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Detection and Identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis* from Blood and Milk of Bovines

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ABSTRACT

Bovine tuberculosis, a chronic disease of animals is caused by species of *Mycobacterium tuberculosis* complex (MTC) and it remains a potential threat to animals as well as humans. Differentiation of the species of MTC is required for epidemiological and diagnostic purpose. The present study evaluated the presence of different species of MTC in bovines using gyrB-restriction fragment length polymorphism analysis. In this study, blood and milk samples from 50 milch animals which were positive reactors of comparative intradermal tuberculin test were collected. Screening of MTC was done by IS6110-PCR using primers INS1/INS2 specific for MTC. The positive samples were further identified using *gyrB*- Restriction fragment length polymorphism analysis. Out of 50 positive reactors to CITT, only 4 (8%) animal were positive for MTC by IS6110-PCR. And *gyrB*-RFLP analysis using *RsaI* and *SacII* showed two positive for *M. bovis* and two animals for *M. tuberculosis*. Thus, *gyrB*-RFLP could be used as an additional tool in differential diagnosis of mycobacterial diseases thereby able to differentiate species of MTC.

Keywords: *Mycobacterium bovis, Mycobacterium tuberculosis* complex, PCR, RFLP.

Bovine tuberculosis, a chronic disease of animals is caused by species of *Mycobacterium tuberculosis* complex and it remains a potential threat to animals as well as humans. Bovine tuberculosis can affect practically all mammals, causing a general state of illness, coughing and eventual death (OIE, 2009).*Mycobacterium tuberculosis* complex comprises of the closely related species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*. These species are the causative agents of tuberculosis (TB) in humans and animals. MTC are genetically closely related though differ in terms of host predilection, pathogenicity, epidemiology and geographical distribution range (Chimara *et al.,* 2004). The disease is distributed worldwide and in India, 300,000 people die from TB annually (TB India, 2012). The disease is of economic and public health importance. In developing countries, 10-15% of human TB while about 1-2% in developed countries is caused by *M. bovis* (Ashford *et al.,* 2001).

Early diagnosis of mycobacterial infections plays a vital role in control of tuberculosis. Diagnosis of bovine tuberculosis is based primarily on conventional methods (culture, acid fast bacilli staining tuberculin test) and

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recently on molecular techniques (Filia *et al.,* 2016; Langnyei *et al.,* 2016). Though culture is the gold standard for diagnosis of tuberculosis, it takes several weeks to identify the species of Mycobacterium (Figueiredo *et al.,* 2009). Tuberculin test is widely used for diagnosis of bovine tuberculosis since it is easy to perform on livestock in a large scale. But the disadvantage of this test is that it lacks both having specificity and sensitivity (Figueiredo *et al.,* 2009).

Various PCR-mediated methods have been developed for the rapid detection and differentiation of mycobacterial species. One of these methods is PCR amplification of a fragment of *gyrB* gene followed by restriction digest of the PCR products. PCR-RFLP of the *gyrB* gene using the commonly used enzyme *RsaI* permits one to easily separate the MTBC species from other atypical *Mycobacterium*. The present study was designed with the objectives to detect and differentiate members of *Mycobacterium tuberculosis* complex in bovines.

MATERIALS AND METHODS

Ethical approval

This study was approved by animal ethics committee of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) (No. IAEC/2016/ 643-675 dated 19/10/2016).

Screening of animals by Comparative Intradermal Tuberculin Test (CITT)

Screening of animal was done from five different organised and unorganised farms in Ludhiana, Kapurthala, and Moga districts of Punjab. A total of 190 animals (131 cattle and 59 buffaloes) more than three years of age from organized and unorganised dairy farms were screened for the cell mediated immune response by Comparative Intradermal Tuberculin test (CITT) (OIE 2009). CITT was performed as per guidelines of OIE (2009)using bovine tuberculin PPD from culture of *M. bovis* (strain AN5, 3000 IU) and avian tuberculin PPD from culture of *M. avium* subspecies avium (strain D4ER, 2500 IU), obtained from Prionics (Switzerland). Bovine and avian tuberculins PPD (0.1ml each) were injected intradermally in the cervical area at least 12cm apart. The correction of the injection was confirmed by palpation of a small pea like swelling at the site. Inflammatory responses were recorded 72 hrs post injection. Animals were considered as positive reactors if the increase in skin thickness at the bovine site of injection was 4 mm or greater than the reaction at the site of the avian injection while no or ≤1 mm difference in the increase in skin fold reaction was considered negative reactors. Difference between 1-4 mm thicknesses was considered as inconclusive.

