

Molecular diagnosis of Zoonotic *Mycobacterium bovis* infection in Melghat, India

Prachi R. Bapat¹, Ashish S. Satav², Seema D. Shekhawat¹, Sonali D. Manke¹, Aliabbas A. Husain¹, Amit R. Nayak¹, Anuja P. Kawle¹, Lokendra R Singh¹, Hatim F. Daginawala¹, Rajpal S Kashyap^{1*}

1-Research Centre, Central India Institute of Medical Sciences, Nagpur, Maharashtra, India

2-Meditations, AIDS, Health, Addiction & Nutrition (MAHAN) Trust, C/O Mahatma Gandhi Tribal Hospital, Karmagram, Utavali, Tahsil Dharni, District: Amravati, Maharashtra, India

*Corresponding author: kashyap_rs@yahoo.co.in

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Summary

Mycobacterium bovis (*M. bovis*) infection in humans is not adequately diagnosed since classical biochemical and cultural tests are both sophisticated and time consuming. However, being intrinsically resistant to Pyrazinamide, the species-specific identification of *M. bovis* is clinically significant. The present study was performed to determine the prevalence of Zoonotic *M. bovis*-induced tuberculosis (TB) in the malnourished tribal population of Melghat using a duplex PCR assay targeting the regions of difference (RD) 1 and 4. A prospective cohort study was carried over a period of 2 years from 2011 to 2013 in the Melghat region of Maharashtra, India. A total number of 347 blood samples were collected from participants recruited through camps organized in 10 different villages of Melghat. The samples were then subjected to duplex PCR assay for differential identification of the mycobacterial pathogens viz., *M. tuberculosis* (*M. tb*), *M. bovis* and *M. bovis* BCG. The duplex PCR assay identified *M. bovis* in 2.59% (9/347) and *M. tb* in 17.29% (60/347) of samples. Altogether the 9 *M. bovis* positive cases had exposure to domesticated animals or consumed raw, unpasteurized milk. This study provided a rapid and cost effective molecular tool for screening of *M. bovis* in the isolated regions of Melghat.

Keywords: Duplex PCR, *Mycobacterium bovis*, Tuberculosis, Zoonotic TB.

Introduction

India remains one of the major Tuberculosis (TB) endemic countries accounting for 2.0-2.3 million TB cases compared with 9 million cases reported worldwide (Husain et al., 2016). The *Mycobacterium tuberculosis* complex (MTC) is consisted of the closely related bacterial sub-species among which

Mycobacterium tuberculosis (*M. tb*) and *Mycobacterium bovis* (*M. bovis*) are the most important pathogens (Alexander et al., 2013). The identification of the closely related members of the MTC has remained a challenging task owing to non-specific clinical presentations and lack of specific diagnostic tests for differentiation in most

diagnostic capacities in high TB endemic regions. The transmission of *M. bovis* from animals to human is either by inhalation of aerosol from infected animals or through consumption of raw unpasteurized milk (Rodwell et al., 2008; Ameni et al., 2013). Despite being recognized as major zoonotic infections, limited diagnostic studies are available on *M. bovis* infections in humans in TB endemic countries like India.

Currently available diagnostics tests for identification of *M. bovis* are based on conventional methods like microbiological features, phenotypic and biochemical tests. However, these tests are slow, unreliable and time consuming. The high degree of variability among these tests warrants the development of molecular biological tools for identification of *M. bovis* infections (Comer, 1994). Recent comparative genome analysis has provided valuable information on the region of difference (RD) in the chromosome of MTC indicating that specific identification of MTC can be achieved by the detection of these regions (Parsons et al., 2002). The genes in the RD1 belong to the early secreted antigenic target -6 (*esat6*) gene cluster. ESAT-6 is a potent stimulator of the immune system, and is an antigen recognizer during the early stages of infection. RD1 of *M. tb* is considered to be the primary attenuating deletion in the related vaccine strain *M. bovis* BCG (Lewis et al., 2003) and thus can be used for differential diagnosis of *M. tb* from other sub-species in the MTC. PCR based methods targeting RDs can be easily performed in local clinical set-ups and are cost effective, which is the foremost

requirement of the studied region due to its geographical remoteness and isolation from the developed world (Huard et al., 2003).

