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Short Paper

Detection of non-tuberculous mycobacterial species using PCR-RFLP analysis in trans-tracheal washes in cattle and buffaloes with respiratory distress

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Abstract

Background: Bovine tuberculosis (bTB) is a chronic disease of cattle with high economic importance in livestock farming caused by *Mycobacterium bovis* and bears a zoonotic potential. There are some non-tuberculous mycobacteria (NTM) which cause disease similar to bTB and interfere with diagnosis of bTB. Non-tuberculous mycobacteria are saprophytic in nature but some of them may cause pulmonary infections, mastitis, lesions in respiratory tract and lymph nodes of cattle, due to which they are being recognized worldwide and interfere with the diagnosis of bTB. **Aims:** The aim of the study was to detect NTM species from cattle and buffaloes with respiratory distress using biochemical test and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (PRA). **Methods:** A total of 50 trans-tracheal washes were collected from cattle (n=41) and buffaloes (n=9) with respiratory distress. The samples were inoculated on Middlebrook *7H10* media after proper decontamination with 4% NaOH. The isolate obtained was identified by biochemical testing. Extracted DNA from samples and isolate was subjected to PRA which involved *hsp*65 gene amplification (439 bp) and RFLP analysis of amplified product. **Results:** Out of 50 trans-tracheal washes only one isolate of *Mycobacterium kansasii* (n=1) (2%) was obtained which was confirmed by biochemical testing and PRA. *Mycobacterium kansasii* (n=4) (8%), *Mycobacterium intracellulare* (n=1) (2%), and *Mycobacterium vaccae* (n=1) (2%) were identified by PRA. **Conclusion:** The study emphasizes the importance of NTM in animals. Polymerase chain reaction-restriction fragment length polymorphism analysis is a more reliable and rapid method for identification of NTM than conventional methods.

Key words: *hsp*65, Identification, Non-tuberculous mycobacteria, Polymerase chain reaction, Restriction fragment length polymorphism

Introduction

The genus *Mycobacterium* contains more than 199 species and most of them are classified as non-tuberculous mycobacteria (NTM) (Tortoli, 2006). The NTM are "atypical mycobacteria" belonging to species other than those classified in the *Mycobacterium tuberculosis* complex (which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*). Some NTM are saprophytic in nature and some may cause disease in animals and humans (Kankya *et al*., 2011). They are a common inhabitant of environment throughout the world which includes natural and municipal water sources, soil aerosols, protozoans, domestic and wild animals, milk and food products. Their ability to cause disease and its clinical relevance varies globally. Their distribution varies geographically providing information regarding geographic-specific exposure e.g., climate, environment and associated host factors, reflecting regional differences in mycobacterial habitats (Honda *et al*., 2018).

The NTM were classified into four categories on the basis of their growth rates, colony morphology and pigment production in the presence and absence of light and more than 140 NTM species have been identified to-date (Porvaznik *et al*., 2017). For a long time these microscopic organisms were ignored as their clinical significance was obscure and were perceived as simply ecological contaminants (Covert *et al*., 1999). Only after the epidemic of acquired immune deficiency syndrome (AIDS), did NTM attract sudden attention in certain countries (Gopinath *et al*., 2010). Non-tuberculous mycobacteria species have been reported to cause cross-reactive immune responses that interfere with the diagnosis of bovine tuberculosis (bTB) in both livestock and wildlife (Gcebe *et al*., 2017).

The exact mode of transmission of NTM is not known, however, transmission can happen through different modes which includes cutaneous, respiratory (retro-bronchial and pulmonary), due to dust inhalation. Non-tuberculous mycobacteria cause pulmonary infections, because of which they are being perceived

worldwide and are most commonly caused by *Mycobacterium avium* complex (MAC), *M. kansasii*, and *M. abscessus*. These sometimes produce asymptomatic pulmonary infection which does not always equate with active infection and are diagnosed with supportive radiographic and clinical findings (Johnson and Odell, 2014). Disease including mastitis in cattle and cutaneous mycobacterial granuloma in cats and dogs is caused by number of organisms such as *M. chelonei*, *M. fortuitum*, *M. phlei*, *M. smegmatis*, and *M. thermoresistible*. *Mycobacterium kansasii* produces lesions in respiratory tract and lymph nodes of cattle and can be isolated from tissue samples of cattle giving a positive tuberculin skin test (TST) (Waters *et al*., 2010). 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 bTB and paratuberculosis. This study was designed with the role of NTM in animals in mind.

