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SHORT COMMUNICATION

Tuberculosis caused by *Mycobacterium orygis* in wild ungulates in Chennai, South India

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Abstract

We report the isolation of *Mycobacterium orygis*, a member of *Mycobacterium tuberculosis* complex (MTBC), from two black bucks (*Antelope cervicapra*) and one spotted deer (*Axis axis*) from the Guindy National Park forest range in Chennai, India. Lung tissue and lymph node samples collected during post-mortem examination were processed using NaOH method and cultured in solid and liquid media. DNA extracted from the cultured isolates was used to amplify the *mpt64* gene by specific primers and the band visualized at 240 bps confirmed the isolates as a member of MTBC. Further examination of these isolates by spoligotyping and whole-genome sequencing confirmed the isolates as *M. orygis* and the phylogenetic tree revealed their well-clustered position with other *M. orygis* isolates around the globe. The deletion of RD7–RD10, RDOryx_1, RDOryx_4, RD12Oryx, RD301 and RD315 further substantiated these isolates as *M. orygis*. The exact source of infection in animals was untraceable and the pairwise comparison of the genomes based on single-nucleotide polymorphisms difference did not detect any events of transmission within the affected animals. Nevertheless, it would be wise to take into account the environment where there exists a high chance of transmission due to the increased human–animal interaction. Since it is well known that the pathogen is capable of causing infection in both human and animal hosts, systematic surveillance and screening of spotted deer, black buck as well as humans in the vicinity is essential for successful implementation of the One Health approach.

KEYWORDS
black buck, deer, *Mycobacterium orygis*, *Mycobacterium tuberculosis* complex (MTBC), tuberculosis, wild ungulates, zoonosis

1 | INTRODUCTION

Mycobacterium orygis is a member of *Mycobacterium tuberculosis* complex (MTBC) capable of causing disease in both humans and animals as reported by several studies (Refaya et al., 2019; Van Ingen et al., 2012). MTBC is composed of several highly genetically related species that have adapted to both human and animal hosts possessing the ability to propagate and transmit in a variety of wild and domesticated animals such as goats, pigs, buffaloes, dogs, primates, badgers, deer, possums and bison (Malone & Gordon, 2017).

In a study conducted in the Netherlands in 1987, MTBC was isolated from an oryx in the zoo which shared a very similar IS6110

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pattern with a human isolate from the same region, suggesting possible human–animal transmission (Van Soolingen et al., 1994). The transmission of *M. orygis* from a human of South Asian origin to a dairy cow in New Zealand has also been documented (Dawson et al., 2012). South Asian link seems to be the prominent factor in majority of the reported *M. orygis* cases (Rahim et al., 2017). The isolation of seven *M. orygis* strains from humans in India reported by Duffy et al., further reinforces the possibility of zoonotic spread which does not limit to *M. bovis* alone (Duffy et al., 2020). Since tuberculosis (TB) infection can traverse the host barrier where the habits and habitats of the host are important factors (especially in case of TB in wildlife like hunting/scavenging patterns or aggregation behaviour, population of host, exposure and susceptibility), it is necessary to address the study of different hosts in tandem (Vicente et al., 2013). Several studies have reported the isolation of MTBC from wildlife around the globe (Lekko et al., 2020; Thapa et al., 2017), especially those belonging to the Cervidae family. They have been isolated from antelopes, gazelles, white tailed deer, red deer, fallow deer and roe deer (García-Bocanegra et al., 2012; Martín-Hernando et al., 2010; Mukherjee et al., 2018; Schmitt et al., 1997). It is possible that TB can spread among wild animals because of mycobacterial shedding into the environment by the infected animals (Dorn-In et al., 2020; Lugton et al., 1998; Santos et al., 2015). While it is possible that some of the hosts may be dead-end hosts (those that do not transmit infection onwards), some of the hosts could be maintenance hosts in which infection can persist with or without the presence of an external source, thus hampering the END TB goal (Lekko et al., 2020).

