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Molecular detection of *Mycobacterium bovis* in goats from Nagpur region of Maharashtra

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Abstract
Tuberculosis is a zoonotic disease caused by bacteria; *Mycobacterium* species. It is distributed worldwide and have the economic and public health significance. The present study was carried out to detect and identify the presence of *Mycobacterium* pathogens using polymerase chain reaction in blood samples from goats. Blood samples from the total of 236 goats suspected of tuberculosis as well as apparently healthy were collected from in and around Nagpur region. The DNA in blood sample was assessed by PCR amplification of *IS6110* and RD1, RD4 gene to detect *Mycobacteria* at genus and species level respectively. The overall positivity of *M. bovis* among goats was recorded as 10.59% (25/236). The study demands thorough testing of the flocks for continuous monitoring for tuberculosis for preventing its further spread.

Keywords: *M.bovis*, goat, blood, Nagpur, IS6110, RD1, RD4, tuberculosis

Introduction
Tuberculosis is a zoonotic disease distributed worldwide, affecting the health of humans and also an economically important disease because of the mortality in animals, loss in productivity, and restrictions on the trade of animals (OIE, 2009) [1]. According to epidemiological reports, tuberculosis in the goat has a global distribution and has been documented in Sudan, Spain, Italy, Portugal, Nigeria, the United Kingdom, Algeria, and Ethiopia (Crawshaw *et al*. 2008; Daniel *et al*, 2009; Zanardi *et al*. 2013) [2-4]. The disease has also been reported in Asian countries like India, Pakistan, Bangladesh, etc., and African countries. Even though a global eradication strategy for zoonotic tuberculosis has been implemented, TB remains endemic in the majority of countries (Zanardi *et al.* 2013) [4]. But in India, there is no specific tuberculosis eradication control program implemented in animals.

Tuberculosis is a chronic, primarily respiratory infectious disease of mammals including humans, wildlife, and domestic animals caused by a group of closely related bacteria known as the *Mycobacterium tuberculosis* (MTB) complex. *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium suricattae*, *Mycobacterium mungi*, *Mycobacterium dassie*, and *Mycobacterium oryx* are all members of the Mycobacterium tuberculosis complex (Van Soolingen *et al*. 1997) [5]. In small ruminants, tuberculosis is predominantly caused by *Mycobacterium bovis* and *M. caprae* (OIE 2009) [1] and also by *M.tuberculosis* (Cadmus *et al*. 2009) [6]. The clinical manifestations of tuberculosis in small ruminants are weakness, weight loss, fluctuating fever, intermittent hacking cough, diarrhea, and large prominent lymph nodes.

Contact with diseased domestic and wild animals spreads the disease, which is infectious. Infection is usually contracted by inhaling infected droplets; ingesting raw milk from infected goats can also infect kids and humans. Before clinical symptoms appear, an animal may spread the disease to many other members of its herd. Hence, the movement of undetected infected domestic animals may also pose the risk of disease transmission (OIE, 2009) [1].

There are various diagnostics techniques to detect tuberculosis which include Tuberculin skin test, Interferon-gamma, serology, acid-fast staining, isolation of bacteria from clinical as well as from necropsy samples, and identification by molecular techniques including PCR and real-time PCR. The identification of cell-mediated immune (CMI) responses (the skin test and -IFN assay) that occur as early as 3 weeks after Mycobacterium infection in cattle is used to diagnose bovine tuberculosis in live animals. *M. bovis* detection based on bacterial isolation and biochemical tests, is considered the "gold standard" for verifying tuberculosis, is time-

consuming can take several weeks and poses a risk to the individuals involved in the isolation procedures and handling and have poor diagnostic accuracy (Mishra *et al.* 2005) [7]. There is no single detection method that is precise and sensitive enough to identify all stages of infection in live animals.

