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SHORT COMMUNICATION

Isolation of *Mycobacterium tuberculosis* from *Antelope cervicapra* and *Gazelle bennettii* in India and confirmation by molecular tests

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Abstract Tuberculosis (TB) in domestic and wild ruminants is mostly caused by *Mycobacterium bovis* (MB). However, the present paper describes the first report of TB of antelopes (*Antelope cervicapra*) and chinkara (*Gazella bennettii*) due to *Mycobacterium tuberculosis* (MT) in India. These wild hosts may represent a vehicle for the dissemination of MT infection to domestic livestock or human. MT was isolated by culture employing the MGIT™ BACTEC™ 960 (Becton Dickinson, BD) system and Middlebrook 7H10 Agar enriched with oleic albumin dextrose catalase (OADC) and Lowenstein-Jensen (LJ) medium supplemented with glycerol, but not sodium pyruvate. The isolates were confirmed as a member of the *Mycobacterium tuberculosis* complex (MTC) by using a commercial nested PCR that targeted the IS6110 sequence. Further confirmation of the isolates as MT strains was achieved by employing commercial line probe assay genotyping kits (Hain Lifescience, Germany) that specifically identifies MT within the MTC group.

Keywords *Mycobacterium tuberculosis* · *Mycobacterium bovis* · Isolation · PCR

Introduction

Bovine tuberculosis is a chronic progressive emaciating bacterial disease caused by *Mycobacterium bovis* (MB) affecting cattle, other domestic animals, certain wildlife populations, as well as humans in large number of countries (OIE 2014; Palmer 2013). The global distribution of *M. bovis* in free living wildlife has been reviewed (De Lisle et al. 2001). However, there are reports of infection due to *Mycobacterium tuberculosis* (MT) in zoo and wildlife species (Montali et al. 2001; Ghodbane and Drancourt 2013). Sporadic cases of infection in domestic (bovines and caprines) and wildlife species (kangaroos, red deer, and roe deer) due to *Mycobacterium avium subspecies avium* (MAA) occasionally resulting into clinical disease has been also described (Thorel et al. 1997; Thorel et al. 2001). Concurrent infection in cattle with MT and MB has been described (Srivastava et al. 2008; Mishra et al. 2005) in India. MT has been also identified from deer (Arora 1993) from a wildlife sanctuary and in zoo in eastern India (Mukhopadhaya 2007). Iso-nicotinic acid hydrazide and rifampicin resistant strain of MT was recovered from a zoo in southern India from a rhinoceros (Valandikar and Raju 1996). It has been shown earlier that badgers, wild bores, and deer can act as maintenance hosts in dissemination of TB in wildlife (Gortazar et al. 2012) in different geographical regions. Despite the information from literature that the members of the *Bovidae* family in the wild boar are mostly affected by *M. bovis*, this paper reports the isolation and molecular confirmation of *M. tuberculosis* from antelope and gazelle in India.

Communicated by C. Gortázar

F. Mukherjee and V. S. Bahekar contributed equally to this work.

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Materials and methods

Animals and samples

The fauna from a wildlife sanctuary in western India consisted of black and white bucks (*Antelope cervicapra*), spotted deer (*Axix axis*), sambar (*Rusa unicolor*), gazelle (*Gazella bennettii*), blue bulls (*Boselaphus tragocamelus*), porcupine, jackal, leopard, lions, crocodiles, and 180 species of birds. The 400-ha sanctuary was divided into eastern and western parts by a river. While the herbivores *A. cervicapra* (black buck $n=150$; white buck $n=30$), *A. axis* ($n=85$), *R. unicolor* ($n=30$), and *G. bennettii* ($n=7$) were kept in large open air enclosures separated by fences (Fig. 1), the *B. tragocamelus* ($n=150$) were free ranging. The carnivores, jackal, leopard, and crocodiles were kept in separate designated pens. The species-wise location of herbivores with respect to each other within the sanctuary is provided in Fig. 1. Fifteen to twenty persons of the sanctuary attended to the herbivores and the carnivores. The movement of each of these persons within the sanctuary was unrestricted. They were freely in direct or indirect contact with each other, and all animal inhabitants of the sanctuary. Progressive emaciation leading to death was first observed among members of *G. bennettii* in 2010 and later among *A. cervicapra* in 2011. Animals showing high morbidity were shifted to isolation pens (Fig. 1). In July 2010, post mortem of a gazelle housed in an isolation pen revealed lung lesions suggestive of TB. Nasal swab, lung, and pre-scapular lymph node were collected at necropsy (Table 1). Before the death of a female black buck in January 2011, a single cervical intradermal test (SCIT) with bovine tuberculin-purified protein derivative obtained from the Indian Veterinary Research Institute, Izatnagar, was conducted as recommended (OIE 2014). The SCIT result was negative. At necropsy nasal swab, lung, and pre-scapular lymph node were collected from the black buck (Table 1). Smears from lung lesions from the gazelle as well as the antelope showed acid fast bacilli (AFB) on Ziehl-Neelsen staining. Nasal swabs and tissues collected at necropsy were transferred into sterile Middlebrook 7H9 broth and 50 ml Falcon tubes, respectively. The samples

