Rev. Sci. Tech. Off. Int. Epiz., 2018, **37** (3), 999-1012

Isolation and analysis of the molecular epidemiology and zoonotic significance of *Mycobacterium tuberculosis* in domestic and wildlife ruminants from three states in India

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Summary

The majority of tuberculosis cases in ruminants are caused by *Mycobacterium bovis* (*MB*). However, in this study, the authors reported the isolation of *Mycobacterium tuberculosis* (*MT*) from bovine milk, nasal swabs and post-mortem tissue samples (*n* = 841) collected from cattle and buffaloes in the states of Telangana, Maharashtra and Gujarat in India in the period from 2010 to 2015. The isolates (*n* = 7) were confirmed as *Mycobacterium* due to their growth characteristics and colony morphology in a commercial liquid medium Mycobacterial Growth Indicator Tube (MGIT)™ employing the BD BACTEC™ MGIT™ 960 system and the Löwenstein-Jensen (LJ) medium supplemented with glycerol but not with sodium pyruvate, and BD-DIFCO™ Middlebrook 7H10 agar containing oleic albumin dextrose catalase (OADC). These isolates were initially identified as members of the *M. tuberculosis* complex (MTC) using a commercial nested polymerase chain reaction (PCR) kit based on the IS*6110* MTC specific nucleotide sequence. The isolates were confirmed as *MT* using three commercial line probe assay kits, were further genotyped, and the spoligotypes identified were of East African Indian (EAI) 3_IND, EAI5, Central-Asian (CAS) 1_DELHI, U and T1 lineages. Two *MT* isolates from one antelope (*Antilope cervipara*) and one gazelle (*Gazella bennettii*) from Gujarat, which were identified previously, were spoligotyped during this study and identified as belonging to EAI3_IND and EAI5 lineages, respectively. The epidemiological significance and zoonotic implications of regional presence and documentation of the same or two different spoligotypes in different species within the family Bovidae as well as humans is discussed.

Keywords

Epidemiology – India – Isolation – *Mycobacterium tuberculosis* – Ruminant – Spoligotyping – Zoonosis.

doi: 10.20506/rst.37.3.2902

Introduction

Tuberculosis is a contagious, chronic, emaciating zoonotic disease that can infect a large number of animal species, mainly mammals, including domestic and wild ruminants, marine mammals and humans (1). Bovine tuberculosis (BT) has been reported as the causative agent for significant economic losses in the dairy industry in Turkey, Ethiopia and Argentina, ranging from US$ 15 to 63 million (2, 3, 4). The disease is mostly caused by infection with a member of the *Mycobacterium tuberculosis* complex (MTC), namely *Mycobacterium bovis* (*MB*), while another member of the MTC, *Mycobacterium tuberculosis* (*MT*), is generally responsible for human tuberculosis (1, 3, 5). However, during the past decade, bovines infected with *MT* have been reported in Ethiopia, Turkey, the People's Republic of China, Nigeria and Spain (6, 7, 8, 9, 10), and also in India (11, 12). In addition, in the district of Gandhinagar, Gujarat state, in western India, captive wild ruminants infected with *MT* have been reported (13). Moreover, the concurrent infection of the bovine population in India with *MT* and *MB* has also been reported (12, 14, 15, 16).

Genotyping of disease strains leads to the discovery of epidemiologically significant information and it is therefore considered an important tool for decision-making with respect to tuberculosis control programmes. Spacer oligonucleotide typing (spoligotyping), based on the analysis of a direct repeat (DR) locus, is one such globally recognised genotyping method (17). Although spoligotyping data on *MT* strains of human origin isolated from various geographical regions in India are available, similar information on *MT* isolates from domestic and wild ruminants from diverse regions of the country has yet to be recorded. In those studies conducted in India to date, only a single report on the genotyping of *MT* isolates from cattle, from the state of Himachal Pradesh, is available (12). This paper reported on the isolation of *MT* in 2006 from a lung lesion taken from a Holstein-Friesian cow, and from the broncho-mediastinal lymph node of a Jersey cow. Both animals were 14 years old and were from the district of Palampur in the state of Himachal Pradesh, northern India. The spoligotype patterns on the X-ray film for the two isolates cited above were further interpreted by the authors at the National Institute for Research in Tuberculosis (NIRT), Chennai, India, and this indicated that these isolates were of MANU1 lineage.

The current study reported on the isolation of *MT* strains from cattle from three different Indian states: Gujarat, Maharashtra and Telangana. The authors also reported on the genotyping of these strains, including those isolates from wild ruminants that had been described by Mukherjee *et al*. earlier (13). *M. tuberculosis* was isolated from the lung tissues collected at necropsy of a gazelle (*Gazella bennettii*) and an antelope (*Antilope cervipara*) in 2010 and 2011, respectively. The animals were inhabitants of a nature park in the district of Gandhinagar, Gujarat. The nature park was home to, among other wild animals, seven gazelles and 150 antelopes that were housed in adjoining fenced enclosures, and free-ranging blue bulls (*Boselaphus tragocamelus*) (*n* = 150).

The nature park attendants lived in the villages surrounding the park located in Indroda, Gandhinagar, Gujarat, and also in villages surrounding Farm 9 in Gandhinagar, located 15 km from the nature park. (Farm 9 is the livestock farm where the *MT* outbreaks in cattle referred to in this study occurred.) With the exception of core staff, the attendants left the park for their homes in the evening, and returned in the morning to resume their duties. The park had a record of persistent tuberculosis (TB), characterised by two episodes that resulted in mortality in eight deer in 2009–2010 followed by 29 deer in 2010–2011; necropsy of three of these wild ruminants revealed lesions suggestive of TB and impression smears from these samples tested positive using acid-fast staining. The representative samples collected from the lesions at necropsy during both episodes, as reported by Mukherjee *et al*. (13), indicated that both outbreaks were due to *MT* infection since only *M. tuberculosis* was isolated, but not *M. bovis*. The main aims of this study were to attempt to:

a) isolate TB from cattle/buffaloes/wild ruminants sourced from farms/abattoirs/nature parks

b) characterise the isolates to the genotype level

c) explore the epidemiological significance of the identified genotypes in the animals examined

d) examine whether a correlation existed between the genotypes identified in the animals and those reported from human beings during the same period, from the same or neighbouring geographical locations.