We had previously reported the prevalence of latent and active TB in Melghat which is a hilly, forested tribal belt in the Maharashtra state of India with a population of nearly 0.3 million people (Bapat et al., 2015; Kashyap et al., 2013). The tribal population of Melghat has poor socioeconomic conditions and lives in close proximity with their livestock. The prevalence of TB among the habitants and their close association with animals suggested the possibility of zoonotic transmission of the disease in this area. Despite the associated risk factors, the epidemiological prevalence of *M. bovis* in the tribal population has not yet been established.

The present study was conducted with an aim to investigate the prevalence of *M. bovis* infection in the tribal population of Melghat. This survey also demonstrates the usefulness of duplex PCR assay using RD targets to identify and simultaneously differentiate between *M. bovis*, *M. tb* and *M. bovis* bacillus Calmette–Guérin (BCG) which can be of commercial diagnostic utility in low resource settings in India.

Materials and Methods

Ethics Statement

The study was approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur and Meditation Addiction Health AIDS Nutrition (MAHAN) Trust, Melghat. The approved study was in accordance with the

Declaration of Helsinki of 1975 (as revised in 2000). Written consents were taken from each participant after detailed oral explanation about the study.

Study Design and Participants

A prospective study was conducted from 2011 to 2013 in the Melghat region, Amravati district of Maharashtra. The study participants were mostly tribal and were recruited from different villages of Melghat (Fig 1) with the help of the MAHAN Trust, which provides medical care to the tribal population in the Melghat region.

Detailed information from all participants was acquired through a structured questionnaire. All participants were recruited using specific inclusion criteria which included risk factors like occupational exposure to domesticated animals, type of livestock reared, and duration of exposure with infected animals and consumption of raw, unpasteurized milk or milk products. Baseline characteristics such as age, gender, height, weight, education, occupation and behavioural factors were also recorded. The information regarding presence of infections or illnesses experienced in the past three months along with previous contact with active TB cases was also included in the questionnaires. BCG vaccination status was based on the assessment of BCG scar on the left forearm. We also assessed the Body Mass Index (BMI) values, percentage weight loss, dietary uptake and physical attributes as determinants of malnourishment among the study participants.

Out of the 438 participants enrolled in this study, 43 participants were excluded from

the study based on their refusal to give blood. Pregnant women (n =12) and children below the age of 10 years (n = 13) were also excluded. Paired sputum and blood specimens were collected from the remaining 370 participants. Among these, 23 participants who had other bacterial (n=9) or fungal/viral infections (n=14) were also excluded. The remaining 347 participants who matched the inclusion criteria were recruited for the final analysis. Figure 2 presents the inclusion/exclusion criteria adopted for recruitment of the study population.

Culture procedure

Decontaminated sputum specimens (2 ml) were inoculated into BacT/ALERT® MP *M. tb* process bottles (BioMerieux, France) with 10 ml supplemented Middlebrook 7H9 broth and incubated at 37°C in BacT/ALERT system (BioMerieux, France). All culture specimens were examined twice a week for 6 weeks. The positivity of culture was defined by the growth of *mycobacteria* in the liquid media.

DNA isolation and quantification

For DNA isolation from peripheral blood, buffy coat was separated by gradient centrifugation using HISTOPAQUE – 1077 (St. Louis, Mo. SIGMA). Six ml of PBS was thoroughly mixed with 2 ml of peripheral blood. The mixture of peripheral blood and PBS (8 ml) was gradually added from the side of the tube to 4 ml of histopaque and centrifuged at 2000 rpm for 10 min. The buffy coat thus obtained and was transferred to a fresh tube and equal volume of PBS was

added and centrifuged again at 2000 rpm for 10 min. Supernatant obtained was discarded and the pellet was used for DNA isolation by phenol chloroform extraction method as described elsewhere (Deshpande et al., 2007; Manke et al., 2017). DNA concentrations for all samples and strains used in this study were determined with the Quant-iT dsDNA HS assay kit using a Qubit fluorometer (Invitrogen).

PCR

For determination to the species level of the mycobacterial pathogens, namely, *M. tb*, *M. bovis* and *M. bovis* BCG, two genetic regions: RD1 and RD4 were selected as the targets for PCR. Primers used in this study are shown in Figure 3A. For RD1 PCR, the reaction mixture contained 10X PCR buffer, 1.5 mM MgCl₂, 0.8 mM dNTPs, 0.3 μM of each primer, 1U of *Taq* polymerase and 2 μl of sample DNA. PCR reaction was initiated by denaturation for 10 mins at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension for 1 min at 72°C with a final extension for 10 min at 72°C. The reaction mixture for RD4 PCR contained 10X PCR buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs, 0.2 μM of each primer, 1 U of *Taq* DNA polymerase and 2 μl of sample DNA. PCR reaction was initiated by denaturation for 90s at 95°C followed by 35 cycles each of denaturation for 30s at 95°C, annealing at 58°C for 1 min and extension at 72°C for 2 min with a final extension for 7 min at 72°C in a thermal cycler (ProFlex PCR system, Applied Biosystems). Primer pairs for RD4 designed by Taylor GM et al (2007) were

