RESEARCH ARTICLE

Molecular Identification of Non-tuberculous Mycobacterial Species in Fecal Samples from Cattle and Buffaloes

Pallvi Slathia, Deepti Narang*, Mudit Chandra

Abstract

The non-tuberculous mycobacteria (NTM) are 'atypical mycobacteria' belonging to species other than those classified in the *Mycobacterium tuberculosis* complex. NTM species have been reported to cause cross-reactive immune responses that interfere with the diagnosis of tuberculosis in both livestock and wildlife and are considered as potential pathogens for animals and humans. The present study was conducted for detection of non- tuberculous mycobacterial species (NTM) in fecal samples with a history of intermittent diarrhea from cattle and buffaloes. Polymerase Chain Reaction and PRA (PCR-RFLP) was performed on 30 fecal samples collected from cattle and buffaloes of organized dairy farms in Ludhiana, for detection of non - tuberculous mycobacterial species (NTM). In the present study *M. fortuitum* was detected in two out of 30 fecal samples by PCR and PCR-RFLP (PRA).

Keywords: *M. fortuium*, Non-tuberculous Mycobacteria (NTM), Polymerase Chain Reaction (PCR), PRA (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis)

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Introduction

A wide range of non-tuberculous mycobacteria (NTM) have been described in addition to the well-known *Mycobacterium tuberculosis* complex (MTBC) members, such as *M. bovis* or *M. tuberculosis* (Procop, 2007). NTM are saprophytic in nature,present in the environment, primarily in soil and water sources and some of them infect both humans and animals (Bercovier and Vincen, 2001). Clinical manifestations of NTM include a wide range of conditions, including lung disease, lymphadenitis, skin infections, soft tissue infections, and visceral or disseminated disease (Tortoli, 2014).They may also interfere with diagnosis of bovine tuberculosis in animals. Therefore, it is essential to identify mycobacteria at the species level in order to assess their clinical importance.

NTM are identified molecularly by amplifying mycobacterial DNA using PCR and genus-specific primers. NTM has an effect that can be either direct, resulting in more or less significant infections and productivity losses, or indirect, hindering the diagnosis and treatment of paratuberculosis and bovine tuberculosis. For identification and differentiation of NTM upto species level, *hsp65* PRA method has been developed. Due to the conserved nature of the *hsp65* gene, differentiation of NTM with the use of this technique can be accomplished by restriction enzyme digestion of PCR products within a day (Devallois *et al*., 1997). The *hsp65* gene's 439 bp amplicon, which is found in all mycobacterial species and has more diversity compared to the 16S rRNA gene sequence and is useful in identifying species that are genetically related. Variation in the sequence of *hsp65* gene can be used to identify both rapid and slow growing mycobacteria up to the species level (Harmsen *et al*., 2003). Keeping in view the role of NTM in animals and the ability of PRA technique to rapidly identify and differentiate non-tuberculous mycobacteria, the present study was designed to identify NTM in fecal samples by Polymerase Chain Reaction (PCR) and PCR-Restriction Fragment Length Polymorphism Analysis (PRA).

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Materials and Methods

Ethical Statement: The approval of research protocol was obtained from IAEC (Registration no. *497/GO/Re/SL/01/CPCSEA*).

Collection of Samples: Fecal samples (n=30) from cattle (n=17) and buffaloes (n= 13) with history of intermittent diarrhea were collected from dairy farms in and around Ludhiana aged between 2 to 10 years. All the fecal samples were primarily stained with Ziehl Neelsen staining and were microscopically examined and were graded as negative or positive (5- >10 bacilli per HP field) on the smears.

Polymerase Chain Reaction:DNA extraction from fecal samples was carried out using HiPure Stool DNA Purification kit (HiMedia) as per the manufacturer's instruction**.** The extracted DNA was amplified using 5 sets of primer sequences (Table 1). For the amplification, 25 µL of the reaction volume consisting of 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, a known positive control DNA from standard cultures of *M.fortuitum* sub spp. *fortuitum* (MTCC929), *M. kansasii* (MTCC3058), *M. smegmatis* (MTCC6), *M. vaccae* (MTCC272) and *M. intracellulare* (MTCC920) (IMTECH, Chandigarh) were also amplified (Slathia *et al*., 2022).

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: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing of primers at 60°C for 1 min except for *M. fortuitum* where it was 62°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min (Park *et al*., 2000). The amplified PCR products were then run on 1.5 % agarose gel electrophoresis and visualized in Gel Documentation System (Alpha Innotech). Amplicons of 152 bp, 571 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* respectively.