In the present study, the significance of the results retrieved in pursuit of the abovementioned four aims is discussed.

Materials and methods

Animals and samples

Samples (*n* = 841) were collected from 733 adult female cattle, 92 male cattle (31 adult, 61 young animals) and 16 buffaloes of one to 12 years of age from the three states (Telangana [*n* = 352], Maharashtra [*n* = 31] and Gujarat [*n* = 458]) from 2010 to 2015. Cattle below one year of age were considered young and those above one year of age as adult, while buffaloes less than two years of age were considered young and those above two years of age as adult. Out of the total of 841 samples, 405 were milk samples,

388 were nasal swabs, and the remaining 48 samples were of lymph nodes or liver lesions collected during post mortem. The basis of sample collection and details associated with sample collection are described in Table I.

Specifically, a nasal swab (pooled from both nostrils) and a milk sample (pooled from all four quarters) were collected from each female animal that was in milk. For those female animals that were not in milk, only a pooled nasal swab was collected. On all but one occasion, only pooled nasal swabs were collected from male animals on farms; in the case of the exception, a pooled nasal swab and lymph node tissue were collected as samples. Lymph nodes were collected from abattoirs as samples since, apart from the exception, no visible lung lesions were found during inspection after slaughter. (In the case of the exception, an abscess was found in the liver of an animal in a Mumbai abattoir.) This method of sampling was followed on 407 occasions, when it was only possible to collect a single sample from an animal in abattoirs and farms (Abattoir 1, n = 16; Farm 2, n = 13; Farm 3, n = 16; Farm 5, n = 59; Farm 6, n = 62; Abattoir 2, n = 31; Farm 7, n = 90; Farm 8, n = 114 and Farm 9, n = 6). On the remaining 434 occasions, it was possible to collect more than one sample from each animal (Table I). Table I also provides the status of samples with respect to: *a*) the presence or absence of a recorded clinical history of TB; and *b*) the completion status of a single intradermal tuberculin test (SITT). The tissues from necropsy and milk samples were collected in sterile 50-ml Falcon tubes, and nasal swabs were transferred to sterile Middlebrook 7H9 broth.

Interpretation of results of the single intradermal tuberculin test

The SITT was conducted as described previously by the World Organisation for Animal Health (OIE) and Mukherjee *et al.* (1, 13), employing bovine tuberculin purified protein derivative (PPD) from Prionics (Schlieren, Switzerland). The SITT results were interpreted according to the criteria described in previous publications (1, 13). In brief, 72 h after inoculation with bovine tuberculin PPD, the skin-fold thickness of each animal was measured at the site of inoculation. An increase in the skin thickness equal to or greater than 4 mm, with or without inflammatory signs at 72 h, compared with the reading prior to inoculation, was interpreted as a positive test result.

Isolation of *Mycobacterium* from clinical samples

The milk samples were decontaminated and processed as previously described by Gao *et al*. (18). Briefly, 50 ml of milk samples were centrifuged at 3,100 × *g* for 30 min to collect a pellet. The pellet and cream layer were decontaminated using hexadecylpyridinium chloride (HPC) at a final concentration of 0.75% for 5 h at 22°C. Next, samples were centrifuged at 1,000 × *g* for 15 min at room temperature,

Table I
Details of the samples collected and processed for isolation from 2010 to 2015

Origin of sample (source, location, state)	Single intradermal tuberculin test (SITT) result [a]	Clinical history of tuberculosis (TB)	Type of sample [b]			No. of isolates (year of sampling and isolation)
			Milk	Nasal swab	Tissue	
Abattoir 1, Hyderabad, Telangana	Not done	Not available	0	0	16	0
Farm 1, Hyderabad, Telangana	0 (64)	No history of TB	64	64	0	0
Farm 2, Hyderabad, Telangana	Not done	No history of TB	13	0	0	0
Farm 3, Hyderabad, Telangana	Not done	No history of TB	4	20	0	0
Farm 4, Hyderabad, Telangana	Not done	History of TB	(1) 11	11	0	1 (2011)
Farm 5, Hyderabad, Telangana	Not done	No history of TB	0	60	1	0
Farm 6, Hyderabad, Telangana	18 (29)	History of TB	(3) 75	13	0	3 (2010, 2010, 2012)
Abattoir 2, Mumbai, Maharashtra	Not done	Not available	0	0	(1) 31	1 (2012)
Farm 7, Anand, Gujarat	0 (150)	No history of TB	60	150	0	0
Farm 8, Bidaj, Gujarat	0 (144)	No history of TB	144	30	0	0
Farm 9, Gandhinagar, Gujarat	Not done	History of TB	(1) 34	(1) 40	0	2 (2010 and 2011)
Total no. of samples: 841			**405**	**388**	**48**	**7**

a) Some or all animals tested in the farms were positive by the single intradermal tuberculin test (SITT) using bovine tuberculin purified protein derivative (PPD) from Prionics, Switzerland; positive and negative test results were interpreted according to the standard interpretation criteria (1, 13); where figures are included, the number of positive animals is followed by the total number of tested animals in parentheses

b) The number of positive isolates from different types of samples, where available, is contained in parentheses; 'tissues' denotes lymph nodes or liver lesions