Wildlife TB in India is an understudied and underexplored area of research. Even if TB-like lesions are observed during post-mortem examinations, they are seldom followed upon by culturing the causative organisms and molecular diagnoses for the disease. Given the endemicity of TB in humans and the paucity of literature regarding the prevalence of wildlife TB in India, it is important to work towards attaining the goal from all fronts. Here, we report the presence of *M. orygis* in the lymph node and lung samples of two black bucks (*Antelope cervicapra*) and one spotted deer (*Axis axis*) from the Guindy National Park (GNP) forest range located in the city of Chennai.

2 | MATERIALS AND METHODS

The deer and black buck carcasses were presented for post-mortem analysis to Madras Veterinary College in Chennai in March 2020 and March 2021, respectively, following natural death. The animals were suspected for TB on the basis of the lesions present in the internal organs. The lung tissue and the lymph node samples were collected, homogenized, decontaminated using 4% NaOH and neutralized using 1× phosphate-buffered saline (Egbe et al., 2016; OIE Terrestrial Mannual, 2019). Smears of both the homogenate and the decontaminated deposit were made and the slides were stained using Ziehl–Neelson method. The deposits were inoculated in Lowenstein–Jensen (LJ) slants (with and without sodium pyruvate [SP] supplement) and in BACTEC 960 Mycobacteria Growth Indicator Tubes (MGIT) (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). The presence of MTBC in typical mycobacterial colonies that appeared on LJ slants as well as in MGIT was confirmed using BD MGIT™ TBc identification test strips (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA). Briefly, for colonies that appeared on LJ slants, bacterial suspension was prepared and placed onto the sample well of the test device, while samples from MGIT cultures were placed directly into the sample well for identification (Abe et al., 1999; Mycobacteriology Laboratory Manual, 2014). Drug susceptibility testing was carried out with standard first-line drugs using the BD BACTEC™ MGIT™ 960 SIRE Kit (Becton Dickinson Diagnostic Systems). The lyophilized drugs were reconstituted in sterile distilled water and 0.1 ml of each reconstituted drug was added into separate MGIT tubes to achieve final drug concentrations as follows: streptomycin, 1.0 μg/ml of medium; isoniazid, 0.1 μg/ml of medium; rifampicin, 1.0 μg/ml of medium; and ethambutol, 5.0 μg/ml of medium. The drug sensitivity was determined upon comparison of culture growth with growth control tube as per the manufacturer's instructions. DNA was isolated from the cultures using MagGenome XpressDNA Bacteria kit (MagGenome Technologies Pvt Ltd, Chennai, India) according to the manufacturer's instructions. The quality and quantity of isolated genomic DNAs were checked by measuring the $A_{260/280}$ ratio using the NanoDrop method. The isolated DNAs were subjected to polymerase chain reaction (PCR) using *mpt64* primers (FP: 5′-TCCGCTGCCAGTCGTCTTCC-3′; RP: 5′-GTCCTTCGCGAGTCTAGGCCA-3′). Further, the samples were amplified using the DRA (5′-GGTTTTGGGTCTGACGAC-3′) and DRB (5′-CCAAGAGGGGACGGAAAC-3′) primers, and the amplification product was loaded onto the spoligotyping membrane. The patterns obtained were compared with those available in the SpolDB4 database (Brudey et al., 2006; Kamerbeek et al., 1997).

The sequencing library was prepared using a TruSeq Nano DNA LT library prep kit as per the manufacturer's protocol in Illumina Hiseq 2500 instrument with a read length of 151 bp. Genome sequences were assessed with Galaxy/vSNP that locate and validate single-nucleotide polymorphisms (SNPs) in bacterial samples and produce annotated SNP tables and corresponding phylogenetic trees (https://github.com/USDA-VS/vSNP; Stuber, 2022). Briefly, the raw sequences were filtered using trimmomatic (version 0.39) with a framework of minimum phred quality score and read length set to 30 and 80, respectively. The filtered reads were aligned to the reference genome *M. orygis* 51145 (CP063804) using BWA-MEM algorithm (version 0.7.17.1) (Li & Durbin, 2009) and SNPs were called using Freebayes (version 1.3.1) and validated using IGV (Tange, 2011). In order to compare the newly sequenced genomes with the existing sequences available in the NCBI SRA database, we downloaded 48 sequences with fasterq-dump tool from the SRA toolkit (version 2.9.6). All the downloaded sequences and the study sequences were run through vSNP. Phylogenies were constructed with RAxML (version 8.2.4) using the aligned whole-genome SNP sequences under a GTRCATI model of substitution and a maximum-likelihood algorithm with a bootstrap replication of 1000 (Stamatakis, 2014). Tree visualization, annotation, and editing were performed with integrated tree of life (iTOL) (Letunic & Bork, 2019). RD-Analyser and RDScan were used to localize the

