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# Molecular detection of *Mycobacterium tuberculosis* from bovine milk samples

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## ABSTRACT

***Mycobacterium tuberculosis* and *Mycobacterium bovis* are the major causes of tuberculosis. These may infect many animal species, and are likely to be the main source of infection in humans. A total of 181 bovine raw milk samples and 123 pre-scapular lymph node biopsy samples were collected and subjected to acid fast staining, fluorescent staining, isolation and identification. Genus specific PCR to identify the *Mycobacterium tuberculosis* complex (MTBC) organism, and multiplex PCR (mPCR) were done to differentiate *M. tuberculosis* and *M. bovis*. Among the milk samples tested, only one sample was culture-positive for *M. tuberculosis*. Four samples were positive by MTBC-PCR and mPCR; all these four were proved to be *M. tuberculosis*. It is quite likely that animals can be infected with human-originated *M. tuberculosis*, which in turn may act as a source of infection in humans, becoming a reverse zoonosis. Hence, control strategies for human tuberculosis caused by *M. tuberculosis* should necessarily include the control strategies in animals too.**

## Keywords

Bovine milk, *Mycobacterium tuberculosis*, Multiplex PCR, Reverse zoonoses

## ARTICLE HISTORY

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## INTRODUCTION

Tuberculosis (TB) is an important zoonotic disease, and its causal agent belongs to *Mycobacterium tuberculosis* complex (tubercle bacillus) comprising *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti* and *Mycobacterium canetti* (Van Soolingen et al., 1997). *M. tuberculosis* and *M. bovis* are the major causes of tuberculosis, which are highly pathogenic that may infect many animal species and thus are likely to be the source of tuberculus infection in humans. The highest prevalence of human TB is found in the Asia; where China, India, Bangladesh, Indonesia and Pakistan collectively make up over 50% of the global burden (Mathema et al., 2006).

Humans are considered as the principal reservoir hosts for *M. tuberculosis*. The human-to-human infection cycle rotates; however, tubercle bacilli have a wide host range, and *M. tuberculosis* has been detected in fish, reptiles, birds, and mammals including marine animals. Naturally, the first contamination of these animals with *M. tuberculosis* is caused by humans, and then infection may occur between animals, which in turn become the source of infection in humans (Unea and Mori, 2007).

Human infection with *M. bovis* is mostly caused by the intake of contaminated milk or dairy products. Transmission by direct contact or droplet transmission is also possible among high-risk people, such as veterinarians and animal keepers, who are in frequent contact with animals (Unea and Mori, 2007). *M. Bovis*

TB comprised 1.4% of all TB cases in humans in the Netherlands during 1993-2007 (Majoor et al., 2011). In Punjab, India, 15,737 animals were tested for bovine tuberculosis using single intradermal tuberculin test over a 23-year period (1986-2009); among these animals, 847 showed positive reactions with overall prevalence being 5.38% (Sharma et al., 2011). An histry of transmission of *M. Bovis* from animal to human and back to animal was documented where tuberculosis was developed in cattle after being exposed to a patient infected with *M. bovis*; the person was reported to have been exposed and infected during childhood. The strains isolated from cattle and the patients were identical (Fritsche et al., 2004).

Although human-to-cattle transmission of *M. tuberculosis* has been reported (Ayele et al., 2004), the isolation of *M. tuberculosis* from any species other than human, especially from cattle, is interesting and important. *M. tuberculosis* infection has been reported in a wide range of domestic or wildlife animal species, most frequently those living in close contact with humans for a long time. In most cases, infection caused by *M. tuberculosis* has been identified in cattle, among the animals (Ocepek et al., 2005). *M. Tuberculosis* causes less severe disease in cattle than that caused by *M. bovis* (Francis, 1958). It is imperative to see that the source of the pathogen is the cattle as there is a possibility of cross contamination of the samples (Cadmus et al., 2006).

## MATERIALS AND METHODS

**Mycobacterial reference strains:** The reference *Mycobacterial* strains used in this study were *M. tuberculosis* H37Rv (MTCC) and *M. bovis* BCG vaccine strain. The reference strains were obtained from the Serum Institute of India.

**Samples:** A total of 181 bovine raw milk samples and 113 pre-scapular lymph node (PSLN) biopsy samples were collected aseptically from Teaching and Research Hospital, Madras Veterinary College, Chennai, University Research Farm (Kattupakkam), Slaughter Houses (Perambur, Chennai), and also from individual small-holder farmers in the districts of Dharmapuri and Tanjore for screening of zoonotic tuberculosis. All procedures were done after appropriate approval from the Institutional Animal Care and Use Committee at Tamil Nadu Veterinary and Animal Sciences University as well as the Institutional Review Board.