after which the supernatant was decanted (18). Nasal swabs and tissue samples collected during post mortem were decontaminated and processed using a modified version of Petroff's method. In short, tissue samples and nasal samples were decontaminated through the addition of an equal volume of 4% sodium hydroxide, and were allowed to stand for 20 min. Next, they underwent two washings with 10 ml of sterile phosphate buffer solution (PBS) at pH 6.8 and finally the samples were centrifuged at 3,000 × *g* for 15 min, and the supernatant decanted (19). Sediments obtained following decontamination of the milk samples, nasal swabs and tissues, as described above, were suspended in 2.0 ml of Middlebrook 7H9 broth. From each decontaminated suspension, 0.5 ml was inoculated into a BD BACTECTM MGITTM (Mycobacterial Growth Indicator Tube) 960/320 7-ml Tube containing 7 ml of modified Middlebrook 7H9 broth base already incorporated with BD BACTECTM MGITTM 960/320 Growth Supplement (GS) and 0.8 ml of the lyophilised antibiotic mixture – PANTA reconstituted with GS from BD BACTECTM MGITTM 960/320 Growth Supplement kit (Becton Dickinson [BD], Wokingham, United Kingdom) and the tubes were incubated in a BD BACTECTM MGITTM 960 system for 49 days at 37°C. In each case, from the remaining suspension, three tubes were inoculated: *a)* 0.1 ml was placed in a tube containing albumin dextrose catalase (OADC)-supplemented BD-DIFCOTM Middlebrook 7H10 agar (BD); *b)* 0.1 ml in a tube containing Löwenstein-Jensen (LJ) medium (HiMedia, Mumbai, India) supplemented with glycerol, and *c)* 0.1 ml in a tube containing LJ medium supplemented with sodium pyruvate; and these inoculated tubes containing three different media were incubated for eight weeks at 37°C in an ordinary air incubator.

Identification of *Mycobacterium* from culture

Heat-fixed smears prepared from MGIT cultures declared as positive by the BD BACTECTM MGITTM 960 system and typical growths on Middlebrook 7H10 and LJ media were screened for the presence of acid-fast bacilli (AFB). The heat-fixed smears were stained for AFB using two commercial staining kits (TB Quick Stain Kit [BD, India, Gurgaon, India], for the identification of AFB, and the TB Fluorescent Stain Kit M, using auramine-rhodamine [BD, India]). The smears were processed for staining according to the manufacturer's instructions.

Molecular identification of acid-fast-bacilli-positive isolates

DNA extraction from MGIT liquid culture and colonies on Middlebrook 7H10 agar/LJ media was performed according to the protocol recommended for the GenoType® MTC kit (Hain Lifescience, Nehrem, Germany). To identify isolates as members of the MTC, the extracted DNA was amplified according to the protocol specified for the single-tube-nested polymerase chain reaction (PCR) kit (GeNei, Bangalore, India). In the single-tube, two-step assay, the first positive amplification was determined by a 220-base pair (bp) PCR product amplified from the IS*6110* region, followed by amplification of a 123-bp-nested amplicon. For the identification and confirmation of strains, three commercial line-probe assay kits recommended by the World Health Organization (WHO) based on the polymorphism in the *gyr*B gene were employed (Genotype® MTC, Genotype® Mycobacterium CM and Genotype® Mycobacterium AS; Hain Lifescience). As well as identifying the presence of MTC, these kits can differentially identify *M. tuberculosis* from other members of the MTC. The Genotype® MTC kit can differentiate *M. tuberculosis/M. cannetti*, *M. africanum*, *M. microti*, *M. bovis*, *M. caprae* and Bacillus Calmette-Guérin (BCG) strains of the MTC from each other. The Genotype® Mycobacterium CM kit detects 'Common Mycobacteria' (CM) including *M. avium* complex (MAC), and 27 clinically relevant mycobacteria that are classified as non-tuberculous mycobacterium (NTM) or as mycobacterium other than tuberculosis (MOTT). The Genotype® Mycobacterium AS kit detects 'additional species' (AS) of *Mycobacteria* that include another 19 clinically relevant MOTTs/NTMs, but not those that belong to MAC. In this study, the MTC kit was used as the primary test and the CM kit was used to double-check or reconfirm that the isolates really belonged only to the MTC group, whereas the AS kit was used to rule out the presence of NTMs/MOTTs in cultures.

Spoligotyping of *Mycobacterium* strains isolated from domestic and wild ruminants

Primers DRa (0.2 µmol/µl) and DRb (0.2 µmol/µl) were used for spoligotyping (20). The spacers between the direct repeats in the target region were amplified using two 18-nucleotide primers (primer 5'-CCAAGAGGGGACG GAAAC-3' and biotinylated primer 5'-GGTTTTGGGTCTGA CGAC-3'). The PCR products were then hybridised to a membrane (Ocimum Biosolutions, Hyderabad, India). Hybridised DNA was detected using an enhanced chemiluminescence kit (Bio Basic, Israel), with exposure to X-ray film producing a pattern or profile reminiscent of a barcode. The hybridisation pattern was analysed in SPOTCLUST using the SpolDB3-based Model (17). SPOTCLUST 'clusters' spoligotype data for tuberculosis using mathematical models for genotyping, incorporating biological information on spoligotype evolution and epidemiology gained from epidemiological data (http://tbinsight.cs.rpi.edu).

Epidemiological analysis of *Mycobacterium tuberculosis* strains from ruminants with respect to prevailing human strains

Since a check on the *Mycobacterium tuberculosis* molecular markers database (SITVIT) for the origin and distribution of

the characterised strains showed that they correlated with an Indian origin of strains, the ruminant *MT* spoligotypes were compared with human strains from India, using information available from the period of 2004 to 2017 (21, 22, 23, 24, 25, 26, 27, 28, 29) to create a unified information base for a region- and period-specific comparison. The results of the drug sensitivity test (DST) of the human *MT* strains of Indian origin belonging to various spoligotype lineages published previously (22, 27, 28, 29, 30) were analysed for information on the susceptibility or resistance of these strains to drugs.