used as a primer mixture for RD4 PCR and primer pairs for RD1 were designed by Halse et.al (2011). RD4 is a region of difference in the bovine lineage. The use of RD4 flanking primers made sure that the PCR products were formed only if the deletion was present. Figure -3B depicts the position of the primers in the genome sequence.

Duplex PCR

The two target regions were amplified using a duplex PCR. The optimized protocols for RD1 and RD4 PCR assays were combined to reduce cost, time and sample use. The duplex PCR reactions were carried out using a 10X PCR buffer, 1.5 MgCl₂, 0.8 mM dNTPs, 0.4 μM of RD1F/R and 0.2 μM of RD4F/R and 1.25U of *Taq* DNA polymerase. The amplification procedure consisted of initial denaturation at 95°C for 7 min and 35 cycles each of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min followed by a final extension step at 72°C for 10 min. A positive control, DNA extracted from *M. bovis* (ATCC BAA-935) culture, and a negative- no template control were included in each run.

Detection limit of the duplex PCR assay

The limit of detection of the duplex PCR assay was determined using ten-fold serial dilutions from 10⁷ fg to 10 fg of DNA (equivalent to 10⁶–10⁰ genome copies) isolated from *M. tb* (ATCC 25177), *M. bovis* (ATCC BAA-935) and *M. bovis* BCG Pasteur (ATCC 35734) reference strains. To assess the specificity, the concentration of

the DNA templates from each reference strain was adjusted to 10 ng/μl and subjected to PCR.

Interpretation of the Results of Duplex PCR

The PCR amplicons were examined on a 2% agarose gel, stained with ethidium bromide and visualized under UV light (BioRad, USA). The duplex PCR was considered as positive for *M. bovis* when bands of both 176 bp and 110 bp were seen; positive for *M. tb* when band of only 110 bp was seen and positive for *M. bovis* BCG when band of 176 bp was visualized on the gel (Jabbar A et al,2015) (Fig 4A).

DNA Sequencing Analysis

The PCR products were purified and sequenced by Sanger's dideoxy chain termination method at the SciGenom Labs, Cochin India. Sequences were verified by BLAST search using the NCBI website.

Results

Analysis of demographic factors

Out of the 438 participants that were enrolled, a total of 347 participants who met the eligibility criteria were included in the study for analysis. Baseline data of study participants is shown in Table 1. Among the 347 participants, 191 (55%) were males and 156 (45%) were females, 210 participants (60%) belonged to the 18-40 years age group while 187 participants (54%) were malnourished. At the time of evaluation, 73 participants (21%) had respiratory symptoms for more than two weeks and had

clinical course consistent with active TB (fever, weight loss, chest pain, abdominal pain, suggestive X-ray). Nearly 242 participants (70%) lived in animals sheds. Based on the information from the questionnaires, 280 participants (80%) lived in close contact with their animals and had high exposure to them. Thirty nine participants (11%) had previous contact with an active TB case. A total of 94 participants (27%) consumed raw, unpasteurized milk. The BCG vaccination status was confirmed only in 110 (32%) participants.

Culture isolation of mycobacteria from sputum samples

Nine percent (32/347) of the sputum samples were found to be culture positive. All samples were further processed for the detection and differentiation of *M. tb*, *M. bovis* and *M. bovis* BCG by the duplex PCR assay.

*Detection and identification of *M. tb* and *M. bovis* in clinical samples by duplex PCR*

DNA isolated from blood samples of 347 participants were subjected to the duplex PCR assay. Determination to the species level of the mycobacterial pathogens was established by molecular size analysis of the duplex PCR products electrophoresed on a 2% agarose gel with appropriate controls. The duplex PCR assay identified *M. bovis* in 2.59% (9/347) samples, *M. tb* in 17.29% (60/347) samples and *M. bovis* BCG in 0.58% (2/347) samples (Table 2).