FIGURE 1 Macroscopic calcified pale-yellow lesions observed during post-mortem in (a) lungs of black buck 1, (b) lungs of black buck 2, (c) lungs of spotted deer and (d) cut surface of lung of spotted deer showing purulent lesions

TABLE 1 Details regarding animals and phenotype data for the samples

S. no.	Sample source	Age/Sex	Body condition	Smear results	Culture results			Drug sensitivity			
					LJ	LJ-SP	MGIT	SM	H	RIF	E
1	Spotted deer	5 Y/F	Animal emaciated, superficial LNs enlarged	Pos	Pos	Pos	Pos	S	S	S	S
2	Black buck 1	4 Y/M	Animal emaciated, superficial LNs enlarged	Pos	Pos	Pos	Neg	S	S	S	S
3	Black buck 2	7 Y/M	Animal emaciated, superficial LNs enlarged	Pos	Neg	Pos	Pos	S	S	S	S

Abbreviations: E, ethambutol; F, female; H, isoniazid; LN, lymph node; M, male; Neg, Negative; Pos, Positive; R, resistant; RIF, rifampicin; S, sensitive; SM, streptomycin; Y, years.

regions of difference within the sequences (Bespiatykh et al., 2021; Faksri et al., 2016).

3 | RESULTS AND DISCUSSION

The animals presented for post-mortem looked emaciated during preliminary analysis, and further examination revealed the presence of macroscopic calcified lesions in the lungs and lymph nodes consistent with severe TB infection (Figure 1). The age and sex of the animals and the initial observations regarding their body condition can be seen in Table 1. Ziehl–Neelson staining was positive for all the three isolates, and typical mycobacterial colonies were observed on LJ, LJ SP and MGIT media. All the three isolates were sensitive for the first line anti-TB drugs—streptomycin, isoniazid, rifampicin and ethambutol (Table 1). Amplification bands of size 240 base pairs (bps) suggestive of MTBC were observed during PCR. Spoligotyping pattern exhibited the spoligotype 587 (ST587) as compared to the SpolDB4 database (Figure 2).

All three isolates were successfully sequenced generating an average of 10,078,963 read pairs with mean read length of 151 bp. The total number of reads varied from 2,886,500 to 16,187,488. Almost 99.9% of the reference genome was covered, and the average coverage depth of all isolates was 243.23 (ranging from 87.41 to 330.40, median

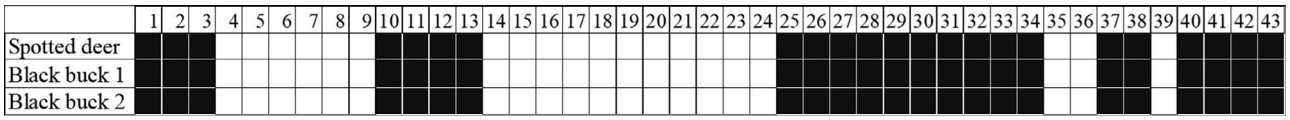

FIGURE 2 Spoligotype pattern observed for the three isolates. Black colour indicates the presence of spacer region, while the white colour represents the absence of spacer region.