Results

Isolation of *Mycobacterium* strains and their confirmation as *Mycobacterium tuberculosis*

Mycobacteria were isolated from seven of the 841 samples, comprising 4/405 milk, 2/388 nasal swabs and 1/48 post-mortem tissue samples from all the three Indian states examined (Gujarat, Maharashtra and Telangana) in MGITs, on Middlebrook 7H10 agar, and on LJ medium supplemented with glycerol, but not on LJ media supplemented with sodium pyruvate. Of the seven isolates, three were recovered from Farm 6 in Telangana, two from Farm 9 in Gujarat, one from Farm 4 in Telangana and the remaining isolate from an abattoir in Maharashtra (Tables I and II). 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 the isolates yielded both the 220- and 123-bp amplicons characteristic of MTC members. The hybridisation pattern generated by the Genotype® MTC kit identified all seven isolates as MT. The results of the Genotype® Mycobacterium CM kit reconfirmed the isolates as members of the MTC and ruled out the presence of *M. avium* strains, while the Genotype® Mycobacterium AS kit confirmed the absence of MOTT strains (Tables I and II). The results of identification by culture, nested PCR and hybridisation pattern generated by the three commercial line-probe assay kits did not indicate the presence of any other member of the MTC (*M. bovis*, *M. bovis* BCG, *M. caprae*, *M. cannetti*, *M. africanum* or *M. microti*) except *M. tuberculosis*. Moreover, none of the battery of tests described above indicated the presence of members of *Mycobacteria* belonging to the MAC or MOTT groups.

Spoligotyping of *Mycobacterium tuberculosis* strains using SPOTCLUST

The spoligotyping analysis indicated the presence of *MT* strains belonging to the East African Indian (EAI) 5 family (Octal code – 474377777413771) from cattle and a gazelle (Octal code – 454377777413761) in Gujarat. The EAI3_IND family was identified in cattle and an antelope from Gujarat, and also from a liver lesion from a male adult bovine in Maharashtra (Octal code – 477777777413071). Three distinct families, U (Octal code – 477776077411771), CAS1_DELHI (Octal codes – 703777740000771 and 702777340000571), as well as T1 (Octal code – 777717777760761), were identified from cattle in Telangana (Tables II and III).

Epidemiological analysis of *Mycobacterium tuberculosis* strains in ruminants and humans

The results of the study indicated the presence of the same spoligotypes in ruminants and humans living in the same and adjoining geographical regions, during the period under study and seemed to suggest the existence of an active 'spillover' mechanism of *MT* infection from human to domestic and wild ruminants. The evidence for this is as follows: between 2010 and 2012, human *MT* isolates were identified as belonging to the EAI5 family in Gujarat (21) and in Mumbai in the adjoining western state of Maharashtra (22, 23). Moreover, the same EAI5 family was identified from a sample from a lung lesion taken from a gazelle (*G. bennettii*) in 2010 and from nasal swabs of cattle in 2011 in Gujarat. Similarly, the EAI3_IND family was reported from human *MT* isolates in Gujarat in 2010–2011 (21), from extra-pulmonary lesions in Mumbai, Maharashtra, in 2012 (23), and, in the present study, EAI3_IND was identified in an antelope (*A. cervipara*) and in cattle in Gujarat in 2011 and 2010, respectively.

In addition, the same spoligotype was identified in a strain isolated from a bovine liver in Mumbai, Maharashtra, in 2012. Furthermore, the U family (ST 1429 and ST 124) was reported in humans from Bhopal, Madhya Pradesh, during 2007–2011 (26), from Puducherry in 2015 (in extra-pulmonary lesions) (28), and from Vellore, Tamil Nadu, in 2017 (29). (The southern state of Tamil Nadu adjoins the state of Telangana in the north.) Kandhakumari *et al.*'s report, published in 2015 (28), was the first time that the U family had been identified from human strains in India. In this study, an *MT* isolate from cow's milk from Telangana was identified in 2010 as belonging to the U family, probably indicating its prior presence in the southern region of India. *M. tuberculosis* isolates from human pulmonary lesions, belonging to the CAS1_Delhi family, were reported in Tamil Nadu in 2010 (21) and Hyderabad, Telangana, in 2011 (27). The same family was identified from cow's milk samples from Hyderabad in 2010 and again in 2012. In addition, T1–T2 and T1 families were reported in human *MT* isolates from: the southern state of Tamil Nadu in 2010–2011, Bhopal in central India, Madhya Pradesh in 2007–2011, and Hyderabad, Telangana, in 2011 (21, 26, 27). In the present study, the T1 family was identified from nasal swabs of cattle in Hyderabad in 2011. Details of the above analysis are summarised in Table III. Drug sensitivity testing (DST) was not performed on isolates of animal

Table II
Details of *Mycobacterium tuberculosis* field isolates from domestic and wildlife ruminants and spoligotyping results

Sample ID no.	Species and age	Gender	Sample	Remarks on isolation	Geographical location of isolates
MCR1	Gazelle, one year	Female	Lung	Previous study (13)	Gandhinagar, Gujarat
MCR2	Antelope, one year	Female	Lung	Previous study (13)	Gandhinagar, Gujarat
MCR3	Cattle, six years	Female	Milk	This study	Farm 9, Gandhinagar, Gujarat
MCR4	Cattle, eight years	Female	Milk	This study	Farm 6, Hyderabad, Telangana
MCR5	Cattle, nine years	Female	Milk	This study	Farm 6, Hyderabad, Telangana,
MCR6	Cattle, seven years	Female	Nasal swab	This study	Farm 9, Gandhinagar, Gujarat
MCR7	Cattle, ten years	Male	Liver	This study	Abattoir 2, Mumbai, Maharashtra
MCR8	Cattle, seven years	Female	Milk	This study	Farm 6, Hyderabad, Telangana,
MCR9	Cattle, eight years	Female	Nasal swab	This study	Farm 4, Hyderabad, Telangana

Open squares: lack of hybridisation; represented from 1 to 43
Solid squares: hybridisation with designated spacer probe
CAS: Central and Middle Eastern Asia
EAI: East African Indian
T1: Tuscany
U: unidentified

The *Mycobacterium tuberculosis* (*MT*) isolates in wild female ruminant species were reported during a previous study (13) and the spoligotyping results of these isolates were reported during this study. The *MT* isolates from cattle were identified and spoligotyped in this study. All isolates were recovered from cultures in the BACTEC™ MGIT™ 960 system (Beckton Dickinson, Wokingham, United Kingdom), BD-DIFCO™ Middlebrook 7H10 agar supplemented with oleic albumin dextrose catalase (OADC) and glycerol, and Löwenstein-Jensen medium supplemented

origin in this study. Of the seven animals that were positive for MT, post-mortem examination could be conducted only on one animal; post-mortem examination revealed lesions in the liver.