Out of other diagnostic methods, PCR has proved to be a promising and better diagnostic technique for a variety of infectious diseases caused by fastidious or slow-growing bacteria. The molecular diagnosis of tuberculosis can be performed using *IS6110* gene is targeted to detect Mycobacterium Tuberculosis Complex (Ortu *et al*. 2006) [8]. *IS6110* is a repetitive element that is specific for MTBC, frequently used as a marker for the presence of MTBC**.** To identify and differentiate the species in the MTBC, RD1 and RD4 duplex PCR is used, as it is very reliable to detect various species in a single reaction (Taylor *et al.* 2007, Halse *et al.* 2010) [9, 10].

Though bovine tuberculosis is endemic in cattle, the status of TB in goats has not been well studied in India, despite their close contact with cattle. Goats and cattle often share watering and grazing areas, allowing close interspecies contact between these domestic animals and thus raising the risk of *Mycobacteria* transmission (Biffa *et al*. 2010; Gumi *et al*., 2011; Kassa *et al*., 2012) [11-13]. Since goats are India's primary meat-producing animal, and milk producer; it is critical to research the prevalence of zoonotic infections including tuberculosis in goats. In developing countries like India, tuberculosis as a zoonotic infection is a major concern for the human population since the human and animals share the same microenvironment and lives in close proximity with each other. Since both *Mycobacterium* species (*M.tuberculosis* and *M. bovis*) pose a threat to animal health and are therefore capable of infecting humans (reverse zoonosis), early detection of the bacteria is needed.

Taking into account all these, the current study aimed to look into positivity of *M. bovis* in goats in and around Nagpur region.

Materials and Methods

Samples

A total of 236 blood samples from the goats reared at different farms and smallholder farmers from in and around Nagpur, Maharashtra were collected. Out of 236 samples, 85 samples were collected from tuberculosis suspected goats having a history of cough, loss of appetite, lethargy, weight loss, and the rest of the 151 samples from apparently healthy animals. The blood samples were collected in sterile EDTA vacutainers transported to the laboratory under chilling condition and stored at 4º C until further processing.

DNA Isolation and quantification

The DNA from blood samples was isolated by QIAamp DNA mini kit (QIAGEN) as per the manufacturer's instruction. The QIAamp DNA purification consists of four steps and is carried out by using a Mini spin column containing silica membrane. Briefly, the blood sample was subjected to lyse, then treated with ethanol and transferred to the mini-column which helps DNA to bind to the column. Then two-step washing was given to mini-column with different washing buffers to remove the residual contaminants and finally mini-column was subjected to elution of DNA with the help of elution buffer. The purity and concentrations of DNA were evaluated by Nano drop Bio spectrometer (Eppendorf), and isolated DNA from blood samples was stored at -20ºC until further analysed.

Molecular Characterization by PCR

a. IS6110 MTBC

IS6110 gene (Ortu *et al*. 2006) [8] is targeted to detect Mycobacterium Tuberculosis Complex (MTC) which was amplified at 123 bp. The details of the primer sequence are given in Table 1. The PCR for MTC was carried out in 25 µl volume mixture containing 12.5 µl of 2X GoTaq® Green master mix (Promega), 0.5µl of 10pmol/µlof each reverse and forward primer, 2µlof DNA template, and 9.5µlof nuclease-free water. The cycling conditions for *IS6110*gene were initial denaturation at 95ºC for 3 minutes followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 56ºC for 1 min, extension at 72ºC for 45 sec, and final extension at 72ºC for 10 min. The PCR products were analyzed by gel electrophoresis using 2% agarose gel, visualized, and photographed under a UV transilluminator, Gel Doc system (Bio-Rad).

b. Duplex PCR (Species specific)

