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Detection of Nontuberculous Mycobacterial Species from Tissue Samples of Cattle and Buffaloes by PCR and PRA (PCR-RFLP)

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ABSTRACT

Background: Nontuberculous mycobacteria are opportunistic pathogens and some of them may cause disease in humans and animals causing pulmonary infections, mastitis, lesions in respiratory tract and lymph nodes of cattle, due to which they are being recognized worldwide and also interfere with the diagnosis of bovine tuberculosis.

Methods: The present study was conducted for detection of nontuberculous mycobacterial species (NTM) in tissue samples (with and without tubercle lesions) in cattle and buffaloes from postmortem hall GADVASU, Ludhiana. Polymerase Chain Reaction and PCR-RFLP which involved *hsp*65 gene amplification (439 bp) and restriction analysis of amplified product was performed on 30 tissue samples for detection of nontuberculous mycobacterial species.

Result: Three out of 30 samples showed *hsp*65 gene amplification and 2 were identified as *M. kansasii* using restriction analysis technique and one could not be identified as the RFLP patterns was different from other known PCR-RFLP profiles. NTM such as *M. kansasi* may cause infection in animals and PRA (PCR-Restriction Fragment Length Polymorphism Analysis) technique was found to be a rapid tool for identification and differentiation of NTM upto species level.

Key words: *hsp*65 gene, *M. kansasii*, PCR, PRA (PCR-RFLP).

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are the 'atypical mycobacteria' belonging to species other than those in the *Mycobacterium tuberculosis* complex. Although most NTM are known to be saprophytic in nature, some NTM species are known to cause pulmonary infections (Griffith *et al.,* 2007) due to which they are being recognized worldwide. These organisms are efficient in causing pulmonary disease, disseminated disease or localized lesions in both immuno-competent and immune-compromised hosts (animals as well as humans) (Jarzembowski and Young, 2008). NTM can activate non-specific immune response which leads to false positive reactions in tuberculin testing (Bouts *et al.,* 2009; Kazda and Cook, 1988) and causes disease, lymphadenitis, soft tissue infections, skin infections and visceral or disseminated disease (Chan and Iseman, 2013). Pulmonary infections are most commonly caused by *Mycobacterium avium* complex (MAC), *Mycobacterium kansasii* and *Mycobacterium abscessus.* These infections are sometimes asymptomatic which does not always equate with active infection and are diagnosed with supportive radiographic and clinical findings (Johnson and Odell, 2014). NTM such as *Mycobacterium chelonei, Mycobacterium fortuitum, Mycobacterium phlei, Mycobacterium smegmatis, Mycobacterium thermoresistible* cause disease including mastitis in cattle and cutaneous mycobacterial granuloma in cats and dogs. Lesions in respiratory tract and lymph nodes of cattle are produced by *M. kansasii* and can also be isolated from tissue samples of cattle giving a positive TST (tuberculin skin testing) (Waters *et al*., 2006; 2010).

Identification of NTM species can be done based on their phenotypic characteristics of biochemical testing, pigment production, growth characteristics and colonial morphology but these traditional methods are time-consuming (Witebsky and Kruczak-Filipov, 1996; Metchock *et al.,* 1999). Thus, more advanced techniques for rapid identification of NTM such as commercial nucleic acid probes, 16S ribosomal DNA sequencing (Turenne *et al.* 2001), high-performance liquid chromatography (HPLC) (Butler and Guthertz, 2001) and PCR-restriction enzyme pattern analysis (PRA) (Telenti *et al*., 1993) methods have been developed.

Molecular detection of NTM by PCR based amplification of mycobacterial DNA with genus-specific primers has been used. The identification is done on the basis of comparison between nucleotide sequence and reference sequences. The most commonly used target gene is the gene coding 16S ribosomal RNA. In addition to this, many other genes have been targeted for this purpose (gene encoding 32kDa

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protein (Soini *et al.,* 1994), the 65 kDa heat shock protein (Telenti *et al.,* 1993) and the 16S-23S ribosomal RNA internal transcribed spacer (Roth *et al.,* 1998).