Discussion

Natural infection of cattle, buffaloes and other ruminants with *MB* has been reported in India. *M. bovis* has been isolated from lymph nodes (5), lung lesions (12), milk (14, 15, 16), and blood from cattle in the states of Himachal Pradesh (5, 12), Uttarakhand (14), Uttar Pradesh (15) and several other states in northern India (16), as mentioned in the reports published from the period from 2005 to 2015. However, the concurrent infection of cattle with *MB* and *MT* has also been reported in India (12, 14, 15, 16). Against this background, infection with *MT* alone, in animals belonging to the Bovidae family, is rarely reported in this country (11, 31, 32). Therefore, the authors were surprised to find only *MT* present in all seven isolates in this study, as well as in the wild ruminants previously reported by Mukherjee *et al.* in 2015 (13). These findings become more significant when one considers that *MT* was isolated from three species of ruminants (cattle, antelope and gazelle) from various geographical regions of India.

In this report, the authors used the highly sensitive and recommended commercial liquid culture system, BD BACTECTM MGITTM 960 (33, 34), as well as reverse slot blot line probe *Mycobacterium* species identification assays (35) for the isolation and confirmation of seven *MT* isolates collected from different locations in India. The identification and confirmation of *MT* infection alone in the different types of samples (milk, nasal swab and liver), originating from diverse geographical locations in India, had not previously been reported. The isolation from ruminants of *M. avium* and those strains grouped as MOTT has been described earlier (35, 36, 37). To examine this possibility, the authors used two commercial line probe assay kits to rule out the presence of *M. avium* and MOTT strains, and the existence of a concurrent infection from the source.

In 2012, Thakur *et al.* reported on the identification of an *MT* strain in cattle from the northern Indian state of Himachal Pradesh in 2006 using the spoligotyping approach (12); interpretation of the spoligotype patterns by the authors indicated that two of the *MT* isolates belonged to MANU1.

Octal code	SpolDB3-based lineage	Binary spoligotype patterns
454377777413761	EAI5	
477777777413071	EAI3_IND	
477777777413071	EAI3_IND	
477776077411771	U	
703777740000771	CAS1-Delhi	
474377777413771	EAI5	
477777777413071	EAI3_IND	
702777340000571	CAS1-Delhi	
777717777760761	T1	

with glycerol but not sodium pyruvate, and were identified as members of the *Mycobacterium tuberculosis* complex (MTC) group by IS6110 commercial nested polymerase chain reaction (PCR) (GeNei, Bangalore, India).

All isolates were confirmed as *MT* by a specific pattern of reverse slot blot hybridisation of the line probe assay using Genotype® MTC kit (Hain Lifescience, Nehrem, Germany). The spoligotype patterns were identified among *MT* isolates. Clade designations were made according to SpolDB3. Genotyping of all these isolates were performed in this study

In the present study, the authors report, for the first time, on the presence and the regional distribution of different *MT* spoligotypes in cattle, from 2010 to 2015. In addition, the authors also present spoligotyping data on *MT* isolates from captive wildlife ruminants, *G. bennettii* and *A. cervipara*. Regional evidence seems to indicate the persistence of spoligotype lineages of East African Indian origin – the EAI5 and EAI3_IND family – in the western states of Gujarat, and EAI3_IND in Maharashtra. A different set of lineages represented as CAS1_Delhi, U and T1 spoligotypes was identified in the southern state of Telangana in the period under study. The authors also report the simultaneous existence of two different *MT* cattle isolates with spoligotype lineages EAI3_IND and EAI5 in the same location in Gujarat (Farm 9 – Table I), responsible for tuberculosis outbreaks in 2010 and 2011, respectively. Similarly, the authors noticed the existence of two different spoligotypes in two different ruminant species identified as EAI5 in *G. bennettii* and EAI3_IND in *A. cervipara* from the same nature park in Gujarat (laboratory identification no. MCR1 and MCR2 – Tables II and III) in 2010 and 2011, respectively. Since five *MT* spoligotypes from seven ruminants in India were identified in this study, it is possible that more *MT* strains exist in ruminants in the subcontinent. A similar observation has previously been made based on the existence of three *M. bovis* spoligotypes in cattle in Ethiopia in just 11 cattle (7).

Although there is an abundance of information available related to the genotypes of human strains of *MT* prevalent in India (21, 22, 23, 24, 25, 26, 27, 28, 29, 31), similar reports on ruminants from this country are scarce. Only two previous reports (12, 13) have identified the existence of different genotypes of the *MT* strain in ruminants, so over all, including the current study, six *MT* lineages (MANU1, EAI3_IND, EAI5, CAS1_Delhi, U and T) have been detected from ruminants to date from a total of just three studies. Moreover, recently, in January 2017, *MT* isolates from cattle of the MANU1 lineage were reported in Tamil Nadu (32). This preliminary information derived from the limited number of studies in ruminants does not reveal the complete picture regarding the number of human strains of *MT* that exist in India's entire ruminant population. It does, however, indicate that, in order to reveal the actual situation, a large number of further studies would be necessary. This supposition is strengthened by previous reports of the existence of different *MT* genotypes in cattle herds (7, 34) and the fact that in high-prevalence settings or where there is a high TB burden, humans and animals living in close contact for a prolonged period are quite likely

Table III
Details of *Mycobacterium tuberculosis* field isolates from domestic and wildlife ruminants and spoligotyping results as compared to the regional presence of human strains