Extraction and detection of DNA from blood and milk samples

Whole blood samples (5ml) from all the 50 animals positive by CITT were subjected to DNA extraction using DN easy Blood and Tissue kit (Qiagen). One ml of blood was taken and centrifuged at 14,000 rpm for 15 min. The pellet was suspended in lysis buffer, and further DNA extraction was done as per the manufacturer's protocol. DNA was eluted and stored at −20°C till further use.

Milk sample (20 ml) was transferred to centrifuge tube and centrifuged at 4000 rpm for one hour. After centrifugation whey was discarded while pellet (SNF) and cream were pooled and collected in 1.5 ml eppendorf tube. Pooled cream and SNF were transferred to column of 0.9 per cent HPC and were subjected to decontamination with constant shaking at 120 rpm in shaking incubator at room temperature for 4 hours. The tubes were kept undisturbed overnight at room temperature to allow the separation of cream and SNF. After separation, HPC was discarded and cream and SNF were pooled together in 1.5 ml eppendorf tube. Pooled mixture was given four washings with 1ml PBS by centrifugation at 5000 rpm, each washing was of 5 min. DNA was extracted from the mixture by using both conventional (Van Soolingen *et al.,* 1997) and Kit method (PROMEGA, Wizard® Genomic DNA Purification Kit).

Polymerase chain reaction (PCR)

PCR reactions were performed on both blood and milk DNA (Hermans *et al.,* 1990; Rodriguez *et al.,* 1999). Reaction mixture (25 µl) containing 12.5 µl of Taq PCR master mix (Qiagen), 5 µl of DNA template and 0.2 µM of each primers INS1 (forward) 5'-CGTGAGGGCATCGAGGTGGC-3', INS2 (reverse) 5'-GCGTAGGCGTCGGTGACAAA- 3' for MTC. Along

with sample DNA, a known positive control DNA and a negative control was also amplified. Thermal cycling was performed in T Gradient Thermocycler (Biometra, Germany) with the following cycling parameter for INS1 and INS2: 94 for 5 min followed by 30 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C followed by final extension of 7 min at 72°C. PCR products were run by agarose gel electrophoresis using 1.5% agarose gel and visualized by gel documentation system (Bio-Rad).

Amplification by gyrB-PCR

The target DNA for amplification was 1,020 bp fragment of the *gyrB* gene, which was used to identify members of the *M. tuberculosis* complex. The primers used were MTUB-f (5'-TCG GAC GCG TAT GCG ATA TC-3') and MTUB-r (5'-ACA TAC AGT TCG GAC TTG CG-3') (Chimara *et al.,* 2004; Niemann *et al.,* 2000) which amplify a gene sequence *gyrB* for 1,020 base pair fragment in MTC PCR. Briefly, a ready to use *Taq*PCR Master Mix (Qiagen) was used. A reaction volume of 50 µl was made, containing 25 µl of *Taq*PCR Master Mix, 2 µl of forward primer (10 pmol/µl), 2µl of reverse primer (10 pmol/µl), 11µl of nuclease free water and 10 µl of DNA template. Thermal cycling was performed in T. Gradient Thermo cycler (Biometra, Germany). The cycling conditions were as follows, initial denaturation at 95 ℃ for 10 min, followed by 35 cycles for denaturation at 94 ℃ for 1 min, annealing of primers at 65 ℃ for 1 min, extension at 72 ℃ for 1.5 min and final extension at 72 ℃ for 7 min. PCR products were run by gel electrophoresis and visualized by gel documentation system (Bio-Rad).

