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Detection of Nontuberculous Mycobacterial Species in Mastitic Milk Samples from Cattle and Buffaloes by PCR and PCR-Restriction Fragment Length Polymorphisms (PRA)

P. Slathia, D. Narang*, M. Chandra and A. Kumar[1]

Department of Veterinary Microbiology College of Veterinary Science
[1]Department of Veterinary Medicine, College of Veterinary Science
Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 001, India
**E-mail: deeptivet@rediffmail.com*

***Abstract*:** The present study was conducted to detect nontuberculous mycobacterial species (NTM) in milk samples with a history or incidence of mastitis from cattle. A total of 30 milk samples were collected from cattle and buffaloes from dairy farms in Ludhiana. The presence of NTM species was detected by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphisms (PRA) using two restriction enzymes *BstEII* and *HaeIII*. Out of 30 milk samples, *M. smegmatis* (n=2) and *M. kansasii* (n=1) were detected positive by both PCR and PRA. The results show the role on NTM in mastitis in which could also lead to a possible transmission to human being.

***Keywords*:** *M. kansasii*, *M. smegmatis*, Nontuberculous Mycobacteria (NTM), PCR, PRA

Nontuberculous mycobacteria (NTM) are ubiquitous organisms and cause disease in animals and humans (Kankya et al 2011). Some NTM species are zoonotic in nature with a wide range of mammalian hosts (Konuk et al 2007). They are widely distributed in environment mostly found in soil and water sources and have also been considered as potential pathogens for animals and humans (Bercovier and Vincen 2001). NTM species have also been reported to cause cross-reactive immune responses that interfere with the diagnosis of bovine tuberculosis (bTB) in both livestock and wildlife (Gcebe and Hlokwe 2017). In addition, these organisms have been recognized as significant cause of infection in both immunocompetent and immunocompromised patients (Bodle et al 2008). The transmission to humans from the environment can occur by ingestion of contaminated milk, food, water etc. (Konuk et al 2007). Faulty pasteurization, contamination during milking and bottling process may favor the survival of certain bacterial species including nontuberculous mycobacteria (Sgarioni et al 2014). Pulmonary infection caused by nontuberculous mycobacteria are recognized worldwide. Disease including mastitis in cattle and cutaneous mycobacterial granuloma in cats and dogs is caused by number of organisms such as *Mycobacterium chelonei, M. fortuitum, M. kansasii, M. phlei, M. smegmatis* and *M. thermoresistible* (Waters et al 2006). The NTM species can be identified based on their phenotypic characteristics of biochemical testing, pigment production, growth characteristics and colonial morphology but these traditional methods are time-consuming. Thus, more advanced techniques for rapid identification of NTM such as commercial nucleic acid probes, 16S ribosomal DNA sequencing, **(**Butler and Guthertz 2001) and PCR-restriction enzyme pattern analysis (PRA) methods have been developed. The emergence of NTM, as significant environmental pathogens, has attracted more attention (Moore et al 2010). Research on NTM transmission sources and mechanisms will help epidemiologists better understand the diseases carried on by these mycobacteria. Some works show that animal products such as milk, seems to be reservoir of mycobacteria and may pose a risk to the public (Carvalho et al 2009). Keeping in view the possible role of NTM in causing mastitis was evaluated in this study

MATERIAL AND METHODS

Sample collection: Raw milk samples (n=30) from cattle (n=18) and buffaloes (n=12) with a history or incidence of mastitis were collected from dairy farms Ludhiana.

Molecular Diagnosis

Polymerase chain reaction (PCR): DNA from the milk samples was extracted using PowerFood Microbial DNA isolation kit (MoBio). The extracted DNA was then amplified using specific primers for *M. kansasii*, *M. smegmatis*, *M. vaccae*, *M. fortuitum*, *M. intracellulare* (Table 1). In addition to

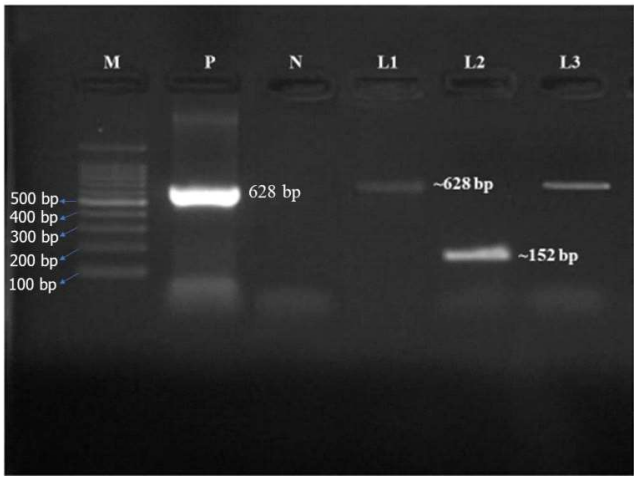
the test sample DNA, a known positive control DNA was also amplified using specific primers in a reaction volume of 25 µl, which also contained 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol/l), 1 µl of reverse primers (10 pmol/l), 2.5 l of nuclease-free water, and 8 µl of DNA template.. 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, 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 and visualized in Gel Documentation System (Alpha Innotech).

