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Research Article

IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIA IN FAECES OF CATTLE AND BUFFALOES USING POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

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ABSTRACT

A rapid method for the identification and differentiation of non-tuberculous mycobacteria in the faeces of cattle and buffaloes was employed on the basis of restriction pattern shown by different species of mycobacteria. The method involves amplification of *hsp*65 gene (heat shock protein gene) encoding 65 kDa heat shock protein by polymerse chain reaction followed by restriction enzyme analysis of the PCR products. Polymerase chain reaction-restriction fragment length polymorphism (PRA) was performed on the DNA extracted from faecal which were positive for *hsp*65 gene. Out of total 200 faecal and 200 milk samples, eleven faecal samples were detected positive for non-tuberculous mycobacteria by targeting *hsp*65 gene in a PCR assay which were then differentiated to species level using restriction analysis technique. M. vacca was detected in four faecal samples and M. kansasii was detected in one faecal sample. Six isolates could not be identified at the species level because their RFLP patterns were different from other known PCR-RFLP profiles. There were different *hsp*65 gene PCR RFLP profiles produced by digestion with BstEII and HaeIII.

Key words: non-tuberculous mycobacteria, *hsp*65, polymerase chain reaction, restriction fragment length polymorphism

The non-tuberculous mycobacteria include those Mycobacterial species that are not the members of the *Mycobacterium tuberculosis* complex and thus are termed as non-tuberculous mycobacteria (NTM; Desikan *et al*., 2017). Several mycobacterial species have been described that are potential pathogens causing pulmonary and cutaneous diseases, lymphadenitis and disseminated infections (Adjemian *et al.*, 2012; Tortoli, 2014). Molecular techniques like polymerase chain reaction (PCR), real-time PCR, multiplex real-time PCR and restriction fragment length polymorphism (RFLP) have been applied for the identification and differentiation of NTM (Esfahani *et al.*, 2012; Choi *et al.*, 2012). Genes encoding the conserved and nonconserved regions in 65-kDa heat shock proteins of mycobacteria, have been found to be suitable for the identification of NTM (Telenti *et al*., 1993). Sequencing of the 16S rRNA gene is a powerful technique for differentiating mycobacterial species, however, it is labor-intensive and difficult to perform as a routine technique in clinical laboratories (Wu *et al*., 2014). For the detection of NTM, PCR-based sequencing consisting of amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons have been used. Identification of the organism is made by comparing the nucleotide sequence of the organism with reference sequences. The most commonly targeted gene codes for the 16S ribosomal RNA. With the application of 16S ribosomal DNA sequencing, high-performance liquid chromatography (HPLC) and 65 KD gene polymerase chain reaction-restriction fragment length polymorphism analysis (PRA), the identification of different species of NTM has risen in recent years (Tortoli, 2010; De Zwaan *et al*., 2014).

Several other genes have also been targeted like genes coding for the 32 kDa protein (Soini *et al*., 1994), the 65 kDa heat shock protein (Telenti *et al*., 1993), and the 16S 23S ribosomal RNA internal transcribed spacer (Hashemi-Shahraki *et al*., 2013). Presently more than 150 species are recognized as NTM in the genus *Mycobacterium* (Falkinham, 2015). Saifi *et al*. (2013) developed an assay that employs amplification of a *Mycobacterium* genus-specific amplicon with a RFLP analysis to differentiate the most commonly encountered non-tuberculous mycobacterial pathogens to the species level. The 439bp amplicon of *hsp*65 gene, which is present in all mycobacterial species, shows more variability than 16S rRNA gene sequence and is therefore helpful in identification of genetically related species. Variation in the sequence of *hsp65* gene can be employed to identify both slowly growing and rapidly growing mycobacteria to the species level (Harmsen *et al*., 2003). For the identification of NTM, *hsp65* PRA technique has been widely used and based on this an algorithm has been developed for differentiating 34 mycobacterial species. With the help of this algorithm differentiation of NTM can

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be done within a day by restriction enzyme digestion of PCR products due to the conserved nature of the *hsp65* gene (Devallois *et al*., 1997). So the present study is designed for rapid identification and differentiation of non-tuberculous mycobacteria from the faecal and milk samples of cattle and buffaloes by PRA as this technique is quite reliable and rapid in detecting NTM.

MATERIALS AND METHODS

Collection of Samples: Faecal (n=200) and milk (n=200) samples from cattle and buffaloes with a history of chronic intermittent diarrhea were collected from dairy farms in and around Ludhiana and Teaching Veterinary Clinical Complex (TVCC), GADVASU, Ludhiana.