The *M. bovis* positive cases were investigated individually to determine their degree and level of contact with

domesticated animals (Table 3). Of these, five cases were bacteriologically confirmed with pulmonary TB and the remaining cases had a clinical course suggestive of active TB. Of the 9 cases, 4 were females and 5 were males. Six cases had an animal exposure of more than 10 hrs per day and lived in close proximity at night. Four participants consumed raw milk and 4 had a TB positive family member. The cattle owned by these positive cases were in contact with the wildlife and shared watering points with wild animals. Based on the physical examination by local veterinarians, 12 of the 43 animals (28%) had poor body condition, showed low productivity and were weak and emaciated. Table 4 shows the characteristics of cattle (n=43) owned by the 9 cases detected positive for *M. bovis*.

Analytical Sensitivity

Ten-fold serial dilutions (in a range of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg) of DNA from *M. bovis*, *M. tb* and *M. bovis* BCG reference strains were used to assay the analytical sensitivity of the duplex PCR. DNA of the *M. bovis* and *M.tb* strains were detected up to 100 fg by duplex PCR, whereas DNA of *M. bovis* BCG was detected up to 10 fg. The duplex PCR assay showed 100% analytical specificity when tested with DNA of reference strains of the MTC (Fig 4B).

Sequence analysis

DNA sequencing analysis of duplex PCR products confirmed that the 176 bp and 110 bp products were identical with the *M. bovis* RD4 and RD1 regions respectively (Fig 5). Thus, it was concluded that the duplex PCR

products of these samples were derived from *M. bovis*.

Table 1. Characteristics of 347 subjects from Melghat are listed in the table. Percentages in different categories are indicated in parentheses. Group of exposure was determined on the basis of type of activity, duration and conditions of exposure to animals.

Characteristics	Level	No. (%)
Age	Below 18	37 (11)
	18-40	210 (60)
	Above 40	100 (29)
Gender	Males	191 (55)
	Females	156 (45)
Malnourished	Yes	187 (54)
Symptoms of Active TB	Yes	73 (21)
Living inside a cowshed	Yes	242/347 (70)
Daily hours of contact with animals *	Yes	6 (0-8)
Group of exposure	High	280 (81)
	Medium	45 (13)
	Low	22 (6)
Consumption of unpasteurized milk	Yes	94/347 (27)
Previous contact with TB case	Yes	39/347 (11)
BCG scar	Yes	110/347 (32)

* Daily hours of contact with animals are represented as median (interquartile range)

Table 2. Summarized results of BACT/Alert culture and molecular analysis for identification and differentiation of *Mycobacterium bovis* (*M. bovis*), *Mycobacterium bovis* BCG (*M. bovis* BCG) and *Mycobacterium tuberculosis* (*M. tb*).

Test	No. of positive cases
BACT/Alert culture	32
RD4+/RD1+ (<i>M.bovis</i>)	9
RD4+/RD1- (<i>M.bovis</i> BCG)	2
RD4-/RD1+ (<i>M.tb</i>)	60

Table 3. Overall characteristics of *M. bovis* positive cases

Case No.s.	Village	Age (yrs)	Gender	No. of animals / species (Breed) *	Time spent with animals	Physical contact with animal	Close proximity at night	Consumption of raw milk	TB positive family member	Cattle in contact with wild life	Cattle sharing watering points with wild animals
1	Tarubanda	27	F	2 oxen (Indian)	10-12 hrs	Yes	Yes	Yes (Sometimes)	Yes (Husband)	Yes	Yes
2	Tarubanda	39	M	3 cows (Indian)	10 hrs	Yes	Yes	No	Yes (Father)	Yes	Yes
3	Kot	66	M	2 oxen (Indian)	No contact	No	No	No	Yes (Son)	Yes	Yes
4	Kot	25	M	20 buffaloes (Indian)	6-8 hrs	Yes	Yes	Yes (Sometimes)	No	Yes	Yes



Fig. 1. Map of Melghat. Inset shows its geographical location with respect to the state of Maharashtra, in the Indian subcontinent. Map indicates the 10 villages in Melghat where the study participants were recruited from.