The impact of NTM is both direct, causing more or less severe infections and loss of productivity, or indirect, by interfering with diagnosis and control of bovine tuberculosis and paratuberculosis. In this study, highly conserved 65 kDa heat shock protein was used for NTM detection by PRA as a target.

MATERIALS AND METHODS

Lymph nodes, lungs tissue samples (n=30) suspected of bovine tuberculosis from cattle (n=20) and buffaloes (n=10) with gross lesions/anomaly (tubercle lesions, enlarged lymph nodes, abscess) and without any gross lesions/ anomaly were collected from postmortem hall GADVASU, Ludhiana. DNA from tissue samples as well as from standard cultures was extracted using NucleoSpin Tissue DNA extraction Kit (Machery-Nagel) as per the manufacturer's protocol. Later, PCR and PRA (PCR-RFLP) was conducted on the extracted DNA for identification of presence of NTM in these samples.

Polymerase chain reaction

The extracted DNA was amplified using 5 sets of primers (Table 1).

PCR protocol

For the amplification, the reaction volume of 25 µl was made containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primers (10 pmol/µl), 2.5 µl of nuclease free water and 8 µl of DNA template along with the test sample DNA, known positive control DNA from standard cultures of *M. kansasii* (MTCC3058), *M. smegmatis* (MTCC6), *M. vaccae* (MTCC272), *M. fortuitum* subspp. *fortuitum* (MTCC929) and *M. intracellulare* (MTCC920) (IMTECH, Chandigarh) were also amplified.

Thermocycling conditions

The thermocycling conditions for *M. kansasii M. smegmatis M. vaccae* and *M. intracellulare* were same. Thermal cycling was performed in research thermal cycler and cycling conditions were as follows, initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing of primers at 60°C for 1 minute and 62°C for 1 minute for *M. fortuitum*, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplified PCR products were then run by agarose gel electrophoresis using 1.5 per cent agarose and visualized in Gel Documentation System (Alpha Innotech).

PCR-Restriction fragment length polymorphism analysis (PRA)

PRA uses the polymerase chain reaction to amplify the selected DNA regions (the internal transcribed spacer ITS regions) (Park *et al.,* 2000). These PCR products are then digested by restriction enzymes and visualized on an agarose gel. This method was used to differentiate various species of NTM from samples collected.

Amplification of *hsp*65 gene

A highly conserved heat shock protein 65 portion gene of Mycobacteria was amplified using primer sequence forward (TbII) 5′- ACCAACGATGGTGTGTCCAT-3′ and reverse (TbI2) 5′- CTTGTCGAACCGCATACCCT-3′ of 439 bp (Schinnick, 1987) (Applied Biosystem). For the amplification, the reaction volume of 25 µl was made containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primers (10 pmol/µl), 2.5 µl of nuclease free water and 8 µl of DNA template. Along with the test sample DNA, known positive control DNA of *M. kansasii* (MTCC3058), *M. smegmatis* (MTCC6), *M. vaccae* (MTCC272), *M. fortuitum* subspp. *fortuitum* (MTCC929) and *M. intracellulare* (MTCC920) (IMTECH, Chandigarh) were also amplified. The reaction was subjected to 45 cycles of amplification which includes denaturation for 1 min at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C and the final extension was done at 72°C for 10 min.

Restriction fragment length polymorphism (RFLP)

The amplified PCR product (439 bp) was then digested with two enzymes *BstEII* and *HaeIII* (promega). RFLP of the standard cultures of *M. kansasii, M. smegmatis, M. vaccae, M. fortuitum* and *M. intracellulare* was also done. For the digestion of PCR product with *BstEII,* 10 µl of PCR product was added directly to the mixture containing 1 µl (5 U) of

Table 1: Primer sequences and their sizes of PCR product in different NTM species.