Laboratory identification no.	Species [a] [b]	Tissue	Geographical location of isolates	Year of isolation	Clade [c]	Regional presence of families of *Mycobacterium tuberculosis* human isolates [d]			
						Spoligotype/ clade	Year of isolation/ reporting	Location, region (tissue)	References
MCR1	Gazelle	Lung	Gandhinagar, Gujarat	2010	EAI5	EAI5	2010, 2011; 2012	Gujarat; Maharashtra, Mumbai; Maharashtra, Mumbai	(21, 22, 23)
MCR2	Antelope	Lung	Gandhinagar, Gujarat	2011	EAI3_IND	EAI3_IND	2010, 2011	Gujarat	(21)
MCR3	Cattle	Milk	Gandhinagar, Gujarat	2010	EAI3_IND	EAI3_IND	2010, 2011	Gujarat	(21)
MCR4	Cattle	Milk	Hyderabad, Telangana	2010	U	U	2007–2011, 2015	Bhopal, Madhya Pradesh; Tamil Nadu, Puducherry; Vellore, Tamil Nadu	(26, 28, 29)
MCR5	Cattle	Milk	Hyderabad, Telangana	2010	CAS1-Delhi	CAS1_Delhi	2011; 2008–2009; 2014–2015; 2010	Tamil Nadu; Tamil Nadu, Tiruvallur; Southern India; Telangana, Andhra Pradesh (pulmonary)	(21, 24, 25, 27)
MCR6	Cattle	Nasal swab	Gandhinagar, Gujarat	2011	EAI5	EAI5	2010; 2011	Gujarat; Maharashtra, Mumbai	(21, 22)
MCR7	Cattle	Liver	Mumbai, Maharashtra	2012	EAI3_IND	EAI_IND3	2012	Maharashtra, Mumbai (extra-pulmonary)	(23)
MCR8	Cattle	Milk	Hyderabad, Telangana	2012	CAS1-Delhi	CAS1_Delhi	2008–2009; 2014–2015; 2010, 2011	Tamil Nadu; Tamil Nadu, Tiruvallur, Southern India; Telangana, formerly Andhra Pradesh (pulmonary)	(21, 22, 24, 25)
MCR9	Cattle	Nasal swab	Hyderabad, Telangana	2011	T1	T1 T1–T2	2011; 2007–2011 2010; 2014–2015	Tamil Nadu; Bhopal, Madhya Pradesh Hyderabad Telangana, formerly Andhra Pradesh, Southern India	(21, 22, 25, 26)

EAI: East African Indian
CAS: Central and Middle Eastern Asia
U: Unidentified
T1: Tuscany

a) Sample identification numbers MCR1 and MCR2 were *Mycobacterium tuberculosis* (*MT*) isolates from wild female ruminant species reported during a previous study (13) and the spoligotyping results of the same were reported during this study

b) Sample identification numbers MCR3, MCR5, MCR6, MCR7, MCR8 and MCR9 were *MT* isolates from female cows, apart from MCR4 which was from a male; these isolates were identified and spoligotyped during this study. All isolates were recovered from cultures in the BACTEC™ MGIT™ 960 system (Beckton Dickinson [BD], Wokingham, United Kingdom), BD-DIFCO™ Middlebrook 7H10 supplemented with oleic albumin dextrose catalase (OADC) and glycerol, and Löwenstein-Jensen medium supplemented with glycerol but not sodium pyruvate, and were identified as members of the *Mycobacterium tuberculosis* complex (MTC) group by IS6110 commercial nested polymerase chain reaction (PCR) (GeNei, Bangalore, India). All isolates were confirmed as *MT* by a specific pattern of reverse slot blot hybridisation of the line probe assay using the Genotype® MTC kit (Hain Lifescience, Nehrem, Germany)

c) The spoligotype patterns were identified among *MT* isolates; clade designations were made according to SpolDB3

d) Regional presence of spoligotypes of *Mycobacterium tuberculosis* human isolates (28) are as mentioned in the text (21, 22, 24, 25, 26, 27, 28, 29)

to acquire *M. tuberculosis* infection (7, 16, 34, 38, 39, 40) due to their genotypes being the same or closely related as a result of spillover or spill-back mechanisms (38). It is probably through this mechanism that ruminants/cattle acquire infection as there are several *MT* strains of human origin worldwide, whereas *MT* variants do not occur in cattle.

Mycobacterium tuberculosis infection in animals can spread from one geographical area to another, due to the unrestricted movement of infected animals or contact with infected humans. The chronology of *MT* outbreaks in different ruminant species recorded in the area where Farm 9 and the nature park are located (13) probably indicates that the same spoligotype lineage EAI3_IND that infected cattle in 2010, infected *A. cervipara* in the nature park in 2011. Similar *MT* spoligotypes were observed when the EAI5 identified in *G. bennettii* in 2010 was recovered from cattle in Farm 9 in 2011. Large-scale unrestricted road migration of farmers/transients along with their

sick, unproductive, infected and stressed animals, due to drought and shortage of fodder, is common from Gujarat to the adjacent state of Maharashtra. In this manner, infected animals gain access to a new location and new hosts. Such unproductive animals ultimately end up in the abattoirs of Mumbai. It seems, perhaps, that this could be one of the probable mechanisms through which *MT* infection in cattle, reported from Gandhinagar, Gujarat in 2010 due to EAI3_IND lineage, spread to Mumbai, Maharashtra, in 2012. In contrast, the authors observed two different *MT* strains, U and CAS1_Delhi, in the same farm (Farm 6) in Telangana, also in 2010. Moreover, yet another spoligotype variant, T1, was reported in another farm (Farm 4) in Telangana in 2011. The reason for the predominant presence of the CAS spoligotype in humans in Hyderabad in recent times, a strain mostly reported from northern India, could be due to the increasing influx and convergence of non-resident Indians (NRI) working in the Middle East and the migrant population from northern India which now resides in Hyderabad, the commercial capital of Telangana (38). This study's observation of the existence of the CAS1_Delhi spoligotype in cattle in 2010 could be due to the spillover effect from humans.