Two genetic areas, RD4 and RD1, were used to determine the species level of *Mycobacterium* pathogens, namely *M. tuberculosis, M. bovis* and *M. bovis* BacilleCalmette–Guerin (BCG) by using a duplex technique **(**Taylor *et al*. 2007, Halse *et al.* 2011**)** [9, 10]. RD4 is a Region of Difference in the bovine lineage. The details of the primer sequence are given in table no. 1. The duplex PCR was carried out in 25 µlvolume mixture containing 12.5 µl of 2X GoTaq® Green master mix (Promega), 0.75 µlof 10pmol/µlof each reverse and forward primer for RD1 and 0.5 µlof 10pmol/µlof each reverse and forward primer for RD4, 2µlof DNA template and 8µlof nuclease-free water. The cycling conditions for duplex PCR were initial denaturation at 95ºC for 7 minutes followed by 35 cycles of denaturation at 95ºC for 1 minute, annealing at 59ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 10 mins. Positive control for *M.bovis, M.tuberculosis* and *M.bovis* BCG strain were used along with the negative control.

The PCR products were analyzed by gel electrophoresis using 2% agarose gel, visualized and photographed under UV transilluminator (Bio-Rad). The result for PCR of RD1 and RD4 duplex PCR was read as, the PCR product amplified at 110 bp and 176 bp was positive for *M.bovis,* at 176 bp only, positive for *M. bovis* BCG strain and at 110 bp only, positive for *M.tuberculosis.*

Table 1: Details of Primers for *IS6110* and RD1, RD4 duplex PCR

Target gene	Primers	Primer sequence (5'- 3')	product size	Reference
MTCspecific insertion sequence (IS6110)	Forward	ACCTGAAAGACGTTATCCACCAT	123bp	Ortu *et al.* 2006 [8]
	Reverse	CGGCTAGTGCATTGTCATAGGA		
RD4	Forward	AATGGTTTGGTCATGACGCCTTC	176 bp	Taylor *et al.* 2007 [9]
	Reverse	CCCGTAGCGTTACTGAGAAATTGC		
RD1	Forward	CCCTTTCTCGTGTTTATAGTTTGA	110 bp	Halse *et al.* 2011 [10]
	Reverse	GCCATATCGTCCGGAGCTT		

Results

In the present study, 236 blood samples were collected from goats, suspected of tuberculosis based on the symptoms and from apparently healthy animals. For Mycobacterium Tuberculosis Complex PCR, gene IS6110 amplified at 123 bp (Fig. 1). In tuberculosis suspected flock, out of 85 goat blood samples about 25 (33.33%) samples were turned positive. Among healthy flocks, none of the samples turned positive for MTBC PCR giving 0% prevalence. The 25 DNA samples which turned positive for MTBC PCR were subjected to duplex PCR of RD1 and RD4 to detect the *M.bovis* and *M.tuberculosis* (Fig. 2). All of the 25 samples amplified at 110 bp and 176 bp i.e. identified as *M.bovis* and none of MTBC positive sample turned positive for *M. tuberculosis.* These results indicated the presence of the *M. bovis* circulation in tuberculosis suspected flock. The overall prevalence of *M. bovis* was recorded as 10.59% (25/236). The details of the results are given in Table 2.

All the samples which turned positive for *M.bovis* belong to the same area, which indicates the presence of circulatory tuberculosis in that particular region only.

Table 2. Prevalence of *M.bovis* in goats

Species	Health Status of animal		MTBC positive	*M. bovis*	*M. tuberculosis*
Goat	Healthy	151	0	0	0
	Suspected	85	25(33.33)	25 (33.33)	0
	Total	236	25 (10.59)	25 (10.59)	**0**