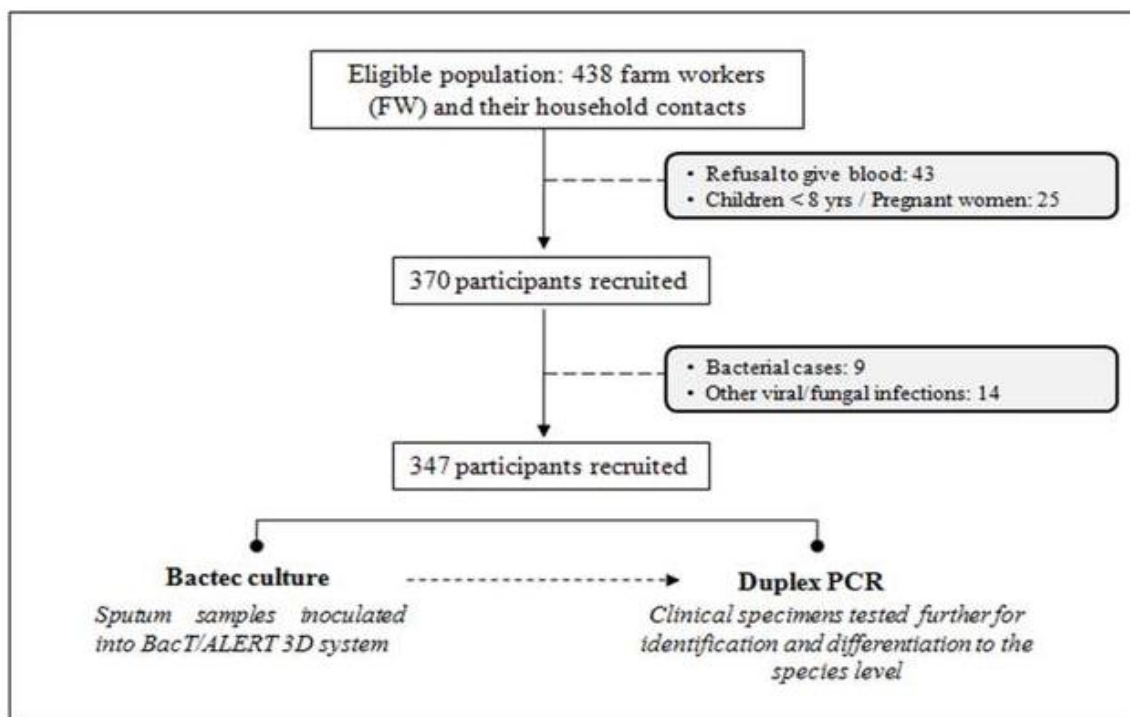


Fig. 2. Study design. The figure represents the inclusion/exclusion criteria (indicated in the grey box) adopted for recruitment of the study population.

Table 4. Characteristics of animals owned by *M. bovis* positive cases. Body condition of animals determined based on physical examination by veterinarian. Animals were classified as having poor body condition if they showed low productivity, were weak, emaciated or diseased

Variable	Category	* Animals (n = 43) owned by <i>M.bovis</i> positive cases
Gender	Female	24
	Male	19
Body condition	Poor	12
	Medium	23
	Good	8
Age class	<12 months	2
	≥ 12 months -3 yrs	14
	> 3 yrs - 10 yrs	17
	>10 yrs	10