Organism	Primer	Primer sequence	Size of PCR product	Reference
M. kansasii	Forward (ITS)	5′- GCAAAGCCAGACACACTATTG-3′	152 bp	Esfahani *et al.* (2012)
	Reverse (ITS)	5′- AAGAACACGCTACCCGTAGG-3′		
M. smegmatis	Forward	5′- ACCATGTCTATCTCAGTGTGCT-3′	628 bp	Brahma *et al.* (2017)
	Reverse	5′- ACGCTCGAGGTCCACTACAA-3′		
M. fortuitum	Forward	5′- GACTGCCAGACACACTATTGG-3′	172 bp	Esfahani *et al.* (2012)
	Reverse	5′- GTGAGACCACACGATTCTGC-3′		
M. intracellulare	Forward	5′- CCT TTA GGC GCA TGT CTT TA-3′	450 bp	Park *et al.* (2006)
	Reverse	5′- ACC AGA AGA CAT GCG TCT TG-3′		
M. vaccae	Forward (ITS-F1)	5′- CGAAGCCAGTGGCCTAACCC-3′	500 bp	Park *et al.* (2006)
	Reverse (ITS-R)	5′- TGGATCCTGCCAAGGCATCCACCAT-3′		

enzyme, 2.5 µl of restriction buffer (5 x buffer B) and 11.5 µl of water the mixture was incubated at 60°C for 60 minutes. Similarly, for the digestion of PCR product with *HaeIII*, 10 µl of PCR product was added directly to the mixture containing 1 µl (5 U) of enzyme, 2.5 µl of restriction buffer (5 × buffer B) and 11.5 µl of water the mixture was incubated at 37°C for 60 minutes (Telenti *et al*., 1993). The enzymes and buffers were purchased from Promega.

Evaluation of restriction patterns

After the digestion, 4 µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added and 10 µl of the mixture was loaded onto a NuSieve 3:1 agarose gel (Lonza). A gene ruler DNA™ ladder plus 50 bp (Fermentas) was run along with the test samples. The gel was visualized in Gel Documentation system (AlphaImager 3400HP, AlphaInnotech). The size of the amplicon was determined by comparing it with the standard molecular weight marker. The results were interpreted as per the algorithm used by Telenti *et al.* (1993) and PRA site (http://app.chuv.ch/prasite/index.html).

RESULTS AND DISCUSSION

PCR

In the present study 30 lymph nodes and lung tissue samples were subjected to PCR for detection of NTM. The size of the amplicon was determined by comparing it with the standard molecular weight marker. Amplicons of 152 bp, 628 bp, 172 bp and 450 bp and 500 bp were considered positive for *M. kansasii, M. smegmatis*, *M. fortuitum* and *M. intracellulare* and *M. vaccae*. Out of 30 samples 2 samples were positive for *M. kansasii*, one from tissues with gross lesions and one without any gross lession (Fig 1). In molecular detection of NTM 16S-23S internal transcribed spacer region is the most common genomic loci. In a cross-sectional study conducted by Hoza *et al.* (2016) a total of 744 sputum samples were collected from 372 TB suspects. They were detected by using various methods (16S rRNA and *hsp*65 gene sequencing). The prevalence of NTM was found to be 9.7% of the mycobacterial isolates. A similar study was done by Ghielmetti *et al.* (2018) in which *M. kansasii* was detected from tissue samples with and without having macroscopic lesions.

Differentiation of NTM by PRA

PCR-RFLP (PRA) is a rapid and reliable technique that gives the ability to identify different species of mycobacteria. In PRA method, 439 bp PCR product of *hsp*65 gene was amplified (in both standard culture DNA and samples) and digested with the *BstEII* and *HaeIII* restriction enzymes. The restriction patterns were analyzed for species identification as per Saifi *et al.* (2013). Similar study was conducted by Telenti *et al.* (1993) in which 65-kDa protein (*hsp*65 protein) was used.