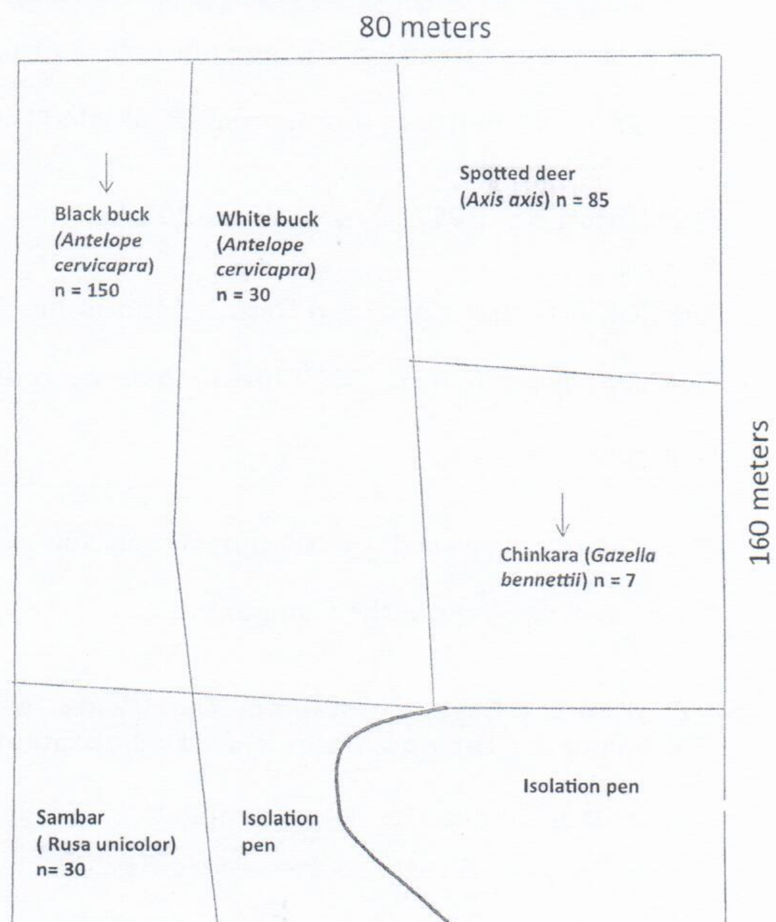

Fig. 1 Relative location of animals housed in the nature park with respect to those species from which *M. tuberculosis* was isolated. This is a unit of the 400-ha nature park in Gujarat divided into eastern and western parts by a river. In addition, the park harbors various carnivores (lion, leopard, jackal, and crocodile), porcupine, and 180 species of birds

Table 1 Results of bacteriological culture and confirmation by molecular methods

Test method	Geographical location: Gujarat, Western India					
	Species *Antelope cervicapra*			Species *Gazelle bennettii*		
	Nasal swab	Pre-scapular lymph node	Lung tissue	Nasal swab	Pre-scapular lymph node	Lung tissue
Bacteriological culture in media						
MGIT BACTEC 960	Negative	Negative	Positive	Positive	Negative	Positive
Middlebrook 7H10 agar supplemented with OADC and glycerol	Negative	Negative	Positive	Positive	Negative	Positive
Middlebrook 7H10 agar supplemented with OADC and sodium pyruvate	Negative	Negative	Negative	Negative	Negative	Negative
Lowenstein-Jensen medium supplemented with glycerol	Negative	Negative	Positive	Positive	Negative	Positive
Lowenstein-Jensen medium supplemented with sodium pyruvate	Negative	Negative	Negative	Negative	Negative	Negative
Molecular confirmation						
Confirmation of culture as a member of the MTBC: commercial nested PCR targeting the IS6110	ND	ND	Positive	Positive	ND	Positive
Confirmation of culture as MT by Genotype® MTBC[a]	ND	ND	Positive[d]	Positive[d]	ND	Positive[d]
Confirmation of culture as MT but not *M. avium* by Genotype Mycobacterium® CM[b]	ND	ND	Positive[e]	Positive[e]	ND	Positive[e]
Confirmation of culture as MOTT by Genotype Mycobacterium® AS[c]	ND	ND	Negative[f]	Negative[f]	ND	Negative[f]