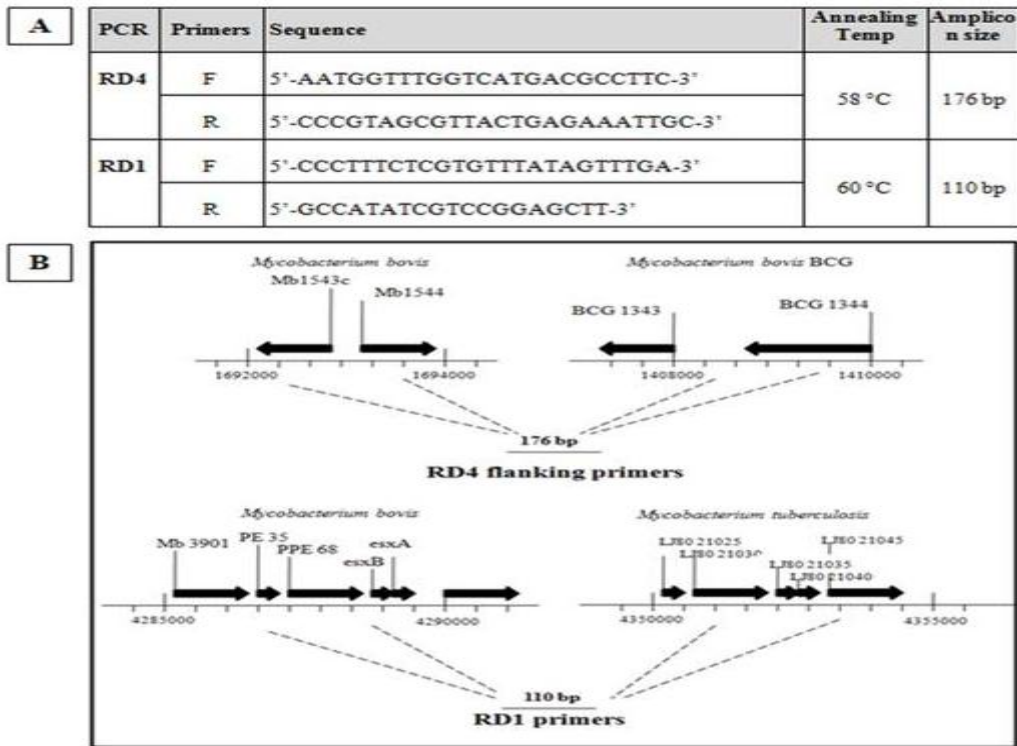


Fig. 3. A: Sequences of primers using RD4 and RD1 regions as targets of duplex PCR. B: Position of the primers in the genome sequence of *M. bovis*, *M. tb*, and *M. bovis* BCG.

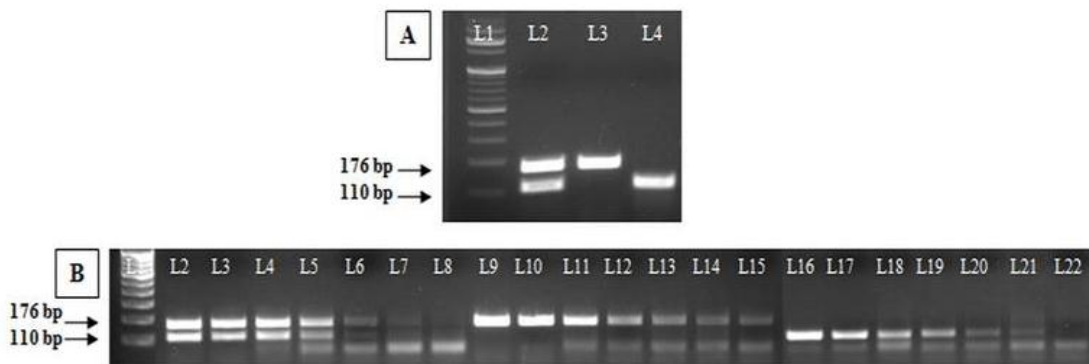


Fig. 4. Duplex PCR for detecting and differentiating *M. bovis*, *M. tb*, and *M. bovis* BCG. A) The ethidium bromide-stained (on 2% agarose gel) amplification products of L1: *M. bovis*, L2: *M. bovis* BCG and L3: *M. tb*. The 176 bp and 110 bp products obtained are indicated. B) PCR sensitivity detection limit done using serially diluted positive controls of *M. bovis*, *M. bovis* BCG and *M. tb*. L1: 100 bp molecular ladder, L2-L8: 10 ng/μl to 10 fg/μl of *M. bovis* positive control (sensitivity up to 100 fg); L9 to L15: 10 ng/μl to 10 fg/μl of *M. bovis* BCG positive control (sensitivity up to 10 fg); L16-L22: 10 ng/μl to 10 fg/μl of *M. tb* positive control (sensitivity up to 100 fg).