**Processing of samples:** From each live animal, milk (2 mL) and the pre-scapular lymph node (PSLN) biopsy were collected. The samples were collected aseptically, homogenized and decontaminated using 0.5% hexa-decylpyridinium chloride for 15 min (OIE, 2009). The suspensions were then centrifuged at 10,000 rpm for 15 min. The pellet was used for making smear, culturing, and DNA extraction.

**Acid fast staining:** PSLN biopsy samples were subjected to centrifugation. Smear of the sediment and milk samples were prepared, dried and fixed by gentle heating. Staining was performed using Ziehl-Neelson's acid fast staining kit (HiMedia), and the smears were examined for the presence of mycobacteria.

**Fluorescent staining (Auramine staining):** Fluorescent staining with auramine O was performed using Mycobacteria fluorescent stain kit (HiMedia), and examined for the presence of mycobacteria.

**Culture:** Briefly, after processing, the samples were inoculated on Lowenstein-Jensen slants, one with glycerol and another with sodium pyruvate. Then the tubes were incubated at 37°C in an inclined position overnight then vertically for at least 6 to 8 weeks with weekly examination starting from three days post inoculation.

**Molecular confirmation by MTBC genus specific PCR:** From the sediment of milk, PSLN biopsy samples, DNA was extracted using QIAGEN DNA kit as per the instructions of the manufacturer. The PCR was performed on a total volume of 20 µL as per the procedure mentioned by Liébana et al. (1995) using the primers mentioned in **Table 1**. The cycling conditions were standardized as initial denaturation at 94°C for 5 min, followed by 30 cycles of a denaturation step at

**Table 1: Oligonucleotides used in the experiment.**

| Primer sequence (5′ - 3′) | Target size | References |
|---|---|---|
| ***IS 6110*** | | LiéBana |
| IS41 (F) 5′-CCT GCG AGC GTA GGC GT-3′ | 317-bp | et al., 1995 |
| IS43 (R) 5′-TCA GCC GCG TCC ACG CC-3′ | | |
| **Hypothetical protein 'Rv1506c'** | | Bakshi et al., 2005 |
| CSB1Common FP- (5′-TTC CGA ATC CCT TGT GA-3′) | | |
| CSB2 *M. bovis* RP- (5′-GGA GAG CGC CGT TGT A-3′) | 168-bp | |
| CSB3-*M. tuberculosis* RP-(5′-AGT CGC CGT GGC TTC TCT TTT A-3′) | 262-bp | |

94°C for 1 min, annealing at 68°C for 2 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. The amplicons were analyzed by electrophoresis using 1.5% agarose gel.

**Multiplex PCR and differentiation of *M. tuberculosis* and *M. bovis*:** Multiplex PCR was performed as per the method described by Bakshi et al. (2005) with the DNA extracted from milk, PSLN biopsy samples, and the primers mentioned in **Table 1**; the primers included CSB1 Common FP, CSB2 *M. bovis* RP and CSB3- *M. tuberculosis* RP. The product size amplified with the primer set CSB-1 and CSB-3 should be 262-bp. The CSB-1 complements 50–66 bases, whereas the primer CSB-2 complements bases 217–202 of the *M. bovis* gene [accession: AJ003103], and the PCR product size generated by the primer set CSB-1 and CSB-2 was 168-bp. The cycling conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of a denaturation step at 94°C for1 min, primer annealing at 52.3°C for 1.30 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The amplicons were analysed by electrophoresis in a 1.5% agarose gel. The unique amplification products of either 168-bp (*M. bovis*-specific) or 262-bp (*M. tuberculosis*-specific) were compared respective reference species (**Figure 2**).

## RESULTS AND DISCUSSION

The primers (IS6110 FP and RP) anneal to a sequence in the repetitive element IS6110 which is specific for *M. tuberculosis* complex (**Figure 1**). Four milk samples were found positive by PCR for Mycobacterium genus. All these samples were subjected to mPCR for differentiation between *M. tuberculosis* and *M. bovis* using CSB1, CSB2 and CSB3 primers (**Table 1**). The size of the PCR product obtained was 262-bp which was specifically positive for *M. tuberculosis* (**Figure 2**). The positive controls in this study consisted of known human and animal tissue samples and *M. microti*, while the negative controls were *M. kansasii* and milk samples from bovines.