Microscopic Examination of Samples: Each faecal sample was subjected to Ziehl Neelsen (acid fast) staining (Quinn *et al.*, 1994). Two gram faeces was finely ground into a paste with 8-10 ml sterile distilled water using a sterile pestle mortar. The fine paste formed was transferred to a 15 ml centrifuge tube and was concentrated by centrifugation at 2500 rpm for 5 min at room temperature. Supernatant was discarded and smear was prepared from interface layer. This smear was then stained by Ziehl Neelsen staining. Milk samples were also subjected to acid-fast staining. Five ml of the pooled milk was centrifuged at 2500 rpm for 15 min and the supernatant was discarded. Smear was prepared from the pellet collected at the bottom of centrifuge tube. The smear was then stained with Ziehl Neelsen staining.

Amplification of *hsp65* Gene: The extraction of genomic DNA from faecal samples was done using commercially available bacterial genomic isolation kit (HiPurA DNA purification kit) as per manufacturer's instructions. Using genus specific primers, a portion of highly conserved heat shock protein (*hsp65*) gene of Mycobacteria was amplified. For the amplification of the desired gene, the composition of the PCR reaction mixture was: GoTaq® Green Master Mix (12.5µl), forward primer (Tb11; 1µl), reverse primer (Tb12; 1µl), nuclease free water (0.5µl) and DNA template (10µl). The reaction was subjected to 45 cycles of amplification (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. 439-bp fragment between positions 398 and 836 of the published gene sequence was amplified by using primers Tb11 (5'-ACCAACGATGGTGTGT CCAT-3') and Tb12 (5'-CTTGTCGAACCGCA TACCCT-3') (Schinnick, 1987). Standard cultures of *M. avium, M. kansasii* and *M. smegmatis* were also amplified using Tb11 and Tb12 primers.

Restriction Analysis: Two restriction enzymes *BstE* II and *Hae* III (Promega) were used for the digestion of the amplified 439bp PCR product. Restriction analysis of the standard cultures of *M. avium*, *M. smegmatis* and *M. kansasii* was also done. For *BstE* II digestion, 10 µl of amplified PCR product was mixed with 1µl (5 U) of enzyme, 2.5 µl of restriction buffer (10 x) and 11.5 µl of water and the mixture was incubated at 60°C for 60 min. Similarly, 10 µl of the amplified PCR product was digested at 37°C in a mixture containing 1 µl (5 U) of *Hae* III enzyme, 2.5 µl of restriction buffer (10 x) and 11.5 µl of water. After the digestion of the amplified *hsp65* gene product, 4 µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was mixed with 8 µl of the PCR product and the mixture was then loaded onto a NuSieve 3:1 agarose gel (Lonza). Digested PCR products of standard cultures, test samples and gene ruler DNA™ ladder plus 50 bp and 100 bp plus (fermentas) were made to run. After electrophoresis, the agarose gels were visualized in Gel Documentation system (AlphaImager 3400HP, AlphaInnotech). The amplicon size was determined by comparing with the standard molecular weight marker. Using the algorithm given by Telenti *et al.* (1993), the results were interpreted after completion of the run.

RESULTS AND DISCUSSION

Among the acid-fast positive faecal (n=173) and milk (n=62) samples, 11 faecal samples tested positive for *hsp*65 gene (Fig. 1; Table 1). All the standard cultures of *M. avium, M. kansasii* and *M. smegmatis* were positive by *hsp65* gene PCR with a specific band of 439 bp. PCR products of the suspected clinical samples (n=11) were subjected to RE digestion using enzymes *Bst*EII and *Hae*III. The samples gave a specific restriction pattern of bands which helped in the species level differentiation of these mycobacteria which are summarized in Table 2, and Figs. 2 and 3). Four samples were detected as *M. vacca* having the RFLP pattern as 164/79 bp when digested by *Hae*III and 439 bp uncut band when digested with *Bst*EII. One sample was detected as *M. kansasii* having RFLP pattern as 149/114/68 bp when digested with *Hae*III and 149/85 bp when digested with *Bst*EII (Fig. 4) and the rest 6 were having variable restriction patterns which could not be speciated . RFLP patterns of three samples were 85 bp with *Hae*III and uncut 439 bp with *Bst*EII, 159 bp with *Hae*III and uncut 439 bp with *Bst*EII and 243 bp with *Hae*III and uncut 439 bp with *Bst*EII. The other three samples were having same RFLP pattern as 301/229/147 bp with *Hae*III and 358/303 bp with *Bst*EII. The

Table 1
***Mycobacterium* positive samples based on amplification of *hsp*65 gene by PCR**

Type of sample	Species	No. of samples processed	No. of positive samples
Faeces	Cattle	61	8
	Buffaloes	139	3
Milk	Cattle	61	0
	Buffaloes	139	0

restriction patterns (Fig. 5) of the standard cultures of *M. avium*, *M. smegmatis* and *M. kansasii* (Micobiologics) were similar to that reported by Telenti *et al.* (1993).