PCR for presence of *hsp65* gene

Among the clinical samples processed 3 out of 30 tissue

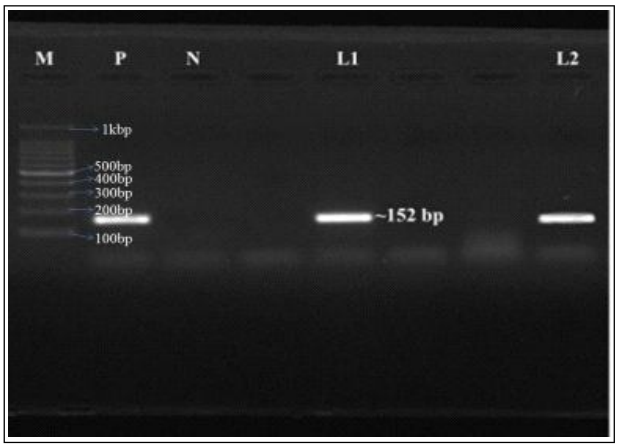

Fig 1: Agarose gel electrophoresis showing an amplicon of 4 152 bp of *M. kansasii* from tissue samples.
M: Marker (100 bp DNA ladder), P: Positive, N: Negative.
L1, L2: Positive sample for *M. kansasii* from tissue samples.

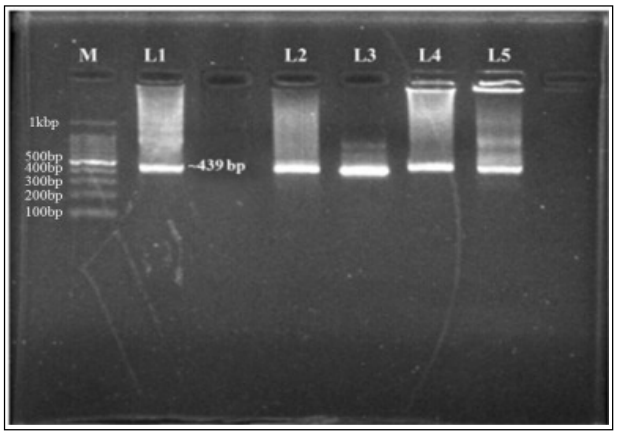

Fig 2: Agarose gel electrophoresis showing an amplicon of 4 439 bp from standard cultures (*M. kansasii, M. smegmatis, M. fortuitum, M. vaccae, M. intracellulare*).
M: Marker (100 bp DNA ladder).
L1, L2, L3, L4, L5= Positive standard for *hsp65* gene PCR (439 bp).

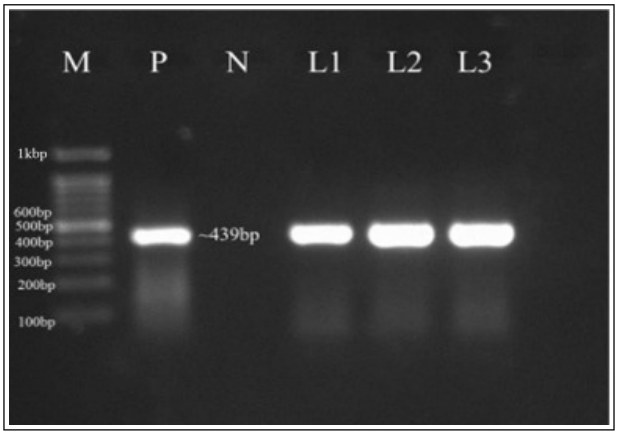

Fig 3: Agarose gel electrophoresis showing an amplicon of 4 439 bp from tissue samples.
M: Marker (100 bp DNA ladder), P: Positive control, N: Negative.
L1, L2, L3: Positive for *hsp*65 gene PCR (439 bp) tissue samples.

samples (10%) were positive for *hsp* 65 gene (Fig 3). The standard cultures (*M. kansasii, M. smegmatis, M. fortuitum, M. vaccae, M. intracellulare*) also showed the 439 bp band of *hsp*65 gene (Fig 2).

Restriction enzyme analysis of the *hsp65* gene

The PCR product of *hsp*65 gene amplicon of standard cultures along with the samples was subjected to digestion with restriction enzyme using *BstEIII* and *HaeIII* (Fig 4). From 30 tissue samples, two were identified as *M. kansasii* (n=2) having the RFLP pattern as 245/220 bp when digested with *BstEIII* and 140/105/70 bp when digested with *HaeIII* (Fig 5) and one could not be identified as the RFLP pattern was different from other known patterns (Fig 6).