PCR-Restriction fragment length polymorphism analysis (PRA): Initial step was the amplification of *hsp*65 gene. For the amplification, the reaction volume of 25 µl was made containing 12.5 µl of GoTaq® Green Master mix, 1 µl each of forward and 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 using specific primers (Table 1). 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 enzymes *BstEII* and *HaeIII* were used to digest the 439 bp amplified PCR product. RFLP of the standard cultures of *M. kansasii, M. smegmatis, M. vaccae, M. fortuitum* and *M. intracellulare* was also done. The digestion of PCR products with two restriction enzymes was done as per Telenti et al (1993). The products were then visualized using 1.5% agarose gel and visualized in Gel Documentation system 40 (AlphaImager 3400HP, AlphaInnotech). The results were interpreted as per Telenti et al (1993) and PRA site (http://app.chuv.ch/prasite/index.html).

RESULTS AND DISCUSSION

Direct detection of NTM from milk samples by PCR : The 30 mastitic milk samples were subjected to PCR for detection of NTM. Out of 30 samples 3 samples were positive. *M. kansasii* (n=1) and *M. smegmatis* (n=2) were detected (Fig. 1). Siqueira et al (2016) also detected *M. smegmatis* from raw milk samples collected from cattle.

Differentiation of NTM by PCR-Restriction fragment length polymorphism analysis (PRA): An efficient and accurate method for identifying various mycobacteria



M: marker (100 bp DNA ladder), P: positive (*M. smegmatis*), N: negative
L1 and L3: Positive sample for *M. smegmatis* from milk samples
L2: Positive sample for *M. kansasii* from milk sample

Fig. 1. Agarose gel electrophoresis showing an amplicon of~ 628 bp of *M. smegmatis* and *M. kansasii*~152 from milk samples

Table 1. Primer sequences of different NTM species and *hsp*65 gene

Organism	Primer	Primer sequence	Product size	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		
*hsp*65	Forward (TbII)	5- ACCAACGATGGTGTGTCCAT – 3	439 bp	Telenti et al (1993)
	Reverse (Tb12)	5-CTTGTCGAACCGCATACCCT – 3		

species is PCR-RFLP (PRA). Based on the separation of DNA segments by restriction endonucleases, PCR-PRA generates fragments that are used to categorize different species of mycobacteria. The nucleotide sequence of locus hsp65 was the method's primary target. In PRA method, 439 bp PCR product of *hsp*65 gene (present in all the mycobacterial species which can be used for the differential identification of Mycobacterial species as per Telenti et al (1993) and Chimara et al (2008) and was amplified and digested with the *BstEII* and *HaeIII* restriction enzymes. The restriction patterns were analyzed for species identification (Saifi et al 2013).

PCR for presence of *hsp65* gene: Among the clinical samples processed, three out of 30 mastitic milk samples (10%), were positive for *hsp*65 gene (Fig. 2). Similar study was conducted by Telenti et al (1993) in which 65-kDa protein (*hsp*65 protein) was used for differentiation of mycobacterial species. Chang et al. (2002) also used *hsp*65 gene PCR product (439 bp) for identification of NTM up to species level. Wang et al (2005) used same procedure for identification of RGM (rapidly growing mycobacteria) from clinical samples.