Restriction fragment length polymorphism (RFLP)

DNA polymorphisms in the 1,020-bp *gyrB* fragment amplified with the primers MTUB-f and MTUB-r were analyzed after digestion with restriction enzymes *RsaI* and *SacII* as recommended by the manufacturer's protocol (Promega).Digestion mixture consist of 16.3 µl sterile, deionized water, 2 µl RE 10X buffer, 0.2 µl of 10 µg/µl Acetylated BSA and 1 µl of 1 µg/µl DNA, mixed by pipetting followed by 0.5 µl of 10 µg/µl Restriction Enzyme. The mixture was incubated at 37 ℃ for 4 hours. The DNA digest was separated in a 2% agarose gel by electrophoresis, and the gels were visualized under a UV transilluminator. A 100-bp DNA ladder was used as a size standard

RESULTS AND DISCUSSION

Mycobacterial infection is considered as a potential threat to both animal and human health. Detection and identification of mycobacterial species were done basically by conventional AFB staining and culture which are usually time consuming and also unable to differentiate M. tuberculosis from other non- tuberculosis Mycobacterium. PCR techniques offer high sensitivity and have been successfully used for diagnosing bovine TB in several types of naturally infected organic materials such as tissue, blood, and nasal exudates (Filia *et al.,* 2016; Langnyei *et al.,* 2016; Coetsie *et al.,* 2000; Ahir *et al.,* 2016).

Diagnosis of bovine tuberculosis in live animals is primarily based on the detection of specific cell-mediated immune (CMI) responses and molecular techniques. In recent years, various attempts have been made to develop alternative and more rapid methods of detection of MTC. In this study, out of 190 animals tested by CITT, 70 (36.84%) animals were positive, 41 (21.57%) inconclusive and 79 (41.57%) were negative reactors. From these 70 positive animals, blood and milk samples from 50 milch animals were screened for *Mycobacterium tuberculosis* complex using INS1/INS2 primers followed by PCR-RFLP. Out of these 50 blood samples, 4 (8%) were positive for MTC (Figure 1) while no milk samples were positive for MTC. The results in the present study are similar to those reported by other workers in organized farms using single intradermal tuberculin test and CITT (Filia *et al.,* 2016; Ahir *et al.,* 2016; Thakur *et al.,* 2010).

For the rapid and specific diagnosis of TB, PCR assays are the most promising alternative method (Figueiredo *et al.*, 2009; Serrano-Moreno *et al.,* 2008). PCR techniques offer high sensitivity and have been successfully used for diagnosing bovine TB in several types of naturally infected organic materials such as tissue, blood, and nasal exudates (Coetsie *et al.,* 2000; Gomez-Laguna *et al.,* 2010). The most commonly used PCR is based on primers that amplify segments of the IS*6110* element, particularly targeting 245 bps fragments. In this study, blood and milk samples from 50 milch animals were screened for *Mycobacterium tuberculosis* complex by IS*6110* PCR amplify an insertion

sequence of 245 bps using INS1/INS2 primers followed by PCR-RFLP. Out of these 50 blood samples, 4 (8%) were positive for MTC while no milk samples were positive for MTC.The results in the present study are similar to those of Filia *et al.* (2016) who observed that 4.23% blood samples were positive by PCR. Basit *et al.* (2015) reported the overall prevalence of tuberculosis was 6.5% by PCR from a total 200 tissue samples of lungs, lymph nodes and liver from cattle, buffaloes, sheep and goats collected from abattoir.

In the present study, as compared to CITT, only 4 blood samples were positive by PCR. CITT has been considered less in sensitivity and specificity. Though PCR-based methods helps in rapid detection of target DNA in suspected clinical samples, the efficacy of PCR assays can be influenced by the quality of target DNA and the presence of sample-derived PCR inhibitors (Schrader *et al.,* 2012). PCR inhibitors found in blood, serum or plasma samples includes substances like IgG, haemoglobin and lactoferrin (Al-Soud *et al.,* 2000; Al-Soud *et al.,* 2001). Most of these inhibitors are presumed to affect the RNA directly and not the enzymes of the reaction (Konet *et al.,* 2000).