M. kansasii is also known to have the potential to interfere with bTB diagnostics and in some cases it may interfere with bTB diagnosis giving false-positive reactions (Vordermeier *et al.,* 2012). Although the infection caused by *M. kansasii* is rare and is often associated with respiratory tract and associated lymph nodes lesions, diagnosed at postmortem. Chang *et al.* (2002) treated 439-bp PCR product of 10 NTM with *BstEII* and *HaeIII* for identification of NTM to species level. Six different RFLP profiles were produced by digestion with *BstEII* and eight different RFLP profiles were produced by digestion with *HaeIII*. From this, 9 of 10 samples of NTM were identified to the species level. Six mycobacterial species were identified, including *M. gordonae* type I, *M. gordonae* type II, *M. gastri*, *M. kansasii*, two *M. fortuitum* subsp. 3rd variant, *M. simiae, M. scrofulaceum* and *M. szuigai*.

M. kansasii and *M. persicum* are known to have the potential to interfere with bTB diagnostics and, in some cases, to cause false-positive reactions leading to considerable economic losses (Vordermeier *et al*., 2007; *et al*., 2006).

The *hsp*6 gene was chosen as the target for amplification because it's highly conserved among all the *Mycobacterium* species as reported by Buchanan *et al.* (1987). Some other PCR based procedures capable of identifying multiple species of Mycobacteria have been classified by Plikaytis *et al.* (1992). The PCR-RFLP procedure by Telenti *et al.* (1993) is more reliable because of its ability to identify the more number of species without the need for probing or sequencing of the amplicons. Similar study by Hafner *et al.* (2004) analysed the heat shock protein 65 (hsp65) gene restriction fragment length polymorphism (RFLP) patterns of some rarely isolated NTM for which patterns were not been published before (*Mycobacterium bohemicum*, *Mycobacterium hassiacum*, *Mycobacterium heckeshornense*, *Mycobacterium monacense* and *Mycobacterium triplex*). Also new hsp65-variants for *Mycobacterium interjectum* (type II), *Mycobacterium mucogenicum* (type V), *Mycobacterium gordonae* (type VIII) and *Mycobacterium paraffinicum* were described. Tortone *et al.* (2018) evaluated the usefulness of molecular methods, especially *hsp*65-PRA (PCR-Restriction Enzyme Analysis). For 56 NTM isolates recovered from 32 (42.1%) positive samples were used in the study and identification upto species level was done using *hsp65*-PRA.

Ong *et al.* (2010) also reported 5 novel restriction patterns, different from any of the patterns of the algorithm. Saifi *et al.* (2013) suggested that PRA is sensitive, specific

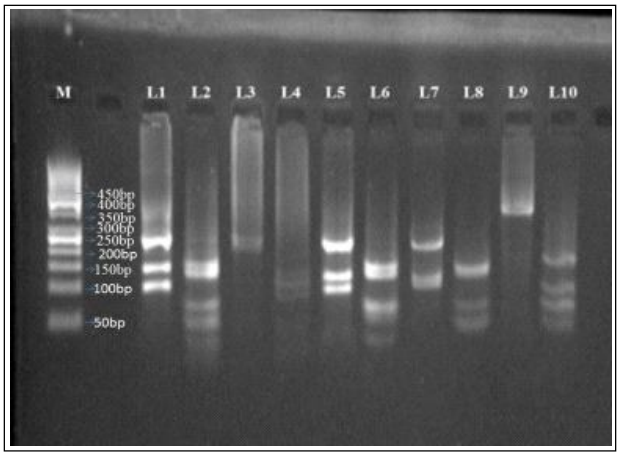

Fig 4: Agarose gel electrophoresis showing RFLP pattern of standard cultures (*M. smegmatis, M. kansasii, M. fortuitum, M. intracellulare, M. vaccae*).