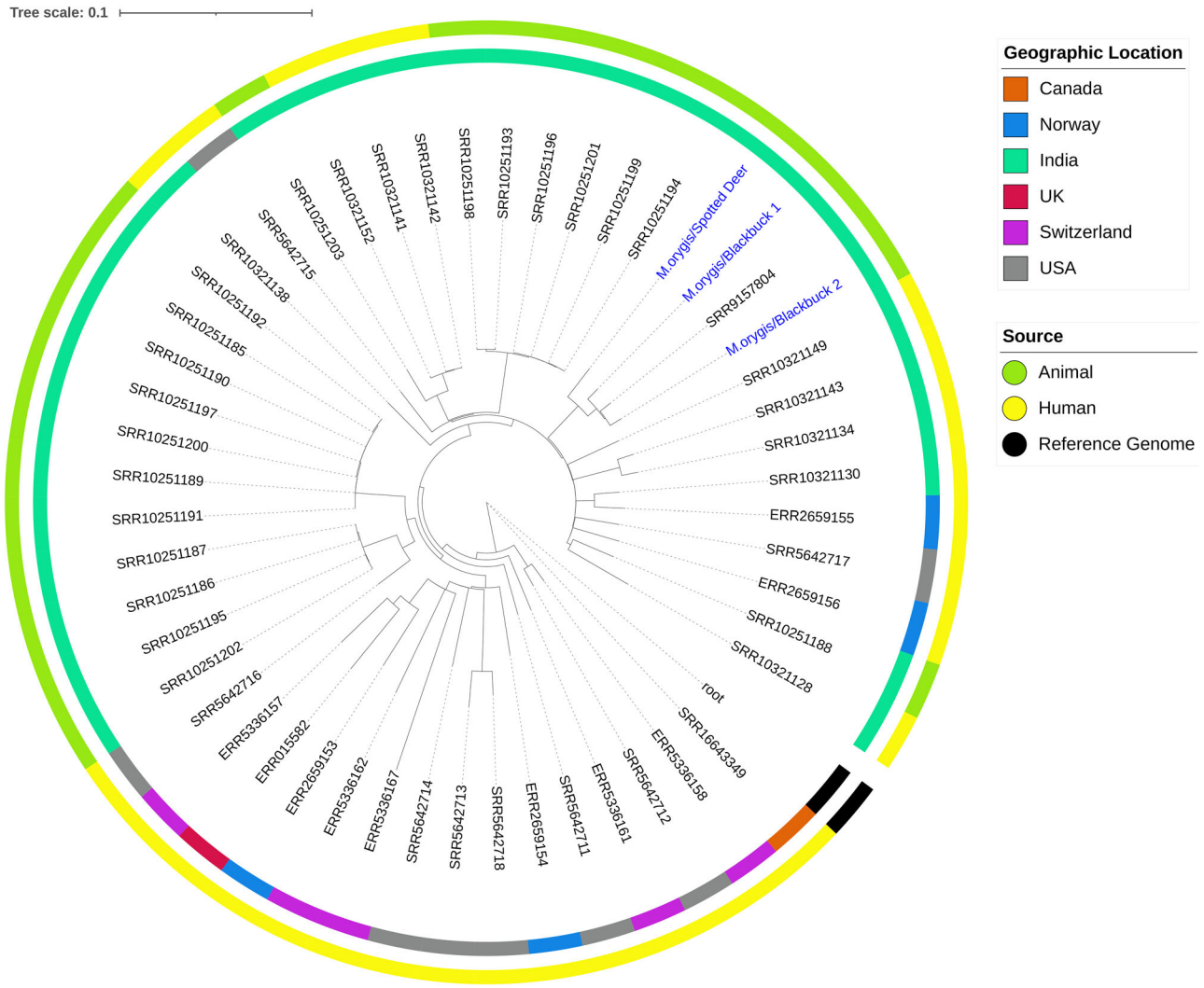

FIGURE 3 Phylogenies of newly sequenced isolates in the context of *Mycobacterium orygis* isolates around the globe: Interpretative phylogenetic tree combining the three study isolates along with 48 *M. orygis* sequences downloaded from National centre for Biotechnology Information Sequence Read Archive (NCBI SRA). The outer band represents the source from which the isolates were derived and the inner band illustrates the geographic location of the isolates. The sequence of the isolates from this study is labelled in blue.

311.88). Here, we observed that all the three isolates share the same genotype, but there is no sufficient data to prove the possibility of infection from the same source. Pairwise comparisons of the genome were done based on SNP differences for detecting the possibility of transmission within the affected animals. It was observed that the SNP difference within the isolates ranged from 40 to 110 SNPs which ruled out the possibility of any transmission as the SNP-based cut-off for detecting transmission within clusters ranges from three to 14 SNPs (Walker et al., 2013). The SNP difference between the study isolates and the reference genome ranged from 281 to 291.