Restriction enzyme analysis of the *hsp65* gene : The PCR product of *hsp*65 gene amplicon of standard cultures along with the isolates was subjected to digestion with restriction enzyme using *BstEIII* and *HaeIII*. Specific band patterns were seen which helped in the species level differentiation of these *Mycobacteria* (as per the algorithm given by Telenti et al. (1993) and PRA site (http://app.chuv.ch/prasite/index.html). The standard cultures used in this study showed the following RFLP patterns, *M. smegmatis* (*BstEIII*) (235/130/85) and *M. smegmatis* (*HaeIII*) (145/125/60), *M. kansasii* (*BstEIII*) (245/220) and *M. kansasii* (*HaeIII*) (140/105/70), *M. fortuitum* (*BstEIII*) (245/125/80) and *M. fortuitum* (*HaeIII*) (155/135), *M. intracellulare* (*BstEIII*) (245/125/100) and *M. intracellulare* (*HaeIII*) (155/150/60), *M. vaccae* (*BstEIII*) (440) and *M. vaccae* (*HaeIII*) (140/115/70) (Slathia et al 2022). From 30 milk samples, 3 samples were positive for *hsp*65 gene out of which 1 sample was identified as *M. kansasii* having the RFLP pattern as 245/220 bp when digested with *BstEIII* and 140/105/70 bp when digested with *HaeIII* and two samples were identified as *M. smegmatis* having the RFLP pattern as 245/145/85 bp when digested with *BstEIII* and 160/130 bp when digested with *HaeIII* (Fig. 3). A collection of opportunistic mycobacterial species that do not belong to the M. TB complex are known as nontuberculous mycobacteria (Brode et al 2017). Prior research on raw milk and dairy products suggested that some developing nations have high levels of NTM species (Konuk et al 2007).

Similar study was conducted by Sgarioni et al. (2014) detected 15 percent *M. smegmatis* from milk samples**.** Siqueira et al. (2016) described *M. smegmatis* as a cause of pyogranulomatous mastitis and isolated *M. smegmatis* from 68.75% milk samples. *M. smegmatis* is widely found in water and soil and makes up the majority of the NTM. *M. smegmatis* has not been mentioned in disseminated infections, even in immunocompromised animals, despite the possibility that this species is connected to posttraumatic soft-tissue infections (Bohsali et al 2010). *M. kansassii* along with other NTM species *viz*., *M. terrae*, *M. agri* and *M. haemophilum* were present in 35 samples of raw milk tested by in Turkey for

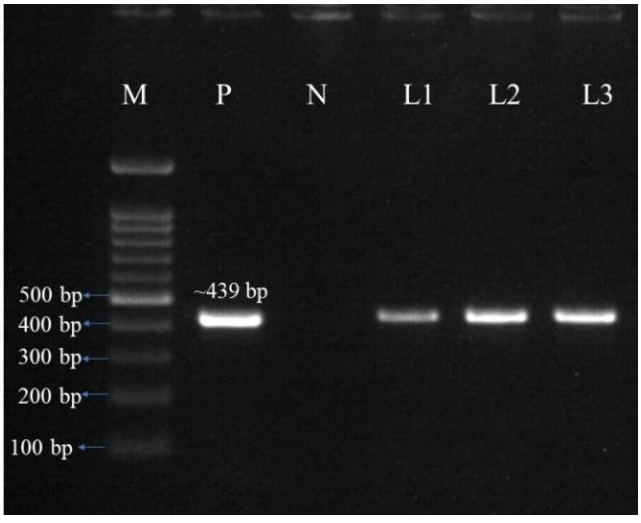
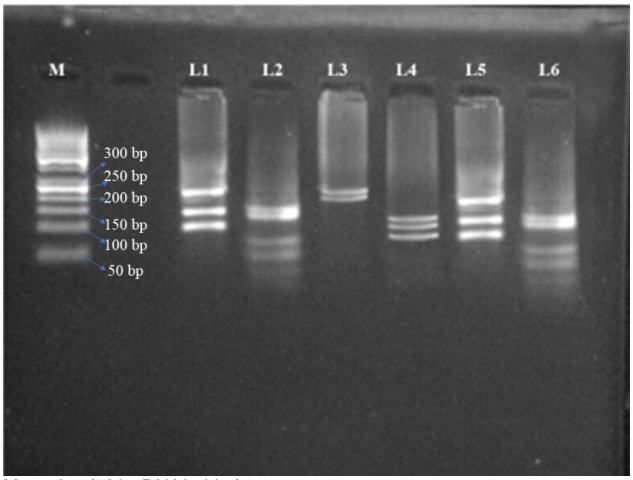


Fig. 2. Agarose gel electrophoresis showing an amplicon of ~439 bp from milk samples



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. smegmatis* (*BstEIII*)(235/130/85)
L6: *M. smegmatis* (*HaeIII*) (145/125/60)

Fig. 3. Agarose gel electrophoresis showing RFLP pattern of NTM species in milk samples

the presence of mycobacteria using phenotypic techniques and verified by PCR-PRA (Konuk et al2007).

CONCLUSIONS

M. smegmatis is the most predominating NTM followed by *M. kansasii* in milk samples. NTM can cause disease in animals and consumption of milk from infected animals remains a risk factor for exposure to NTM. The implementation of measures that prevent milk contamination during and post milking with NTM are also needed to avoid spreading of diseases.

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