However, in the present study, milk samples were found negative by IS*6110* PCR. The absence of *Mycobacterium* species in the milk samples from CIIT-reactive cows by PCR might be due to onetime sampling in the design of the study where some periods of bacterial excretion might have been missed (Carvalho *et al.,* 2014) or due to presence of few numbers of excreted bacteria, or to the presence of dead or non-viable bacilli due to the action of macrophages, or even to the use of the decontamination method (Zumarraga *et al.,* 2006). The intermittent character of bacilli secretion after a short constant post-infection period was also documented (Menzies *et al.,* 2000).

For further confirmation of the species of *Mycobacterium tuberculosis* complex, *gyr*B PCR restriction endonuclease differentiation was carried out. For gyrB-RFLP, these 4 positive DNA samples were further amplified by PCR using the primer pair MTUB-f and MTUB-r specific for amplification of the 1,020-bp fragment of the *gyrB* gene of the *M. tuberculosis* complex (Fig. 2). These four PCR products along with a positive control were digested for 4 hours at 37°C with restriction enzyme *RsaI* and *SacII*. These enzymes digest the DNA of specific species and cut at the restriction site specific for these enzymes. GyrB-RFLP revealed *RsaI* digestion showing bands at 480bps and 385bps for *M. bovis* and at 560bps and 385bps for *M. tuberculosis*. *SacII* shows no digestion confirming presence of *M. bovis* not *M. caprae.* Agarose gel electrophoresis of RFLP is shown in Fig. 3.

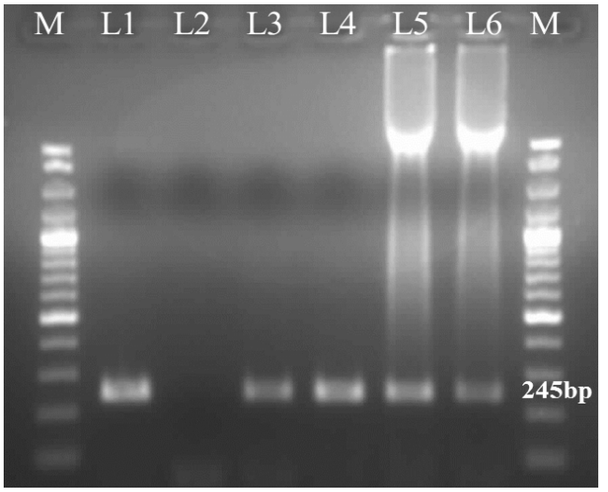

Fig. 1: Agarose gel electrophoresis of the amplified IS6110 gene using INS1/INS2 primers

M: 100 bp plus DNA ladder; **L1:** Positive control; **L2:** Negative control; **L3-L6:** samples

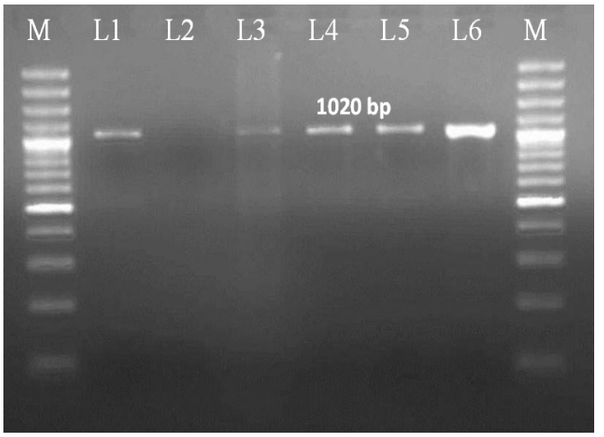

Fig. 2: Agarose gel electrophoresis of the amplified *GyrB* gene by using MTUB1/MTUB2 primers

M: 100 bp plus DNA ladder; **L1:** Positive control; **L2:** Negative control; **L3-L6:** samples