Of the 181 milk samples and 123 PSLN biopsy samples, four samples were positive by both IS6110 and mPCR. Only one milk sample was found positive by culture, indicating the presence of *M. tuberculosis* organisms in milk. Comparing all the diagnostic tests for tuberculosis detection including acid-fast staining, fluorescent staining and culture methods, PCR identified the maximum number of positives (n=4/181); comparing to other tests proved its higher sensitivity. This is in correlation with the study in

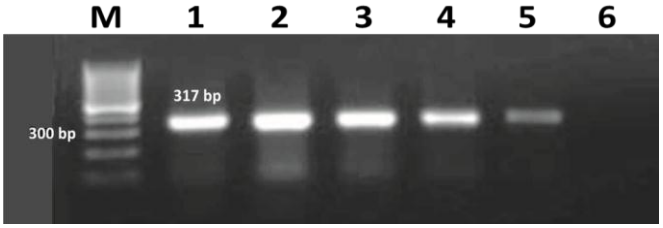


**Figure 1:** Molecular confirmation by MTBC genus specific PCR targeting the IS*6110* region. M-100 bp ladder, Lane 1- Positive *M. tuberculosis*, Lane 2 – Bovine milk sample MTBC positive, Lane 3 – Human sputum sample MTBC positive, Lane 4 – Sheep tissue sample MTBC positive, Lane 5 – *M. microtti*, Lane 6 – *M. kansasii* – Negative control.

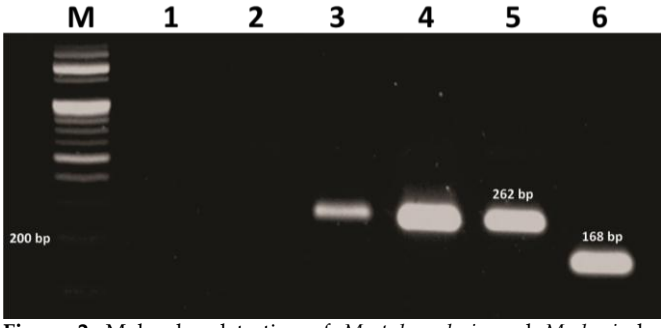


**Figure 2:** Molecular detection of *M. tuberculosis* and *M. bovis* by multiplex PCR and their differentiation targeting hypothetical protein 'Rv1506c'. M-100 bp ladder, Lane 1 – Negative control, Lane 2 – *M. kansasii* – Negative control, Lane 3 & 4 – Bovine milk sample *M. tuberculosis* positive, Lane 5- Positive *M. tuberculosis*, Lane 6- Positive *M. bovis*.

California by Bermudez et al. (2010), where PCR identified an additional 53 culture negative samples, In another study in cattle farms of North India, out of 768 specimens 54 *M. tuberculosis* complex isolates were obtained; 40 of these isolates were identified as *M. bovis* and 14 as *M. tuberculosis* (Srivastava et al., 2008).

The milk samples from individual animal were collected in aseptic manner in sterile containers indicating the excretion of this organism from the cattle through milk. Humans are the initial source of *M. tuberculosis* infection for animals, and there are potentials for this infection being carried back to humans. A study in Ethiopia by Martin (Vordermeier et al., 2012) reported that although the extent and risk of infections caused by *M. bovis* are unclear, the facts that *M. tuberculosis* could be isolated from tuberculus cattle could demonstrate a potential cattle-to-human transmission risk. However, pasteurization and boiling of milk kills this organism; raw milk consumption, improper pasteurization, unpasteurized milk products, and eating raw/partially cooked meat may predispose humans to tuberculosis infection. The impact of this disease can be devastating in those limited-resource countries that are suffering from high burdens of both

TB and human immunodeficiency virus (Parsons et al., 2011). Hence, control of tuberculosis in humans caused by *M. tuberculosis* is dependent not only on the control of transmission between humans but also include breaking the transmission route particularly through bovine milk.

## CONCLUSION

This study describes molecular identification of *M. tuberculosis* complex (MTBC) strains from raw cattle milk in Chennai, Tamil Nadu, and establishes a baseline for future investigations so as to formulate effective control measures against tuberculosis in humans and animals. Further molecular characterization is needed to ensure that correct estimates are made of true burden of infection due to *M. bovis* and *M. tuberculosis* from bovine sources.

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