In the present study, PCR-RFLP was employed to differentiate NTM present in the DNA extracted from faecal samples rather than on the Mycobacterial isolates. *M. vacca* was detected in four faecal samples and *M. kansasii* was detected in one faecal sample. A total of 6 samples were having novel restriction patterns which were different from those given in algorithm. But RLFP pattern cannot be considered the discriminant one, since genetic heterogeneity leading to more than one RLFP pattern in a species cannot be excluded as is repoted by Brunello *et al.* (2001). Ong *et al.* (2010) also reported five novel restriction patterns which were different from any of the patterns given in the algorithm. In 276 isolates, Alcaide *et al.* (1997) reported five different patterns of *M. kansasii* after the PCR-RFLP analysis of the *hsp65* gene. With the *hsp65* gene-based method, 2 and 6 patterns were found in *M. fortuitum* and *M. gordonae* respectively. However, intraspecies variability should not be considered a drawback of the PRA method if the patterns are distinct. This can help us to trace the epidemiology of NTM in terms of both geographical distribution and pathogenicity (Wayne and Sramek 1992). Telenti *et al.* (1993), Taylor *et al.* (1997) and Saifi *et al.* (2013) also employed *hsp65* PCR-RFLP technique in differentiating other Mycobacterial species. The well-characterised species with unique restriction patterns are easy to be identified, however, sometimes it becomes difficult to interpret others which show distinct restriction patterns.

Table 2
Restriction analysis pattern of *hsp*65 gene using HaeIII and BstEII enzymes

Restriction analysis pattern		Species	No.of samples
HaeIII	BstEII		
166/80	No digestion	*M. vacca*	4
86	No digestion	unrecognised	1
168/149/114	149/85	*M. kansasii*	1
159	No digestion	unrecognised	1
243	No digestion	unrecognised	1
301/229/147	358/303	unrecognised	3

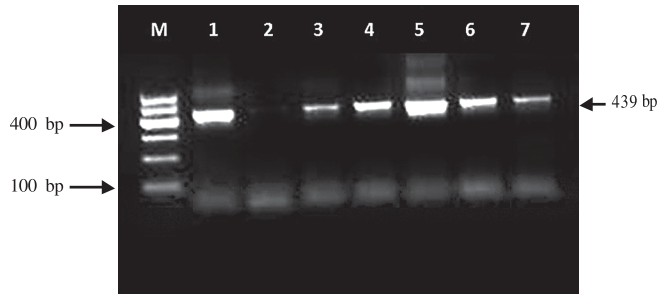

Fig 1. Presence of 439 bp amplified product of hsp65 gene in clinical samples.
M=100 bp marker; Lane 1=439bp positive control; Lanes 2-7=439 bp hsp65 gene in positive samples

Pourahmad *et al.* (2009) reported that only one specific technique cannot be solely used as a tool for identification of rare or novel species of Mycobacteria. Diversity of techniques and tests that are necessary and the time required for a proper identification are the two main limitations to the species level differentiation of mycobacteria. Since the method described here can be completed in a single day, representing a universal system of identifying mycobacteria to the species level and it does not require hybridization to a panel of species-specific probes for differentiating Mycobacterial species, contributes to the solution of these two problems as reported by Kusunoki *et al.* (1991). PRA method has been reported to give favorably concordant results for mycobacterial species and also all mycobacterial isolates could readily be identified by eye, without computer assistance and hence is highly suitable for large-scale use in a clinical laboratory (Wong *et al.*, 2001). The most common genomic loci applied in molecular detection of NTM are the 16S-23S rRNA internal transcribed spacer (Hashemi-Shahraki *et al*., 2013), *hsp65* (Jang *et al*., 2014), 16S rRNA (Wu *et al*., 2014) and rpoB gene (Kim *et al*., 2014). To date, many studies have used multiple loci for the detection of NTM species, and *hsp65* is always included. McNabb *et al*. (2006) reported a 99.1% concordance between biochemical methods or 16S rRNA

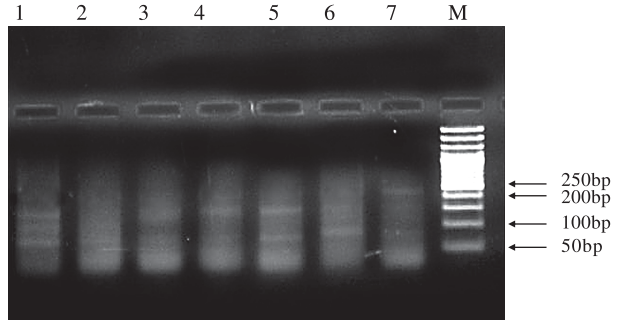

Fig 2. *Hae*lll digested RFLP pattern
M=50 bp plus marker; Lane 2=86 bp (unrecognized); Lanes 1, 4-6=166/80bp (M. vacca); Lane 3=160 bp (unrecognized); Lane 7=250 bp (unrecognized)