Materials and Methods

Trans-tracheal washes collection

Trans-tracheal washes (n=50), from cattle (n=41) and buffaloes (n=9) with a history or incidence of cases of respiratory affections were collected. Thirty samples were collected from Teaching Veterinary Clinical Complex (TVCC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) and 20 samples were collected from bovine tuberculosis (TB) reactor animals (positive for TST) from dairy farms, Ludhiana.

Procedure

The animals (cattle and buffaloes) were properly restrained. The ventral aspect of the neck (where the tracheal rings could be easily palpated) was selected, surgically prepared and was locally anaesthetized with 2-5 ml of lidocaine. With the help of scalpel blade, a small stab incision was given just through the skin. The steel introduction catheter was then inserted on the ventral midline, while stabilizing the trachea with the other hand and passed into the tracheal lumen between two tracheal rings and a flexible flushing catheter was passed towards the lungs so as to reach thoracic inlet. About 50 ml of sterile saline was injected through the catheter into trachea and was immediately aspirated (to recover at least 1/3rd of the injected saline). After the sample collection, the catheter was withdrawn and the site was dressed with pressure bandage for 24 h. The trans-tracheal washes were then transferred into a sterile container for further examination (Sharma *et al*., 2017).

Decontamination and isolation

Isolation of NTM from trans-tracheal washes was done by inoculating the samples on Middlebrook *7H10* media (HiMedia, India) after following appropriate decontamination step with NaOH (Chang *et al*., 2001). For decontamination, 2 ml of trans-tracheal wash was taken in a 15 ml centrifuge tube and the same amount of 4% NaOH was added into the tube and mixed by vortexing. The tube was incubated at room temperature for 15 min for decontamination and liquefaction of sample. After the incubation period, 6 ml of PBS (pH = 6.8) was added to the mixture and the tube was centrifuged at 2300 × g for 30 min at 4°C. The supernatant was discarded and 100 µL of the sediment was inoculated on Middlebrook *7H10* Media and incubated at 37°C for 6 weeks till growth was observed. The colonies obtained after inoculation of samples on Middlebrook *7H10* Media were subjected to acid-fast (Ziehl Neelsen; ZN) staining and different biochemical tests included niacin test, nitrate reduction test, semi-quantative catalase test, heat stable catalase test, pyrazinamidase test (as per the manufacturer protocol HiMedia; India). The organisms were identified based on these tests.

Molecular diagnosis

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (PRA)

Polymerase chain reaction-restriction fragment length polymorphism analysis uses the PCR to amplify the selected DNA regions. These PCR products are then digested by restriction enzymes.

Amplification of *hsp*65 gene

A highly conserved heat shock protein 65 portion gene of mycobacteria was amplified using PCR primer sequence Forward (Tbll) 5´-ACC AAC GAT GGT GTG TCC AT-3´ and Reverse (Tb12) 5´-CTT GTC GAA CCG CAT ACC CT -3´ giving a product size of 439 bp (Schinnick *et al*., 1987). 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), 1 µL of reverse primers (10 pmol), 2.5 µL of nuclease free water and 8 µL of DNA template along with the test sample DNA, a known positive control DNA and negative control was also amplified. Thermal cycling was performed in research thermal cycler (Eppendorf, Germany) and the cycling conditions were: initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing of primers at 56°C for 45 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Standard cultures of *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. intracellulare*, and *M. vaccae* were also amplified using Tb11 and Tb12 primers.