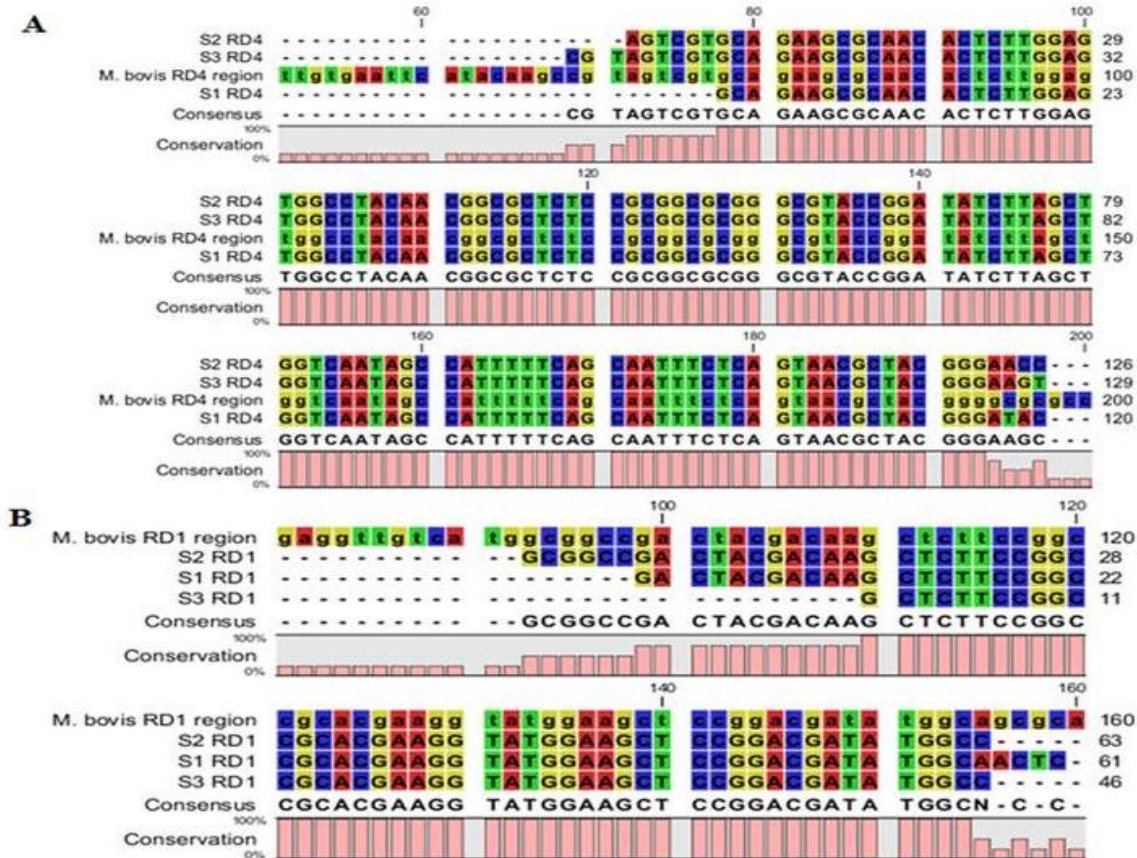


Fig. 5. A) Multiple sequence alignment of *M. bovis* RD4 region and PCR products of RD4 PCR in 3 positive Melghat cases (S1, S2, S3). B) Multiple sequence alignment of *M. bovis* RD1 region and PCR products of RD1 PCR in the same cases (S1, S2, S3). (Multiple sequence alignment done using CLC sequence viewer 6.6.1).

Discussion

The aim of the present study was to report the diagnostic utility of the duplex PCR assay, using RD targets of proven reliability, applicable directly to human clinical samples. The objective was to develop an accurate method for detection and differentiation of *M. tb* and *M. bovis* that could substantially reduce the time and cost of diagnosis in low resource settings of Melghat.

Until recently, the tests used for the identification of *M. bovis* were based on bacterial isolation and biochemical

examinations. Still, the culture isolation of *M. bovis* from clinical samples has low efficiency (Mishra A et al., 2005). Further, TB caused by *M. tb* in humans is clinically and radiologically identical to TB caused by *M. bovis* (Shah NP et al., 2006; Grange JM, 2001). These troubles were overcome by molecular techniques to some extent; however, could not identify the mycobacterial pathogens to the species level. Unlike these targets, the RD targets used in the duplex PCR assay can simultaneously detect and differentiate the closely related sub-species of the MTC,

namely, *M. tb*, *M. bovis* and the attenuated vaccine strain *M. bovis* BCG.

In the present study, 32 culture isolates were obtained by the Bactec culture technique out of the 347 samples examined. However the approach was limited in the ability to identify the mycobacterial pathogen to the species level. The duplex PCR assay was thus used to simultaneously identify and differentiate members of the MTC accurately. Results of the duplex PCR indicated 9 *M. bovis* cases, 60 *M. tb* and 2 *M. bovis* BCG cases out of the total 347 participants.