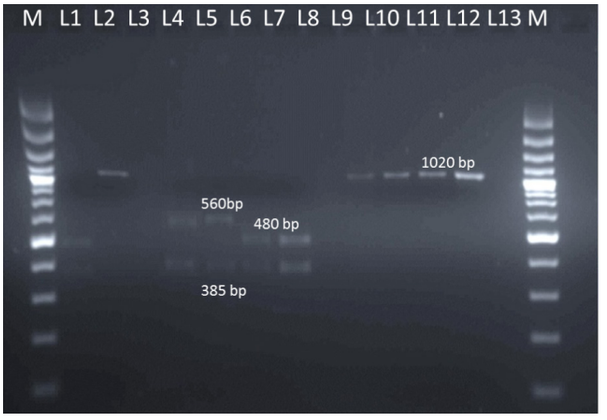

Fig. 3: RFLP patterns of PCR products obtained by RsaI digestion and *SacII* digestion

M: 100 bp plus DNA ladder; **L1**: Positive control digested by *RsaI;* **L2:** Positive control digested by *SacII*; **L4-L7:** Samples digested by *RsaI*; **L9-L12:** Samples digested by *SacII*

PCR-RFLP for detection and differentiation of tuberculosis with selective amplification of the *gyr*B fragment from each species of the *M. tuberculosis* complex were done by various workers (Chimara *et al.,* 2004; Niemann *et al.,* 2000; Abass *et al.,* 2010; Kasai *et al.,* 2000; Goh *et al.,* 2006). In the present study, *gyrB*-RFLP showed that by *RsaI* digestion, bands at 480 and 385 base pair were observed in two samples indicating *M. bovis* while 560 and 385 in another two samples indicating *M. tuberculosis*. Further *SacII* enzyme digestion was done to differentiate *M. bovis subspecies* i.e., *M. bovis* subsp. *bovis and M. bovis* subsp. *caprae*. No digestion was observed in all the samples and this lack of digestion with *SacII* excluded the presence of *M. bovis* subsp. *caprae* confirming the presence of *M. bovis* subsp. *bovis*.

For differentiation of strains of *M. tuberculosis, M. bovis, M. africanum* and *M. microti* Kasai *et al.* (2000) developed gyrB-RFLP. It was found that 1020 bp fragment of the gyrB gene was amplified with specific primers, which do not generate amplicons from other species of mycobacteria followed by digestion of the amplicon with restriction enzymes. Chimara *et al.* (2004) analyzed gyrB-RFLP patterns from reference strains which include *M. tuberculosis H37Rv, M. bovis*, *M. bovis* BCG, *M. canetti, M. africanum* and *M. pinnipedii* isolates. They developed a new table based on band sizes and confirmed by analysis of sequences deposited by Kasai*et al.* (2000). The band pattern of the present gyrB-RFLP was similar to that observed by Chimara and his groups (2004) but differ from Niemann *et al.* (2000) where bands showed different sizes. In our study small band as described by Chimara *et al.* (2004) were not observed. These small bands were also not considered by other workers (Niemann *et al.,* 2000; Kasai *et al.,* 2000).

In the present study, *M. tuberculosis* and *M. bovis* were detected in animals located at different areas. Moreover, two animals were positive for *M. tuberculosis* indicating that the infection was caused from human contact which may be animal handlers indicating a reverse zoonosis in this areas. Many workers have reported that animals can be infected by exposure to any individual shedding the organism (Ayele *et al.,* 2004; Fritsche *et al.,* 2004; Grange *et al.,* 1994). Animals infected with *M. tuberculosis* constitute a potential hazard of transmission of virulent tubercle bacilli back to humans (Mishra *et al.,* 1997). The presence of MTC in animal may raise concerns regarding the zoonotic risk for humans, especially those living at the animal-human interface.

CONCLUSION

Thus, *GyrB*-RFLP showed promising results in diagnosis and further differentiation of MTC in early stage in live animals. *GyrB-RFLP* serves as an additional tool for differentiation of MTC and may help in the epidemiological studies. This can also help in quick segregation of infected animals; restrict transmission and rapid eradication of bovine TB in the country. Further large-scale studies are required for detection, control and eradication of bovine TB in the state.

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Conflict of Interest: The authors declared no conflict of interest.

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