RFLP

The amplified PCR product (439 bp) was later digested with two enzymes *BstEII* and *HaeIII* (Promega, USA). Restriction analysis of standard cultures of *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. intracellulare*, and *M. vaccae* were also done along with the test samples. 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 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 min. 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 x buffer B) and 11.5 µL of water, the mixture was incubated at 37°C for 60 min. 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; India). A gene ruler DNA™ ladder plus 50 bp (Fermentas, USA) was run along with the test samples. The gel was visualized in Gel Documentation system (AlphaImager 3400HP, AlphaInnotech, USA). 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.

The ethical permission number is GADVASU/2018/IAEC/46/18.

Results

From 50 trans-tracheal washes subjected to isolation, only one isolate was obtained which was later confirmed as *M. kansasii* (Fig. 1) from acid fast staining and biochemical tests (Table 1) and PRA.

Microscopic examination

The isolate obtained from samples inoculated on Middlebrook Media stained with Ziehl-Neelsen's (ZN) staining showed acid fast bacilli (Fig. 2).

PRA

Among the clinical samples processed 7 out of 50 trans-tracheal washes were positive for *hsp*65 gene (Fig. 3) and were compared with the standard cultures of *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. vaccae*, and *M. intracellulare* showing the 439 bp band of *hsp*65 gene (Fig. 4).

Fig. 1: Growth of *Mycobacterium kansasii* isolated from trans-tracheal wash on Middlebrook *7H10* media

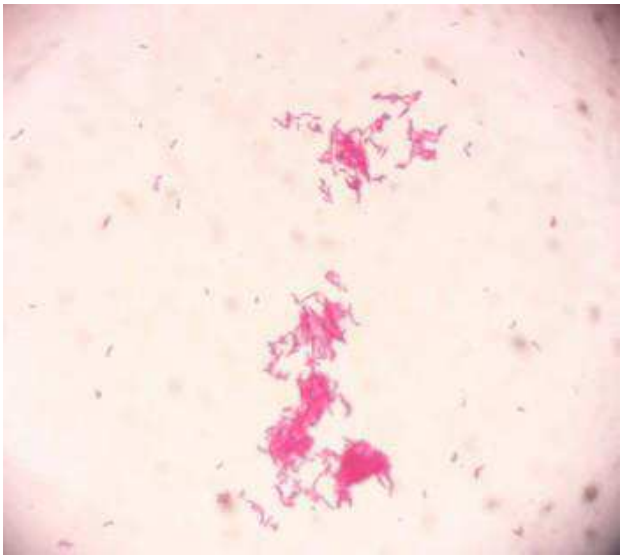

Fig. 2: Clumps of acid fast bacilli from an isolate obtained from trans-tracheal wash

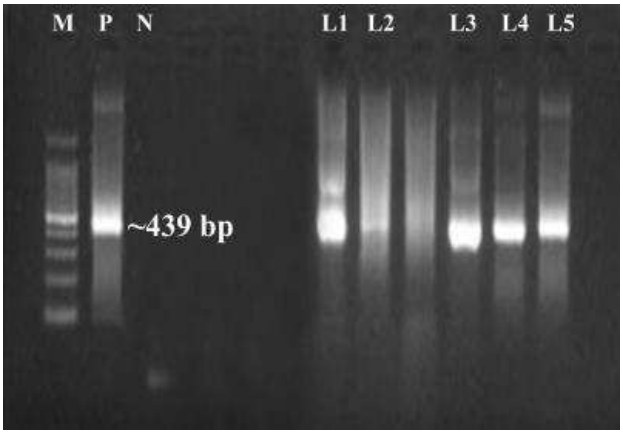

Fig. 3: Agarose gel electrophoresis showing an amplicon of ~ 439 bp from trans-tracheal washes. M: Marker (100 bp DNA ladder), P: Positive standard, N: Negative, and L1-5: Positive sample for *hsp*65 gene