M. bovis is a predominant member of the MTC and can cause TB in a wide range of mammalian hosts including humans, cattle, goats, dogs, deer and ferrets (Kubica T et al., 2003). It is difficult to clinically distinguish between TB caused by *M. bovis* and *M. tb* in patients. Nevertheless, *M. bovis* exhibits an intrinsic resistance to pyrazinamide which is an important first line anti-TB drug. Therefore, recognition of this pathogen would help clinicians to adopt good patient management with regard to the treatment regime. Detailed investigation of the 9 *M. bovis* positive cases revealed that 6 had an animal exposure for more than 10 hrs per day. The increased risk of infection in these cases relates to not only their poor socioeconomic standards, but also their poor nutritional status. It has been reported that malnutrition, including deficiency of nutrients affects the spread and onset of zoonotic disease in humans (Portillo-Gómez L. and Sosa-Iglesias EG, 2011). These people not only had close contact with the animals while feeding, milking, but also

existed in close proximity at night, sometimes even under the same ceiling. Of the 9 participants, 4 consumed raw milk and 4 had a TB positive family member. Consumption of raw milk is often considered as a probable route for transmission of *M. bovis* from cattle to human (Grange and Yates, 1994). During the interviews, it was recorded that many participants preferred unpasteurized milk over boiled milk due to its richer taste. Another common pattern in these villages is sharing shelter with cattle which leads to close exposure and potential for transmission of pathogens. It was noted that the hamlets/ huts; sometimes employed as animal sheds, were constructed in close propinquity to the homes. Also, provisional huts were established along the farms where the farm workers and animals existed in close proximity at night. According to one of the respondents, they share their room with the newly born calves at night to protect them from wild animals. This could be one of the reasons for transmission of *M. bovis* from cattle to humans (Firdessa et al., 2012). Though uncommon, human to human transmission of *M. bovis* can occur, which could be the probable ground for the positivity of Case 3 who had no contact with animals and had no history of ingestion of raw milk (Evans JT et al., 2007). As well, contact with wild animals through water bodies cannot be disregarded in this neighborhood. The study participants lived in rural tribal areas and had poor housing conditions, minimal approach to health care facilities, lacked awareness about the disease and related causes, and had unhygienic

habits. Altogether these factors further promote transmission of pathogens from infected animals to man.

The duplex PCR assay also identified 60 cases with infection due to *M. tb*. These findings are synonymous with our previous reports where TB prevalence in the region was estimated to be nearly 32%. The PCR results also indicated the presence of *M. bovis* BCG in 2 cases. Although the incidence of *M. bovis* BCG in clinical samples is rare, disseminated disease has been reported in immunocompromised hosts. Malnutrition may be one of the likely causes for disseminating BCG infection in these cases.

DNA sequencing of duplex products of all samples that gave amplified products equivalent to 176 bp and 110 bp matched the RD4 and RD1 gene sequences of *M. bovis* respectively. Hence, it was concluded that these individuals were infected with *M. bovis*. The high specificity and sensitivity (detection limit up to 100 fg of DNA) of the duplex PCR thus illustrates the potential utility of this assay as a reliable diagnostic tool.

This is the first report of the duplex PCR assay for the recognition and simultaneous differentiation of *M. tb*, *M. bovis*, and *M. bovis* BCG in the malnourished tribal population of Melghat. A systematic literature search on the occurrence of zoonotic TB by Müller B et al., 2013 showed that there is lack of data for the WHO region of Southeast Asia, including major cattle producing middle- and low-income countries (e.g., India, Bangladesh, Pakistan, Myanmar, and Indonesia).

Recorded incidence rates for zoonotic TB in Europe, the United States, Australia, and New Zealand were consistently below 1/100,000 population/year. The incidence rates were unavailable for other countries. In the Indian context, very few studies have demonstrated the incidence of *M. bovis* in humans; most notably, studies by Shah NP et al., 2006 and Prasad HK et al., 2005 have shown high incidence of *M. bovis* and *M.tb* in extra-pulmonary samples of humans and cattle respectively. A similar study by Mittal M et al., 2014 has demonstrated the importance of screening and differential diagnosis of Mycobacterium tuberculosis complex in humans and livestock.

In its current format, this duplex PCR can be applied in any minimum-setting laboratory. Despite of being an insightful study, it suffers from the limitation of insufficient data on animal health. It was learned that worshipping of cattle has spiritual significance in this area, therefore; withdrawing blood or doing any tests on their animals was not possible. Nevertheless, we attempted to gather data regarding certain animal health parameters including productivity and body condition through the local vets. In spite of this, the study has limitations regarding the accurate data on health of animals which may have entered in an asymptomatic phase after infection with *M. bovis* wherein no clinical signs were developed (Phillips CJ et al., 2003).

Conclusions

This study provides a rapid and cost effective molecular tool for screening of *M. bovis* in the isolated regions of Melghat.

Further investigative studies would assist in clarifying the dynamics of *M. bovis* transmission and would help in constituting appropriate strategies for prevention of zoonotic TB in neglected regions like Melghat.

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