The burden of *M. orygis* in the human population is evasive as only very few cases (~7) were reported so far, and none of the cases have originated from Tamil Nadu (Duffy et al., 2020). Phylogenetic tree was generated with the sequences downloaded from NCBI SRA along with the study isolates, which reveals that the isolates belong to *M. orygis* as evidenced by their well-clustered positions with the other

M. orygis sequences present in the database (Figure 3). Regions of differences (RD) are generally considered as a gold standard genetic marker for classification of MTBC isolates. Two different open-source workflows, RD-Analyzer and RDScan, were used to identify RDs within the genome (Bespiatykh et al., 2021; Faksri et al., 2016). RD-Analyzer confirmed the deletion of RD7, RD8, RD9 and RD10 and the presence of RD1 and RD4 which is characteristic of *M. orygis (van Ingen et al.*, 2012). RDScan identified additional regions of deletions such as RDOryx_1, RDOryx_4, RD12Oryx, RD301 and RD315 which are reported to be unique for *M. orygis* (Bespiatykh et al., 2021). Variant analysis substantiated the previously reported mutations at T38G of Rv2041 and G698C of Rv0444C (Duffy et al., 2020; van Ingen et al., 2012). The genomic analysis along with corroborating spoligotyping results confirms that these isolates are *M. orygis*.

GNP is a 2.7-km^2 protected forest cover in the midst of Chennai city. The forest is home to many species of flora and fauna including the black buck listed as an 'endangered species' according to the Wildlife Protection Act of India, 1972 (Wildlife conservation and management in Tamil Nadu, 2016). For ex situ conservation, 8.9 ha were enclosed as the Guindy Children's Park which is open to public visitation, where we can see black bucks and spotted deer roaming freely in the grounds. The national park is adjacent to the Indian Institute of Technology—Madras (IIT-M) campus, the Central Leather Research Institute (CLRI), Raj Bhavan (residence of His Excellency—the Governor of Tamil Nadu) and Anna University, which are also places where these animals can be seen frequently. These places have a high population of wild ungulates, and here humans and animals coexist making them hotspots for zoonotic TB and reverse zoonotic TB spread. In a field visit to IIT-M campus, we were able to spot spotted deer as well as black bucks near the staff quarters, restaurants, playgrounds and outside university departments. Spotted deer and black buck in the area most often share common grazing grounds where infection can spread at the species level through aerosol transmission. Faecal pellets of the animals can be seen littered on the ground through which mycobacteria might be shed into the environment. Mycobacterial shedding of this kind by infected animals might promote indirect transmission through contamination of the environment. It was experimentally shown by Ghodbane et al. that not only *M. tuberculosis*, *M. bovis* and *M. canettii* can survive in the soil for about 12 months, but they remain virulent and are capable of causing infection in mice suggesting potential sources for reverse zoonotic spread (Ghodbane et al., 2014). Another important factor to consider is the uncontrolled population of feral monkeys in the area that are known to be susceptible to TB and can transmit the infection as well (Thapa et al., 2017). We were not able to identify the source of *M. orygis* infection in the animals, but it would be prudent to take into account the free ranging transmission because of the increased human–animal interaction in the area. Tourists, staff and/or street vendors who are in close proximity to the animals often feed them and pet them. Since it is known that the pathogen is capable of causing infection in both human and animal hosts, systematic surveillance and screening of spotted deer, black buck as well as humans in the vicinity is needed to successfully implement the One Health approach.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical/welfare authority approval was required as all samples were collected during post-mortem examination.

AUTHOR CONTRIBUTIONS

Conceptualization and supervision: Kannan Palaniyandi. *Methodology*: All authors. *Formal analysis*: Ahmed Kabir Refaya, Harini Ramanujam, Mahaprabhu Ramalingam and Kannan Palaniyandi. *Investigation*: Ahmed Kabir Refaya, Harini Ramanujam, Mahaprabhu Ramalingam, Sivakumar Shanmugam, Ganne Venkata Sudhakar Rao and Kannan Palaniyandi. *Funding acquisition*: Kannan Palaniyandi. *Original draft preparation*: Ahmed Kabir Refaya, Harini Ramanujam and Kannan Palaniyandi. *Review and editing*: All authors. *Visualization*: Kannan Palaniyandi. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at https://www.ncbi.nlm.nih.gov/ BioProject ID: PRJNA789714.

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