PCR-Restriction Fragment Length Polymorphism Analysis (PRA)

Amplification of *hsp*65 gene

A highly conserved heat shock protein 65 portion gene of Mycobacteria was amplified using primer sequence forward (Tbll) 5'- ACCAACGATGGTGTGTCCAT – 3' and reverse (Tbl2) 5'-CTTGTCGAACCGCATACCCT – 3' of 439 bp (Applied Biosystem) (Telenti *et al*., 1993). For the amplification, the total reaction mixture of 25 µL consisted of 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, a known positive control DNA was 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)

After the amplification of *hsp65* gene, two restriction enzymes *BstEII* and *HaeIII* (Promega) were used to digest the amplified PCR product. RFLP of the standard cultures of *M. kansasii, M. smegmatis, M. vaccae, M. fortuitum* and *M. intracellulare* was also carried out. For the digestion of PCR product with *BstEII* and *HaeIII,* in separate test tube 10 µL of PCR product was added directly to the mixture containing 1 µL (5 U) of each enzyme , 2.5 µL of restriction buffer (5 x buffer B) and 11.5 µL of nuclease free water and the mixture was incubated at 60°C for 60 minutes. (Telenti *et al*., 1993). The evaluation of restriction patterns was done using gel electrophoresis. The gel was visualized in Gel Documentation system (AlphaImager 3400HP, AlphaInnotech) and the results were interpreted as per the algorithm used by Telenti *et al.* (1993).

Results and Discussion

Out of 30 fecal samples collected 19 showed the presence of acid-fast bacilli. Among these (n=19), 2(10.52 %) fecal samples were found for *M. fortuitum* by PCR (Figure 1). A similar study was performed by Ghielmetti *et al.* (2017) in which *M. vaccae* along with other NTM was detected from cattle fecal samples.. Kankya *et al.*(2011) reported the presence of *M. fortuitum* from fecal samples along with other NTM from different source.

PCR-RFLP Analysis (PRA)

To distinguish NTM in the DNA isolated from fecal samples in the current study, PCR-RFLP was performed. Among the clinically 19 positive samples 2 (10.52 %) were positive for *hsp*65 gene (Figure 2). The standard cultures (*M. kansasii, M. smegmatis, M. fortuitum, M. vaccae, M. intracellulare*) also showed 439 bp band of *hsp*65 gene. The standard cultures were subjected to restriction enzyme digestion to standardize the protocol (Slathia *et al*., 2022). The *hsp*65 gene positive samples (n=2) were subjected to RE digestion using enzymes *BstEII* and *HaeIII*. The samples gave a specific restriction pattern of bands which helped in the species level differentiation and were identified as *M. fortuitum* having the RFLP pattern as 245/125/80 bp with *BstEII* and 155/135 bp with *HaeIII* (Figure 3). A similar study was performed by Kankya *et al.*(2011) in which *M. fortuitum* along with other NTM were detected from cattle fecal samples.

The *hsp*65 gene was chosen as the target for amplification because it is highly conserved among all the *Mycobacterium* species (Buchanan *et al*.,1987). The *hsp65* PCR-RFLP approach was also used by Taylor *etal*. (1997) and Saifi *et al*. (2013) to distinguish other Mycobacterial species. It is simple to distinguish between well-characterized species with different restriction patterns. This technique can identify mycobacteria upto the species level and can be completed in a single day and does not require hybridization to a panel of species-specific probes for differentiation (Rasool *et al*., 2017). In a study Rasool *et al*. (2017) amplified the 65 kDa heat shock protein (*hsp65*) gene, using PRA in fecal and milk samples and found that 11 out of 200 fecal samples were found positive for *hsp65* gene. Out of these 11 samples, 4 *M. vacca* and 1 *M. kansasii* were confirmed using restriction analysis technique.

Alcaide *et al*. (1997) found five distinct *M. kansasii* patterns in 276 isolates following the PCR-RFLP investigation of the hsp65 gene. Two and six patterns, respectively, were discovered using the hsp65 gene-based approach in *M. fortuitum* and *M. gordonae*.

Conclusions

PCR-RFLP technique was effective and rapid for identifying and discriminating non-tuberculous mycobacteria up to the species level compared to conventional isolation and sequencing, the methodology also expedites the identification of Mycobacteria. Additionally, it was found that *M. fortuitum* may contribute to diagnosis of the diarrheal conditions in cattle and buffaloes.

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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	Park *et al.* (2000)
	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′		