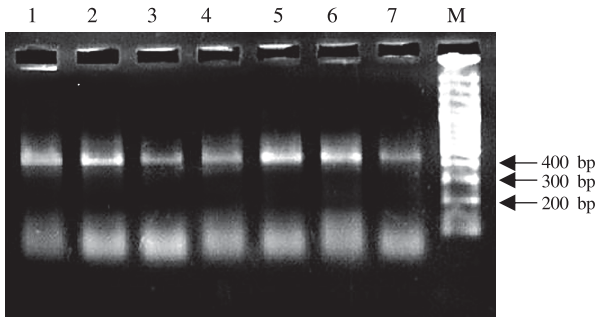

Fig 3. *Bst*Ell digested RFLP pattern
M=100 bp plus marker; Lanes 1-7=uncut 439 bp band

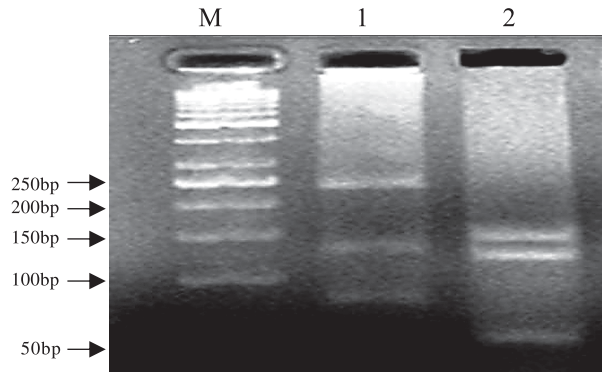

Fig 4. Restriction analysis pattern of *M. kansasii*
M=50 bp marker; Lane 1=*Bst*EII digested (195/149/85); Lane 2=*Hae*III digested (149/144/68)

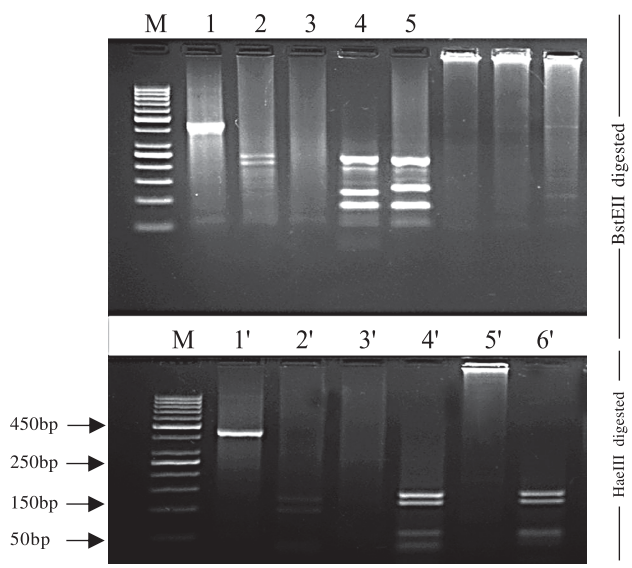

Fig 5. Restriction analysis pattern of standard cultures
M=50 bp marker; L1 and L1'=Undigested 439bp hsp65 PCR product; L2=245/200 bp *M. avium*; L2'=120/100 bp *M. avium*; L4=245/130/80 bp *M. kansasii*; L4'=130/110/70 bp *M. kansasii*; L5=245/140/80 bp *M. smegmatis*; L6'=140/125/65 bp *M. smegmatis*

gene sequencing compared to identification using hsp65 gene sequencing, suggesting that hsp65 gene (PRA method) is a sensitive, specific and effective assay for detecting mycobacterial species. To classify isolates within the genus *Mycobacterium* efficiently, sequencing based on a 439-bp portion of the *hsp*65 gene has proved to be useful but provides low resolution for distinguishing among members of the *M. avium* Complex particularly *Mycobacterium avium* subsp. *paratuberculosis* (Turenne *et al*., 2005). PCR-RFLP procedure reported by Telenti *et al.* (1993) is more reliable other PCR-based procedures capable of identifying multiple species of Mycobacteria because of its ability to identify the large number of species without using probes or sequencing of the amplicons (Saifi *et al*., 2013).

PCR-RFLP technique was found to be a good tool in identifying and differentiating non-tuberculous mycobacteriaupto species level. The technique also takes less time to identify Mycobacteria in contrast to other techniques like conventional isolation, sequencing, etc. It was also seen that *M. vacca* and *M. kansasii* have role in causing diarrhoea in cattle and buffaloes.

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