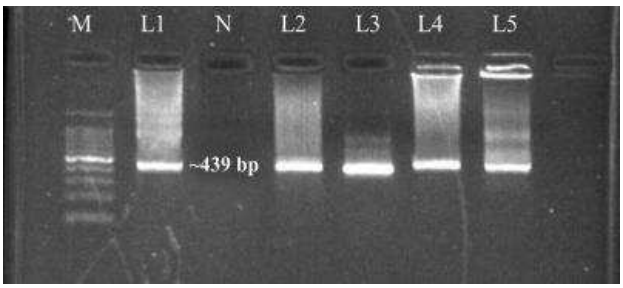

Fig. 4: Agarose gel electrophoresis showing an amplicon of ~ 439 bp from standard cultures (*M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. vaccae*, and *M. intracellulare*). M: Marker (100 bp DNA ladder), N: Negative, and L1-5: Positive standard for *hsp65* gene PCR (439 bp)

Table 1: Biochemical test of isolate

Isolate	Nitrate	Niacin	Semi-quantative catalase	Heat stable catalase	Pyrazinamidase reduction	Thiophene-2-carboxylic acid hydrazide
Mycobacterium kansasii	+	+	Weakly +	+	-	-

RFLP of tracheal lavage samples

From 50 tracheal lavage, 7 samples were positive for *hsp*65 gene amplification from which 4 samples (two from Tb reactor animals) were digested by enzymes identified as *M. kansasii* (8%) having the RFLP pattern as 245/220 bp when digested with *BstEIII* and 140/105/70 bp when digested with *HaeIII* (Fig. 5). One sample was identified as *M. intracellulare* (2%) having RFLP pattern as 245/125/100 bp with *BstEIII* and 155/150/60 bp with *HaeIII*. And one sample was identified as *M. vaccae* (2%) having RFLP pattern of 440 bp *BstEIII* and 140/115/70 bp with *HaeIII* (Table 2, Fig. 6) and one sample was not digested by enzymes thus remained unidentified. These were also compared with the standard cultures of *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. vaccae*, and *M. intracellulare* showing different RFLP patterns (Fig. 7).

Table 2: Identification of NTM species from trans-tracheal washes based on the RFLP pattern

*hsp*65 positive animals	Enzyme		Species identified
	BstEIII	*HaeIII*	
4	245/220	140/105/70	*Mycobacterium kansasii*
1	325	140/115/70	*Mycobacterium vaccae*
1	245/125/100	155/150/60	*Mycobacterium intracellulare*
1	-		Unidentified

Total No. of RFLP positive samples = 7

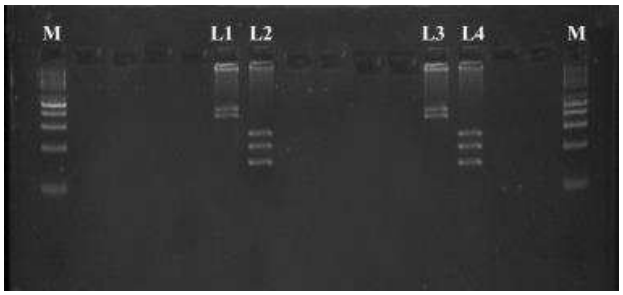

Fig. 5: Agarose gel electrophoresis showing RFLP pattern of NTM species in trans-tracheal washes. M: Marker (50 bp DNA ladder), L1: *M. kansasii* (*BstEIII*) (245/220), L2: *M. kansasii* (*HaeIII*) (140/105/70), L3: *M. kansasii* (*BstEIII*) (245/220), and L4: *M. kansasii* (*HaeIII*) (140/105/70)