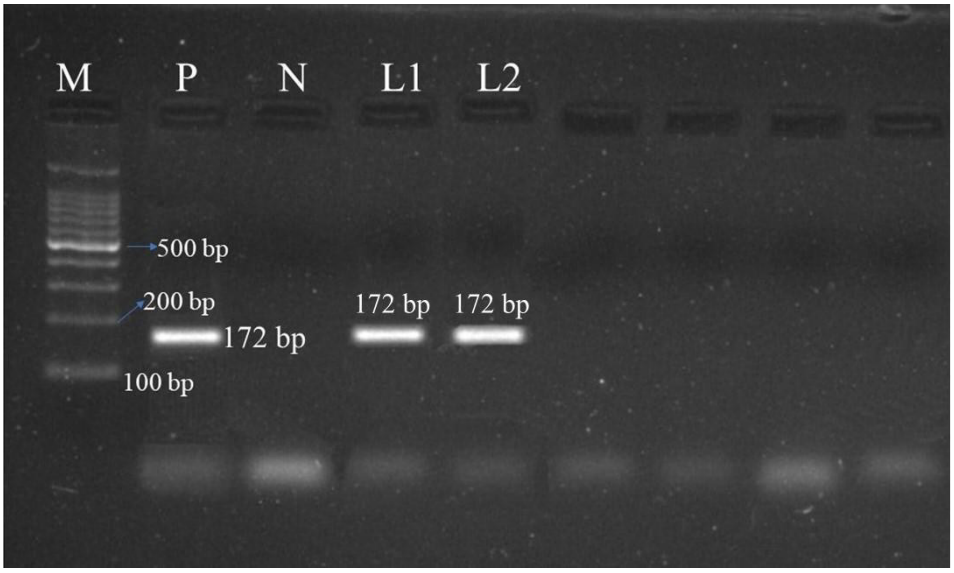

Figure 1: Agarose gel electrophoresis showing an amplicon of ~ 172 bp of M. fortuitum from fecal samples
M: marker (100 bp DNA ladder), P: positive, N: negative
L1, L2: Positive sample for M. *fortuitum* from fecal samples

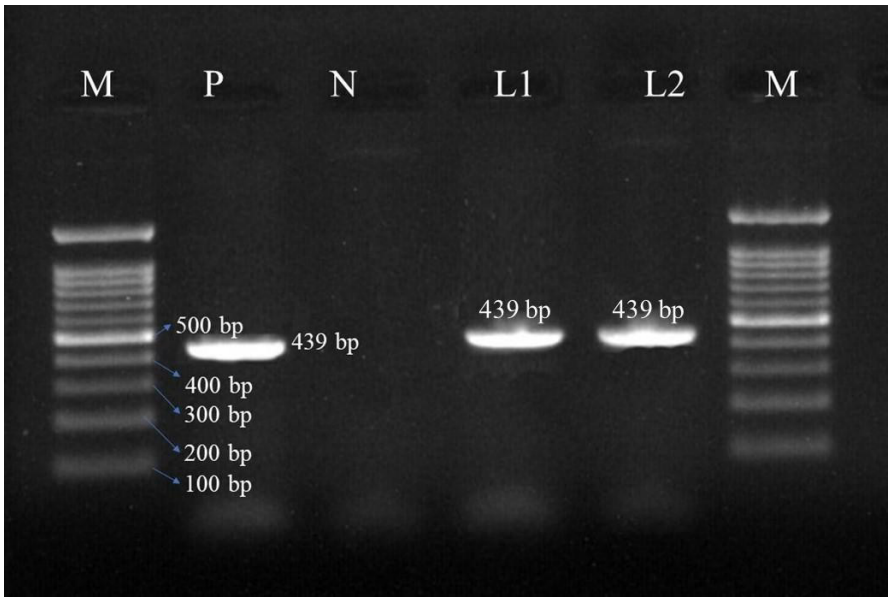

Figure2: Agarose gel electrophoresis showing an amplicon of 439 bp from fecal samples
M: marker (100 bp DNA ladder), P: positive, N: negative
L1, L2: Positive for hsp65 gene PCR (439bp) fecal samples

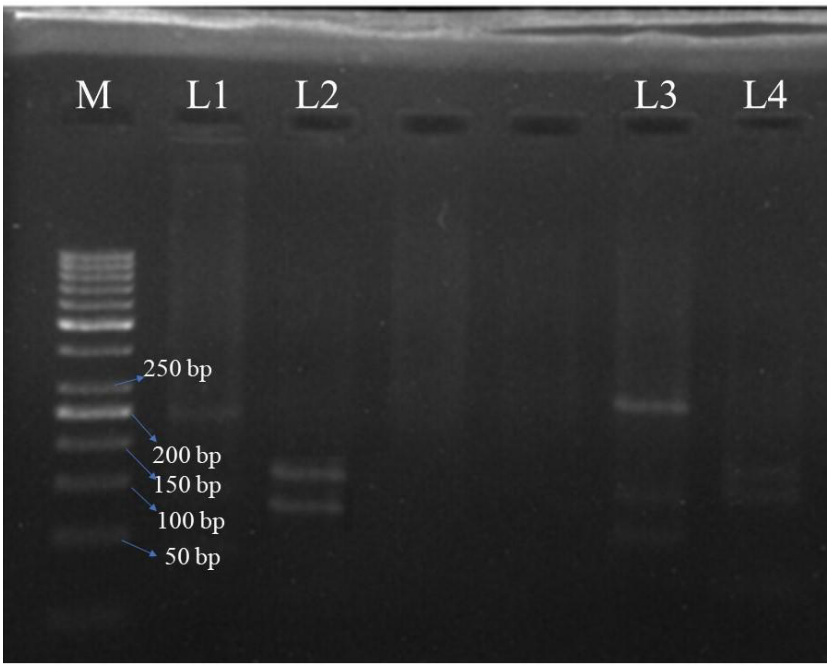

Figure 3: Agarose gel electrophoresis showing RFLP pattern of NTM species in fecal samples
M= 50 bp ladder, L1, L3: *M. fortuitum(BstEIII) (245/125/80), L2, L4: M. fortuitum(HaeIII)*(155/135)

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