In the study of tuberculosis, India stands out as being home to *M. tuberculosis* lineage 1 (Indo-Oceanic or EAI lineage) and lineage 3 (Central Asian or CAS lineage) primarily, which occur at substantially lower frequencies outside the Indian subcontinent. In contrast, lineages 2 (East Asian or Beijing) and 4 (Euro–American) are the most common lineages in Europe, Africa and many other parts of the world. Even within India, the prevalence of lineages varies. Lineage 3 predominates among patients from North and North-West India, and lineage 1 is commonly found among southern Indian patients and at low frequency among patients in other parts of the country. In contrast, lineage 2 has been reported at similar prevalences throughout India, though it does predominate in some north-eastern Indian states (41, 42, 43).

Humans are the maintenance hosts for *MT*; however, cattle and wild ruminants have been identified as spillover hosts (34, 38, 39). It has been observed that spillover hosts are commonly infected when the level of challenge is relatively high (3). A high TB burden in humans infected with *MT* lineages, such as EAI3_IND, EAI5 and CAS1_Delhi prevailing in India may therefore be the reason for such a spillover to ruminants (44). At least, on four occasions, the isolation of *MT* from nasal swabs from cattle in this study (Table II) and from lung samples from wild ruminants in the study by Mukherjee *et al*. (13) seems to indicate the transmission of *MT* infection from humans to ruminants through an aerogenous route. This fact is in agreement with earlier observations (45, 46). Moreover, it was also evident in the current study that *MT* was isolated from milk on at least four occasions, and from liver on one occasion (Table II), probably indicating acquired extra-pulmonary *MT* infection by humans. Transmission of *MT* infection from humans to 'spillover' hosts, such as cattle, from extra-pulmonary sources has been suggested previously (13, 16). Although the precise mechanism cannot be fully explained, the lineages of human *MT* strains isolated from extra-pulmonary tissue sources (19, 43) and the identification of EAI3_IND from extra-pulmonary lesions from cattle in this study strengthen this contention. Similarly, in recent times two separate studies from Ethiopia have indicated that farmers and their cattle were infected with *MT* (7, 40). Both these studies revealed that cattle, in addition to being infected with *M. bovis* and NTM, were also infected with *MT* strains that belonged to three families – CAS, T3-ETH and T1 (7, 40, 47, 48).

The WHO *Global Tuberculosis Report 2015* indicated that India has the largest number of TB cases in the world with 23% (49). Compared to other regions in the world, *MT* infection reported in cattle in India over the last 12 years, with respect to isolation from samples, is quite high, varying from 15% to 84% (32, 50). Since SITT is not able to differentiate *MT* from *MB* infection in cattle, and facilities for the culture of pathogenic *Mycobacterium* species are mostly not available in rural settings in India, over all, the above facts give rise to a crucial diagnostic disadvantage that impacts the control of bovine tuberculosis in India.

The report on the sharing of identical *MT* strains of the same lineage in humans and cattle has been emphasised as an important epidemiological event that should be considered when trying to minimise the risk of transmission of *MT* from humans to cattle and from cattle to humans by spillover and spill-back mechanisms (34, 38). Earlier reports of the isolation of *MT* from bovine milk (11, 16, 50) and from extra-pulmonary lesions in cattle (50) and humans (28, 44), and the practices of drinking unpasteurised and soured milk, and of raising herds of cattle on open pastures in rural India, have brought the authors closer to the realisation that *MT* infection in cattle could pose a new challenge in the control of TB in humans in this country. Taken together, the above observations can impact the control of bovine and human TB.

The relevance of the observed association between virulence intensity in cattle infected with *M. bovis* (51) and *M. tuberculosis* in humans (52) with certain *MT* genotypes has been highlighted. Since several reports, as discussed above (7, 16, 34, 38, 39, 50), have indicated that *MT* strains of the same family are shared among infected ruminants and humans, it would seem that further investigation is necessary to determine whether such *MT* genotypes are present in the ruminants in India. In the present study, it was possible to conduct a post mortem on one animal in a Mumbai abattoir which showed a lesion in its liver; however, the authors were legally restricted from performing the

same on six other *MT* bacterial culture-positive live cattle, owned by private farms (Farms 4, 6 and 9), and therefore were unable to associate the extent and intensity of lesions with culture positivity.

The authors' observations on the distribution of *MT* strains of ruminant origin reported from the regions of Gandhinagar in Gujarat, Mumbai in Maharashtra and Hyderabad in Telangana (formerly in Andhra Pradesh) during 2010 to 2012, when compared to reports on the molecular epidemiology of human strains from 2004 to 2015 (21, 28), probably reflects the parallel existence of *MT* strains of the same family lineage, in the human maintenance host and the spillover ruminant host. The simultaneous existence of the same *MT* families in cattle and in their herders has been recently observed in farms in the southern state of Tamil Nadu (P. Kannan, personal communication). The above observations when combined seem to suggest that a spillover of *MT* strains exists from humans to ruminants on the Indian subcontinent. Drug sensitivity tests of human *MT* strains recovered from ruminants were not undertaken in this study. However, the availability of reports on such tests may add a new dimension to the understanding of the epidemiology of *MT* infection in ruminants; the DST reports on human strains from India that are available indicate a variance in the resistance or sensitivity to antibiotics (22, 27, 28, 29, 30). While pan-sensitivity of CAS1_DELHI (ST 26) and EAI5 (ST 236) was observed in one report (22), for example, in another the majority of human *MT* strains from India of EAI5, EAI3_IND and CAS families were resistant to multiple drugs (27).