M: Marker (50 bp DNA ladder).
L1: *M. smegmatis* (*BstEIII*) (235/130/85).
L2: *M. smegmatis* (*HaeIII*) (145/125/60).
L3: *M. kansasii* (*BstEIII*) (245/220).
L4: *M. kansasii* (*HaeIII*) (140/105/70).
L5: *M. fortuitum* (*BstEIII*) (245/125/80).
L6: *M. fortuitum* (*HaeIII*) (155/135).
L7: *M. intracellulare* (*BstEIII*) (245/125/100).
L8: *M. intracellulare* (*HaeIII*) (155/150/60).
L9: *M. vaccae* (*BstEIII*) (440).
L10: *M. vaccae* (*HaeIII*) (140/115/70).

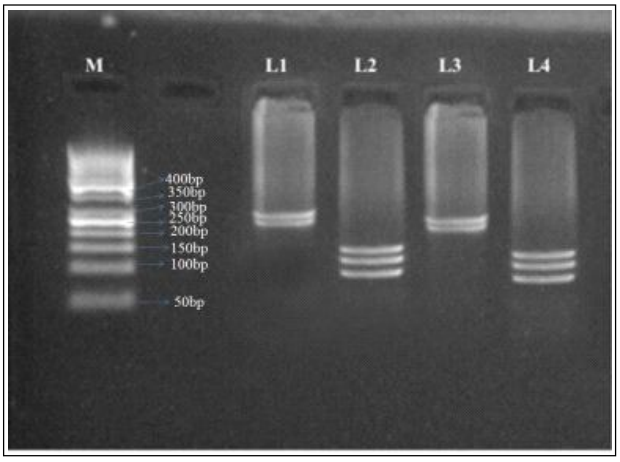

Fig 5: Agarose gel electrophoresis showing RFLP pattern of NTM species in tissue samples (Image 1).

M: Marker (50 bp DNA ladder).
L1, L 3: *M. kansasii* (*BstEIII*) (245/220), L2, L4: *M. kansasii* (*HaeIII*) (140/105/70).

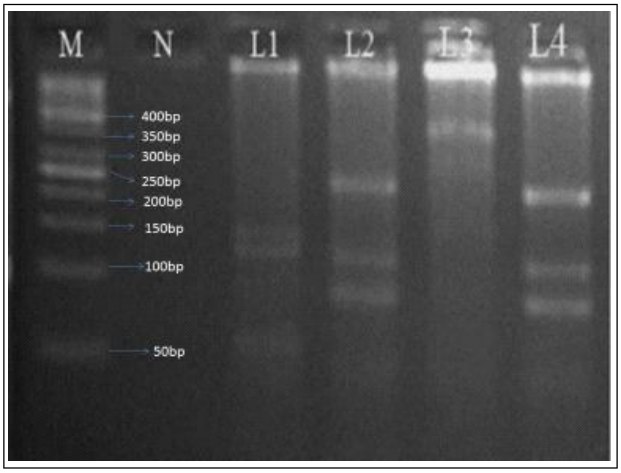

Fig 6: Agarose gel electrophoresis showing RFLP pattern of NTM species in tissue samples (unidentified) (Image 2).

M: Marker (50 bp DNA ladder).

L1: *M. fortuitum* (*HaeIII*) (155/135).

L2: *M. fortuitum* (*BstEIII*) (245/125/80) (standard).

L3: Unidentified (*HaeIII*) (360) (sample).

L4: Unidentified (*BstEIII*) (245/125/80) (sample).

and an effective assay for detection of nontuberculous mycobacterial species than other PCR based techniques and is capable of identification of large number of species without using any probe or amplicon sequencing. A study was conducted by Nour-Neamatollahie *et al.* (2017) in which (PRA) of the hsp65 gene was done on clinical samples (sputum, bronchial lavage, skin samples) of Tb suspected patients as a result of which majority of NTM were obtained along with *M. bovis* and *M. tuberculosis*. The most frequently detected *Mycobacterium* species were *Mycobacterium kansasii*, which was isolated in 5 (45.4%) out of 11 patients with NTM pulmonary disease.

CONCLUSION

NTM such as *M. kansasi* may cause infection in animals and PRA techniques was found to be a rapid tool for identification and differentiation of NTM upto species level.

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