ND not done

[a] For identification of *M. africanum, M. bovis* BGC, *M. bovis* ssp. *bovis, M. bovis* ssp. *caprae, M. microti*, and *M. tuberculosis/M. canettii*

[b] For identification of *M. simiae, M. mucogenicum, M. goodii, M. celatum, M. smegmatis, M. genavense, M. lentiflavum, M. heckeshornense, M. szulgai/ M. intermedium, M. phlei, M. haemophilum, M. kansasii, M. ulcerans, M. gastri, M. asiaticum, and M. shimoidei*

[c] For identification of *M. avium* ssp., *M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. peregrinum, M. marinum/M. ulcerans, M. tuberculosis* complex, *and M. xenopi*

[d] Results of reverse slot blot hybridization patterns generated by Genotype® MTBC confirmed isolates as *M. tuberculosis*

[e] Genotype Mycobacterium® CM reconfirmed that isolates belonging to the MTC group ruled out the presence of *M. avium*

[f] Genotype Mycobacterium® AS out ruled the presence of MOTT strains

were dispatched on ice at 4 °C to the Research and Development (R&D) Laboratory, National Dairy Development Board (NDDB), Hyderabad, India.

Maintenance of reference *Mycobacterium* strains

The *M. tuberculosis* $H37_{RV}$ (ATCC 27294) and *M. bovis* BCG (ATCC 35734) strains obtained from the American Type Culture Collection (ATCC) were propagated and maintained on Middlebrook 7H10 agar or Lowenstein-Jensen (LJ) medium supplemented with glycerol and on 7H10 agar or Lowenstein-Jensen (LJ) medium slopes supplemented with sodium pyruvate, respectively, and incubated at 37 °C for 6–8 weeks.

Isolation of *Mycobacterium* from clinical samples

The samples were decontaminated and processed by modified Petroff's method (Kent and Kubica 1985). A portion of the decontaminated sediments were inoculated into Mycobacterial Growth Indicator Tubes (MGIT)™ from Becton Dickinson (BD) were inoculated in BACTEC MGIT 960 instrument for 49 days at 37 °C; the remaining sediments were inoculated into one tube each of OADC-supplemented Middlebrook 7H10 agar and Lowenstein-Jensen (LJ) medium with sodium pyruvate and glycerol; each tube was incubated for 8 weeks at 37 °C.

Identification of *Mycobacterium* from cultures

Heat fixed smears prepared from MGIT cultures declared as positive by the BACTEC 960 and typical growths on Middlebrook 7H10 and LJ media were screened for presence of acid fast bacilli. The heat-fixed smears were stained for AFB by using two commercial staining kits (BD, India. Cat. Nos. 212522 and 212519).

Molecular identification of AFB positive isolates

DNA extraction, identification of isolates as a member of MTC and MT

The DNA extraction from MGIT liquid culture and colonies on 7H10 agar/LJ media was performed according to the protocol recommended by GenoType® MTBC kit from Hain Lifescience, Germany (Cat. No. 47013). To identify isolates as members of the MT Complex, the extracted DNA was amplified according to the protocol of single-tube-nested PCR kit (GeNei Amplification Reagent Set for *Mycobacterium tuberculosis* complex (MTBC) group of organism; Cat. No. 105936, Bengaluru). In the single-tube two-step assay, the first positive amplification was determined by a 220-bp PCR product amplified from the IS6110 region, followed by amplification of a 123-bp-nested amplicon. For identification and confirmation of strains within the MTC, a commercial line probe assay kit recommended by the World Health Organization (WHO) based on the polymorphism in the *gyrB* gene was employed (Genotype® MTBC, Hain Lifescience, Germany). In addition, two other line probe assay kits (Genotype® Mycobacterium CM, Cat. No. 47012 and Genotype® Mycobacterium AS, Cat. No. 47011; Hain Lifescience, Germany) were used for identification of *M. avium* and Mycobacteria other than tuberculosis (MOTT).

Results and discussion

Mycobacteria were isolated from lung samples from both animals in MGIT, on Middlebrook 7H10 agar, and on LJ medium supplemented with glycerol, but not on media supplemented with sodium pyruvate (Table 1). In addition, Mycobacteria were also isolated from nasal swab samples from *A. cervicapra* (Table 1). Smears of the growth obtained in every culture system used showed the presence of slender acid fast bacilli upon staining. Nested PCR of DNA from isolates yielded both the 220 and 123-bp amplicons characteristic of MTC members. The hybridization pattern generated by the Genotype® MTBC kit identified all three isolates as MT. The results of Genotype® Mycobacterium AS kit reconfirmed the isolates as a member of the MTC and ruled out the presence of *M. avium* strains, while the Genotype® Mycobacterium AS confirmed the absence of MOTT strains.