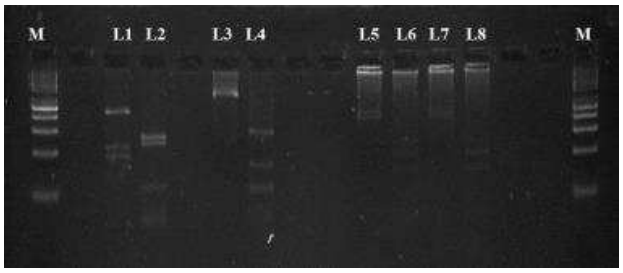

Fig. 6: Agarose gel electrophoresis showing RFLP pattern of NTM species in trans-tracheal washes. M: Marker (50 bp DNA ladder). L1: *M. intracellulare* (*BstEIII*) (245/125/100), L2: *M. intracellulare* (*HaeIII*) (155/150/60), L3: *M. vaccae* (*BstEIII*) (440), L4: *M. vaccae* (*HaeIII*) (140/115/70), L5: *M. kansasii* (*BstEIII*) (245/220), L6: *M. kansasii* (*HaeIII*) (140/105/70), L7: *M. kansasii* (*BstEIII*) (245/220), and L8: *M. kansasii* (*HaeIII*) (140/105/70)

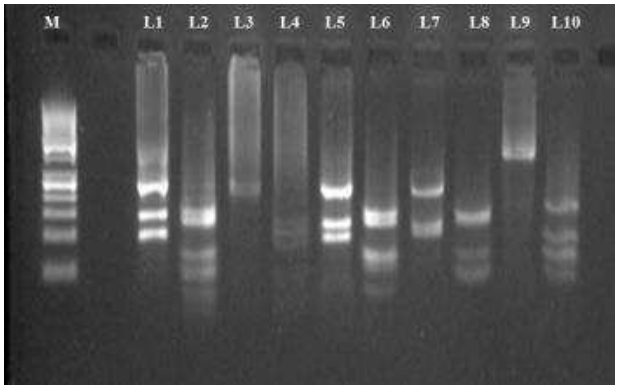

Fig. 7: Agarose gel electrophoresis showing RFLP pattern of standard cultures (*M. smegmatis*, *M. kansasii*, *M. fortuitum*, *M. intracellulare*, and *M. vaccae*). M: Marker (50 bp DNA ladder), L1: *M. smegmatis* (*BstEIII*) (235/130/85), L2: *M. smegmatis* (*HaeIII*) (145/125/60), L3: *M. kansasii* (*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), and L10: *M. vaccae* (*HaeIII*) (140/115/70)

Discussion

In the present study only one isolate was obtained out of 50 samples. The culture isolation is considered as the gold standard test. Culture of *Mycobacterium* is a slow procedure because most *Mycobacterium* species require >6 weeks to grow. The decontamination procedures followed during the sample processing for isolation also decreases the number of viable mycobacteria. Overgrowth of contaminating bacteria and fungus pose a problem while culturing the samples. These factors result in less number of isolates and lesser number of culture positive animals (Whittington and Sergeant, 2001). Telenti *et al*. (1993) reported that mycobacterial species could be identified using 439 bp 65-kDa protein (*hsp*65 protein). Early diagnosis of mycobacterial infection and differentiation of mycobacterial strains from bronchoalveolar lavage specimens could be done by PRA (Cho *et al*., 2009). Similar study was conducted by Nour-Neamatollahie *et al*. (2017) in which (PRA) of the *hsp65* gene was done on clinical samples (sputum, bronchial lavage, and skin samples) of Tb suspected patients, as a result, the majority of NTM were obtained along with *M. bovis* and *M. tuberculosis*. The most frequently detected *Mycobacterium* species were *M. kansasii*, which was isolated in 5 (45.4%) out of 11 patients with NTM pulmonary disease.

The number of *M. kansasii* identified was more than any other NTM *viz. M. intracellulare*, *M. vaccae*, *M. smegmatis*, and *M. fortuitum* in trans-tracheal washes taken from animals with respiratory distress and may interfere with Tb diagnosis in tuberculin skin testing. Polymerase chain reaction-restriction fragment length polymorphism analysis was found to be a good technique for the species level identification and differentiation of NTM. This technique is rapid (requires less time) than other techniques. However, it needs to be studied in more

number of animals.

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Conflict of interest

There is no conflict of interest.

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