Conclusions

The above observations certainly complicate the method of controlling a zoonotic disease, such as TB, in India, since *MT* strains seem to exist and cross-infect hosts without a species barrier at the same time point. It will be worth investigating the spill-back behaviour of these ruminant *MT* strains to humans, as reported in this study. Furthermore, a more intensive molecular epidemiological investigation on the presence of the *MT* strains prevalent in domestic and wild ruminants on the Indian subcontinent, other than those reported herein, is warranted in order to enhance clarity on the magnitude of the spillover effect on ruminants that prevails in India.

Acknowledgements

The authors are grateful to the management of the National Dairy Development Board in Anand for providing the facilities to carry out this work, which was conducted at the Research and Development (R&D) Laboratory, National Dairy Development Board (NDDB), Hyderabad. Mr Vijay Bahekar expresses his gratitude to the NDDB, for providing him with the opportunity to work on the isolation and characterisation of *Mycobacterium* isolates as part of his PhD thesis at the R&D facilities of NDDB at Hyderabad.

Épidémiologie moléculaire et portée zoonotique de *Mycobacterium tuberculosis* isolé chez des ruminants domestiques et sauvages dans trois états de l'Inde

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Résumé

La majorité des cas de tuberculose chez les ruminants sont dus à *Mycobacterium bovis*. Néanmoins, les auteurs rapportent les résultats d'une étude réalisée de 2010 à 2015 en Inde (états de Telangana, Maharashtra et Gujarat), au cours de laquelle *Mycobacterium tuberculosis* a été isolé à partir de lait de vache ainsi que d'écouvillons nasaux et de prélèvements tissulaires post-mortem (*n* = 841) collectés sur des bovins et des buffles. L'appartenance des isolats au genre *Mycobacterium* a été confirmée par l'observation des caractéristiques de croissance des colonies et de leur morphologie dans un milieu de culture liquide du commerce (Mycobacterial Growth Indicator Tube [MGIT]™ : tube avec

indicateur de croissance mycobactérienne) en utilisant l'automate BD BACTEC™ MGIT™ 960 et un milieu de Lowenstein-Jensen additionné de glycérol mais sans pyruvate de sodium, ainsi qu'une gélose BD-DIFCO™ Middlebrook enrichie en acide oléique, albumine, dextrose et catalase (OADC). Dans un premier temps, les isolats ont été identifiés comme étant des membres du complexe *M. tuberculosis* au moyen d'une amplification en chaîne par polymérase nichée ciblant la séquence nucléotidique spécifique IS*6110* du complexe *M. tuberculosis*. Trois kits commerciaux d'analyse de souches ont permis d'identifier les isolats comme étant *M. tuberculosis* ; il a ensuite été procédé à l'analyse des génotypes des souches de spoligotypes, lesquelles appartenaient aux lignées East African Indian (EAI) 3_IND, EAI5, Central-Asian (CAS) 1_DELHI, U et T1. Les spoligotypes de deux isolats de *M. tuberculosis* obtenus précédemment, provenant respectivement d'une antilope (*Antilope cervipara*) et d'une gazelle (*Gazella bennettii*) de l'état de Gujarat ont été analysés lors de la présente étude et identifiés comme étant respectivement de lignée EAI3_IND et EAI5. Les auteurs analysent l'importance épidémiologique et la portée zoonotique de la présence rapportée dans la région du même spoligotype ou de deux spoligotypes différents chez des espèces différentes de la famille des Bovidés ainsi que chez l'homme.

Mots-clés
Épidémiologie – Inde – Isolement – *Mycobacterium tuberculosis* – Ruminant – Spoligotypage – Zoonose.

Aislamiento y análisis de la epidemiología molecular y la importancia zoonótica de *Mycobacterium tuberculosis* en rumiantes domésticos y salvajes de tres estados de la India

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Resumen
La mayoría de los casos de tuberculosis que afectan a los rumiantes son causados por *Mycobacterium bovis* (*MB*). En este estudio, sin embargo, los autores dan cuenta del aislamiento de *Mycobacterium tuberculosis* (*MT*) en muestras de leche, frotis nasales y tejidos obtenidos *post-mortem* (*n* = 841) de ganado vacuno y búfalos de los estados de Telangana, Maharashtra y Gujarat (India) entre 2010 y 2015. Se confirmó que los microorganismos aislados (*n* = 7) eran micobacterias por sus características de crecimiento y la morfología de las colonias cultivadas en medio líquido comercial Mycobacterial Growth Indicator Tube (MGIT)™ empleando el sistema BD BACTEC™ MGIT™ 960 y el medio Löwenstein-Jensen (LJ) suplementado con glicerol, pero no con piruvato sódico, y agar BD-DIFCO™ Middlebrook 7H10 con ácido oleico, albúmina, dextrosa y catalasa (OADC). Mediante una PCR (reacción en cadena de la polimerasa) anidada comercial basada en la secuencia nucleotídica IS*6110* específica del complejo, se empezó por determinar que esos microorganismos pertenecían al complejo *M. tuberculosis* (MTC). Tras confirmar que se trataba de *M. tuberculosis* empleando tres ensayos comerciales con sondas en línea, se procedió a caracterizar su genotipo, lo que sirvió para identificar espoligotipos

correspondientes a los siguientes linajes: East African Indian (EAI) 3_IND, EAI5, Central-Asian (CAS) 1_DELHI, U y T1. Durante el estudio se caracterizaron asimismo los espoligotipos de dos *M. tuberculosis* aislados previamente a partir de un antílope (*Antilope cervipara*) y una gacela (*Gazella bennettii*) de Gujarat, lo que permitió adscribirlos respectivamente a los linajes EAI3_IND y EAI5. Los autores exponen la importancia desde el punto de vista epidemiológico que tiene la presencia comprobada en la región del mismo espoligotipo o de dos espoligotipos diferentes en distintas especies de la familia Bovidae, así como en el ser humano, y las consecuencias que de ahí se siguen por lo que respecta a posibles zoonosis.

Palabras clave
Aislamiento – Caracterización de espoligotipos – Epidemiología – India – *Mycobacterium tuberculosis* – Rumiante – Zoonosis.

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