The MGIT BACTEC 960 liquid culture system used in this study was used to enhance the sensitivity of detection of MTC strain from clinical samples. The use of MGIT BACTEC 960 for efficient and rapid recovery of MT and MB isolates from veterinary specimens has been described earlier (Romero et al. 2011; Robe-Austerman et al. 2013). The gene target IS6110 has been shown to be specific for the identification of strains within the MTC (Githui et al. 1999). The commercial nested PCR described in the current study has been used earlier for direct detection of mycobacteria belonging to MTC group from clinical samples of human patients (Gill et al. 2013). We adopted commercial line probe assays for identification of mycobacteria species. The use of Genotype® MTBC kit manufactured by Hain Lifescience, Germany for speciation of isolates recovered from wildlife and for identification species classified as MOTT has been reported (Gortazar et al. 2011). We used the two latter assays primarily for confirmation of concurrent infection with *M. avium* or any other MOTT strains. Although infection of wildlife mammalian species with *M. avium* has been reported to be sporadic (Thorel et al. 1997; Thorel et al. 2001), it has been isolated from deer in India. It was therefore important to rule out infection in antelope and gazelle with *M. avium*. Accurate identification of mycobacteria strains recovered from infected animals can provide epidemiologically significant information. This assumes more pertinence in light of earlier reports of mixed infection in cattle with MT and MB (Mishra et al. 2005), concurrent infection of cattle and wildlife species with MB and MOTT (Romero et al. 2008; Gortazar et al. 2011), and co-infection of cattle with MT and *M. avium* subspecies *hominissuis* (Romero et al. 2011).

Infection of wild species with MT in captivity is not a novel observation (Montali et al. 2001) and neither is isolation of MT from antelopes (Michel et al. 2013). MT has been isolated from antelopes from zoo and game farms in South Africa (Michel et al. 2013), however, there are no such reports from India so far. An important emerging concern is not whether infection of wildlife species with MT is novel, but recent observations that MT isolates recovered from humans and members belonging to *Bovidae* from the same geographical area share identical spoligotypes and VNTR-MIRU profiles (Malama et al. 2014; Romero et al. 2011). The evidence of molecular epidemiological link provided above, has been suggested as a possible mechanism for spill backs or spill overs of MT from human TB to cattle (Malama et al. 2014; Romero et al. 2011; Michel et al. 2013). The TB infection due to MT in antelope and gazelle identified in the present study may have been due to a number of unique situations coexisting in the sanctuary. Although infected antelope and gazelle were separated by wire fencing, they could have come in direct contact at the fence boundary. Persistence of infection could have played an important role, since 2009–2011 the total number deaths reported in antelopes and gazelles were 37 spread over two episodes (8 in 2009–2010 and 29 in 2010–2011). Necropsy was conducted in some of these animals and gross lesions suggestive of TB were recorded including in three antelopes. The other possible way they could have indirectly acquired infection was through the farms attendants, since their movement between pens housing different wildlife species was unrestricted. The attendants did not reside in the staff quarters but came from different villages surrounding the

sanctuary. They were never screened for TB. Unrestricted movement of free-ranging blue bulls from the sanctuary to adjoining villages and back to the sanctuary had been also observed (Kanani personal communication). During the period of this study (2010–2011), TB due to MT infection in cattle was detected in a village 15 km away from the sanctuary and migration of blue bulls to this village has been also observed (Kanani personal communication). A recent investigation revealed that some of the zookeepers hailed from the same villages from where TB was identified in cattle. These were the same villages where straying of free-ranging blue bulls from the sanctuary was reported. Although we have not yet characterized the MT isolates by MIRU and VNTR, our recent spoligotyping data at the time of writing this report indicates that the two isolates from lung tissues of antelope and gazelle possess distinct spoligotypes. Both spoligotypes were identified from infected cattle from a farm in the village mentioned above (Mukherjee unpublished data). Moreover, the same spoligotype responsible for infecting cattle in 2010 was identified from the infected antelope in 2011. Similarly, the spoligotype that caused infection in the gazelle in 2010 also infected cattle in 2011 (Mukherjee unpublished data).

Taking all the information mentioned above into account, there is sufficient evidence to further explore the potential role of humans in the transmission of MT to wildlife and domestic animals. The study also indicates the necessity for stringent steps to improve monitoring and surveillance against TB in human, wildlife and domestic animals.

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Authors' contribution The authors F. Mukherjee and V. S. Bahekar contributed equally to this work.

Conflict of interest The authors declare that they have no conflict of interest.

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