl-Neelsen (ZN) staining of nasal (**A**), and faecal (**B**) swabs from a sloth bear, revealing pink colored, rod-shaped, and acid-fast bacilli, (ZN, ×1000)

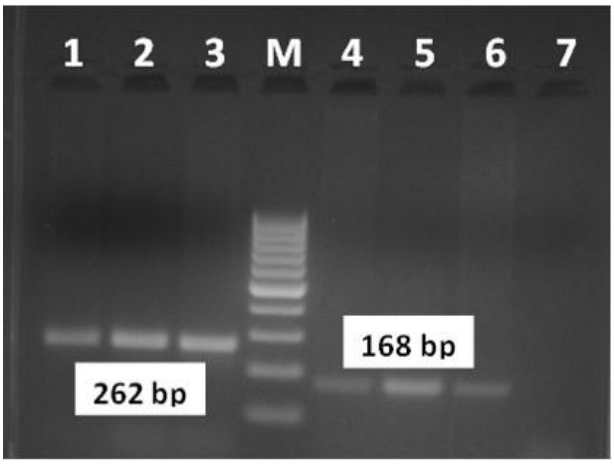

Fig. 5: Agarose gel electrophoresis. Differential amplification of 12.7 kb fragment of MTBC organisms. Lane M: 100 bp ladder. Lane 1: Positive control of *M. tuberculosis*, Lane 2: Sample from sloth bear showing 262 bp amplicon specific for *M. tuberculosis*, Lane 3: Sample from elephant showing 262 bp amplicon specific for *M. tuberculosis*, Lane 4: Positive control of *M. bovis*, Lanes 5 and 6: Samples from spotted deer showing 168 bp amplicon specific for *M. bovis*, and Lane 7: Negative control

Results of different clinical tests were analyzed by a Chi-square test (χ^2) of independence. Based on the statistical analysis, it was concluded that there was a significant difference in the prevalence of TB among different wild animals. Among the clinically infected animals, seven sloth bears, which showed positive results in LFA and PCR, died within 4-6 months after clinical examination, and all of the cases were confirmed to be suffering from TB on the basis of histopathology, tissue acid-fast staining, and molecular techniques.

Discussion

There is a complex pattern of TB transmission among humans, domesticated animals, and wildlife, and the disease is frequently transmitted from one to another (spill-over and spill-back) (Chugh, 2018; Mohamed, 2020). In India, only a limited number of reports are available on the ante-mortem diagnosis of wildlife TB as well as on the transmission pattern at the wildlife-livestock-human interface. Ante-mortem diagnosis of TB often poses a significant challenge in wildlife species due to rarely evident clinical signs (Lekko *et al*., 2021; Thomas *et al*., 2021). In the present study, four sloth bears exhibited clinical signs and symptoms similar to those noticed earlier (Karikalan and Sharma, 2018; Marinaik *et al*., 2022; Sharma *et al*., 2022). Clinical signs in cervids and elephants were not apparent in the present study except for weight loss. Similar observations that most of the cervids and elephants seldom show clinical signs until the terminal stage of disease were also made by other workers (Nugent *et al*., 2015; Songthammanuphap *et al*., 2020).

LFA showed seropositivity for MTBC antigens in 21 (28.76%) cases out of which seven sloth bears that died

during the study period were confirmed as TB positive by conventional and molecular techniques, thus indicating the importance of LFA in clinical diagnosis of wild animals. Various LFA kits are commercially available for wild animals, such as STAT-PAK^{TM} (Chembio Diagnostic Systems, USA), which is validated for use in elephants and non-human primates with sensitivity and specificity of 100%, 95-100% in elephants, 90%, and 99% in non-human primates, respectively (Thomas *et al*., 2021). The MycoPac dual kit, Cisgen Biotech, India, was used in the present study, which claimed to have 95% sensitivity and 98% specificity in sloth bear, elephant, and deer species. This kit uses the cocktail of proteins involved in the pathogenesis of MTBC organisms and other native mycobacterial proteins, which helps in detecting cross-reactivity from NTM (Veerasami *et al*., 2017). ZN staining of smears prepared from clinical samples acts as a quick and economical method that provides a tentative diagnosis for TB. However, it has certain limitations as it cannot distinguish between the members of the *Mycobacterium* genus and other acid-fast organisms like Cryptosporidium and Nocardia (Lawrence *et al*., 2016). Therefore, for confirmation of acid-fast MTBC organisms, concurrent application of other techniques is mandatory. PCR is the most useful molecular technique that helps in species identification of *Mycobacterium*. It is a rapid differentiation technique, hence very useful for routine laboratories (Bakshi *et al*., 2005). TB ante-mortem diagnosis with high sensitivity has been achieved when the combination of techniques is used in wild animals (Thomas *et al*., 2021).

In the present study, *M. tuberculosis* was confirmed as the cause of TB in seven captive sloth bears and two elephants. It might be due to the close association of these bears and elephants with infected humans, as taming the bears for public entertainment was a common practice in India a few years back, along with the major role of elephants in socio-religious activities in the country. This indicates a strong correlation between humans and these captive wild animals with regard to the transmission of *M. tuberculosis*. Detection of *M. bovis* in one spotted deer is indicative of either the role of cervids as the maintenance hosts for *M. bovis* or the case of spillover from domestic livestock living in the periphery of forest-reserved lands.

Thus, from the present study, it is evident that TB is prevalent in free-ranging and captive wildlife in India, encompassing a variety of wild animal species. As supported by the earlier reports, there is a significant spill-over and spill-back of MTBC members at the livestock-human-wildlife interface. The clinical signs are indistinct in wild animals suffering from TB, thus acting as a silent carrier of infection, which poses a serious threat to in-contact humans and animals. Serological assays like LFA were found to be an effective test for screening TB, which when combined with other tests (PCR, ZN staining, etc.), holds good confirmatory diagnostic value in wild animals.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Online Material

Refer to web version on PubMed Central® (PMC) for Supplementary Material.