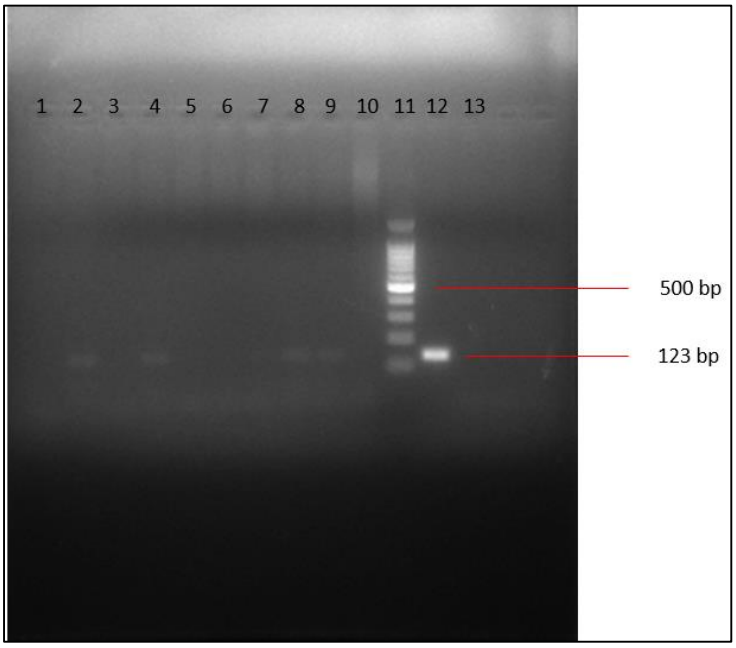

Fig 1: Mycobacterium tuberculosis complex (MTBC) PCR. Lanes 1,3,5,6,7 – Negative DNA samples, Lanes 2,4,8,9 – MTBC DNA samples, Lane 10- Negative control, Lane 12- MTBC positive control (123 bp), Lane 11-100 bp Marker.

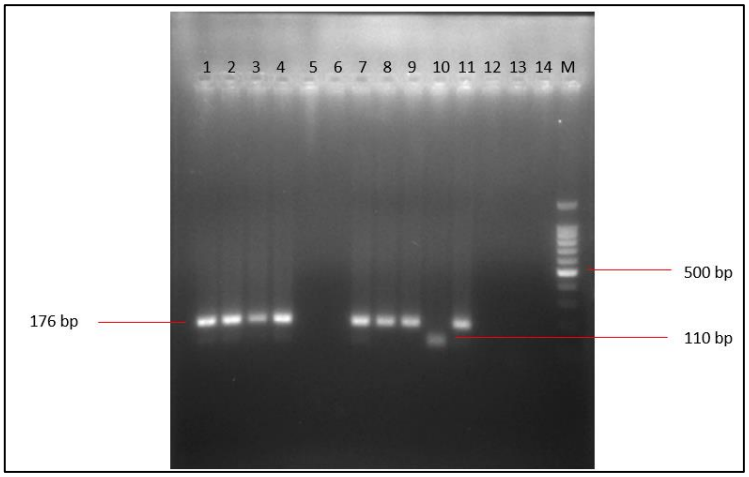

Fig 2: Duplex polymerase chain reaction (PCR) for detecting and differentiating Mycobacterium bovis, Mycobacterium tuberculosis, and M. bovis BCG. Lane M :100 bp Marker, Lanes 1-4: *M.bovis* DNA samples, Lanes 5-6: Negative DNA samples, Lane 7: *M.bovis* Positive control (110 bp +176 bp), Lanes 8-9: M. bovis BCG Positive control (176 bp), Lane 10: *M.tuberculosis* (110 bp) positive control, Lane 12: Negative control.

Discussion

Tuberculosis in small ruminants is a major barrier to the sheep and goat farming industries worldwide. Also, in a tropical country like India, the situation is similar. Tuberculosis is a

notifiable disease that affects people all over the world, resulting in economic losses due to mortality and decreased milk production. To the best of our knowledge, this is the first report of direct detection of bovine TB in blood samples from goats with molecular techniques from in and around the Nagpur region. There are not many studies to compare the result of the present study as most of the study was done in cattle population than the small ruminants and also the frequently taken clinical samples are tissues, that too after the necropsy and mostly subjected to isolations only.

PCR assays are the most promising alternative approach for the rapid and specific diagnosis of tuberculosis (Serrano-Moreno *et al*. 2008, Figueiredo *et al*. 2010) [14, 15]. PCR techniques have been successfully used to diagnose bovine tuberculosis in a variety of naturally infected clinical samples including tissue, blood, milk and nasal exudates (Gomez-laguna *et al*. 2010, Coetsier *et al*, 2000) [16. 17].

The findings of the present study showed that PCR-based direct detection of *Mycobacteria* in the blood is useful in identifying carrier animals quickly. PCR has the advantage of not requiring the organism to be isolated and can detect DNA from both viable and nonviable organisms. Primers that amplify segments of the *IS6110* element are the most widely used method. *IS6110* anneal to a sequence in the repetitive element, which is specific for MTBC, and frequently used as a marker for the presence of MTBC. *IS6110* present in all MTBC species (Bhanurekha *et al.*, 2015) [18]. *IS6110* PCR was used in the present study to amplify an insertion sequence IS6110 of 123 bps in the MTBC directly on DNA collected from blood samples using specific primers. The presence of the *IS6110*indicate the positivity for MTBC.

Furthermore, to detect and differentiate the *M.bovis* from other members of MTC, the duplex PCR of RD1 and RD4 was employed which targets 110 bp and 176 bp respectively.RD1 is present in all *M. tuberculosis* and pathogenic *M. bovis* strains but lack in all BCG strains and almost all environmental *Mycobacteria* (Behr *et al*. 1999) [19]. RD4 is a region of difference in bovine lineage. The use of RD4 flanking primers ensured that only the deletion was present in the amplified PCR products (Taylor *et al*. 2007) [9]. The RD1 region contains genes from the esat6 gene cluster. Early secretory antigenic target-6 (ESAT-6) is an antigen that is recognized during the early stages of infection and is a potent immune stimulator. RD1 region of *M. tuberculosis* is thought to be the main attenuating deletion in vaccine strain of *M.bovis* BCG (Halse *et al*. 2011) [10].

Poor isolation techniques or the stage of infection may explain the PCR assay's superiority to isolation methods. Furthermore, unlike culture approaches, which only detect viable organisms, the PCR assay detects even the tiniest traces of genetic material in samples confirming the exposure to the pathogen.

The overall prevalence of 10.59 % (25/236) in the present study is on the higher side with the observations by Tschopp *et al.* (2011) [20] which was recorded as 0.4% and Hena *et al.* (2012) [21] who reported 1.34% in clinical samples of goats. The same results as of present study was reported by Basit *et al*. (2015) [22] with 10.6% in goat from the clinical sample and on the lower side with Ashenafi *et al* (2013) [23] with 25% of prevalence in clinical samples from goats. The *Mycobacteria* have been more frequently identified in blood than in milk samples (Carvalho *et al*., 2014, Romero *et al*., 1999, Srivastava *et al*., 2008) [24, 25, 26].

Many variables could account for these differences in the result, including differences in disease state among animal populations and diverse environmental factors. Bovine tuberculosis in small ruminants is only a concern when they are in close contact with cattle with a high disease prevalence, according to several previous research (Tschopp *et al.* 2011) [20]. The different prevalence rates reported may be related to management characteristics (Evangelista *et al.* 2013) [27], sampling methods (Katale *et al*., 2013) [28] and disease-control measures adopted in each location (Silaigwana *et al*., 2012) [29].

In the present study, the goats were in close contact with the cattle as many livestock owners have mixed livestock i.e. they rear the cattle and goats on the same farm and also they share the same water sites for drinking water along with the pastures, this could be the reason for high positivity in goats. It was reported that goats acquire tuberculosis when they have close contact with cattle and share pasture with infected cattle (Radostits *et al*. 2000) [30].

Tuberculosis is a zoonotic illness that is a chronic infectious disease. Because milk, meat, and dairy products are the main sources of nutrition and proteins, tuberculosis detection in animals has become critical, but it might also be a major source of TB transmission from animals to humans (Al-Saqur *et al.* 2009) [31].

Conclusions

Based on the present investigation we could conclude that bovine tuberculosis is circulating in small ruminants as well. To combat the zoonotic infection, this study highlights the importance of continuous monitoring of *Mycobacteria* burden especially the *M. bovis* in animals and humans living in close proximity. Thorough surveillance studies like the present one will add into animal TB control programs.

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