



## Screening of Non Tuberculous Mycobacterium Species from Farm Environment

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### ABSTRACT

The ubiquitous presence of NTM species in the environment is one factor which leads to misdiagnosis, treatment failure of tuberculosis throughout the world. The aim of the present study was to screen samples from farm environment to detect the occurrence of NTM species and to analyze their antimicrobial resistance pattern. A total of 218 samples of water, soil, milk, faeces and sewage were collected and processed as per standard isolation protocols for NTM species. Further, the isolates were subjected to biochemical characterization and molecular confirmation was done by PCR targeting genus specific primers like 16SrRNA and *hsp* 65. For species identification, the PCR products were sequenced and assessed the genetic relatedness. Antimicrobial resistance of the isolates was also studied by broth microdilution and disc diffusion method. The present study showed the presence of NTM species from farm settings and the isolates were found to be sensitive to antimycobacterial drugs.

### HIGHLIGHTS

- *M. chelonae* and *M. abscessus* were identified from farm settings.
- Sensitivity to first line antimycobacterial drugs

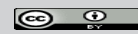
**Keywords:** Non Tuberculous Mycobacteria, Acid Fast Staining, 16SrRNA, *hsp* 65, Antimicrobial resistance.

Non Tuberculous Mycobacteria are opportunistic pathogens, causing mostly TB like pulmonary diseases largely in immunocompromised patients or with pre existing disease conditions and extra pulmonary diseases. NTM incidence is highly underestimated because of similar diagnostic patterns with *Mycobacterium tuberculosis* and *Mycobacterium bovis*. NTM represents more than 160 species which are commonly found in soil and water systems with varying degree of virulence leading to diverse clinical features. From many studies it is evident that NTM species are isolated from domestic and wild animals, pigs, domestic fowls, captive, exotic and wild birds, their excretions including faeces and also from bedding materials. The presence of NTM is documented from perishable foods and vegetables. Human to human transmission of NTM was not reported till date (Sharma *et al.*, 2020). NTM infections can be detected from the clinical and environmental samples by conventional

methods such as acid fast staining, culture in media and based on biochemical parameters. Molecular confirmation is primarily done by PCR targeting various identification markers *viz.*, 16S rRNA, *hsp65* and *rpoB* genes or multi locus sequence typing (MLST) and DNA hybridization techniques. Antimicrobial resistance is also evidenced in NTM organisms. NTM species also share same antimicrobial resistance patterns with the *M. tuberculosis* and *M. bovis* including MDR and XDR. From several studies, it has been noted that NTM species are discrete in susceptibility mechanisms from *M. tuberculosis*. Literature survey has showed the ubiquitous presence of NTM species in various ecosystems. In India, the actual bio burden of NTM species is lacking. The present

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research was envisioned to study the occurrence of NTM from both organized and unorganized farm environment to identify the most predominant NTM species circulating in the study area and to identify the antimicrobial pattern among the circulating isolates.

## MATERIALS AND METHODS

A total of 218 samples from livestock and farm environment (n=218) including water (50), soil (50), milk (55), faeces or dung (50) and sewage (13) were collected in a sterile container under aseptic conditions and transported to laboratory under refrigerated conditions and further processed as per Yajko *et al.* (1995). The samples were subjected to decontamination protocols using 5% oxalic acid – 4% NaOH and 1% CPC and incubated for 30 minutes and 24 hours respectively at 37°C, along with VAN supplement. Decontaminated samples were subjected to acid fast staining and culture isolation was done using Herrold's Egg yolk media and Middlebrook 7H9 broth and subsequently cultured on MB 7H10 agar. The presumptive colonies were subjected to biochemical characterization *viz.* growth on MacConkey agar, growth in 5% NaCl, iron re-uptake from the medium, catalase test and Thiophen – 2 Carboxylic Hydrazide test. The presumptive NTM isolates were then confirmed by Polymerase Chain Reaction targeting 16SrRNA and *hsp* 65 genes and the PCR products from the 16SrRNA was sequenced. NTM isolates obtained in the study were subjected to Antimicrobial testing by broth microdilution method for the first line antimycobacterial drugs like rifampicin, isoniazid, ethambutol and pyrazinamide. Disc diffusion method was carried out against amikacin, azithromycin, cotrimaxazole, ciprofloxacin, enrofloxacin, imipenam, doxycycline and rifampicin and the sensitivity pattern and resistance pattern were recorded.

## RESULTS AND DISCUSSION

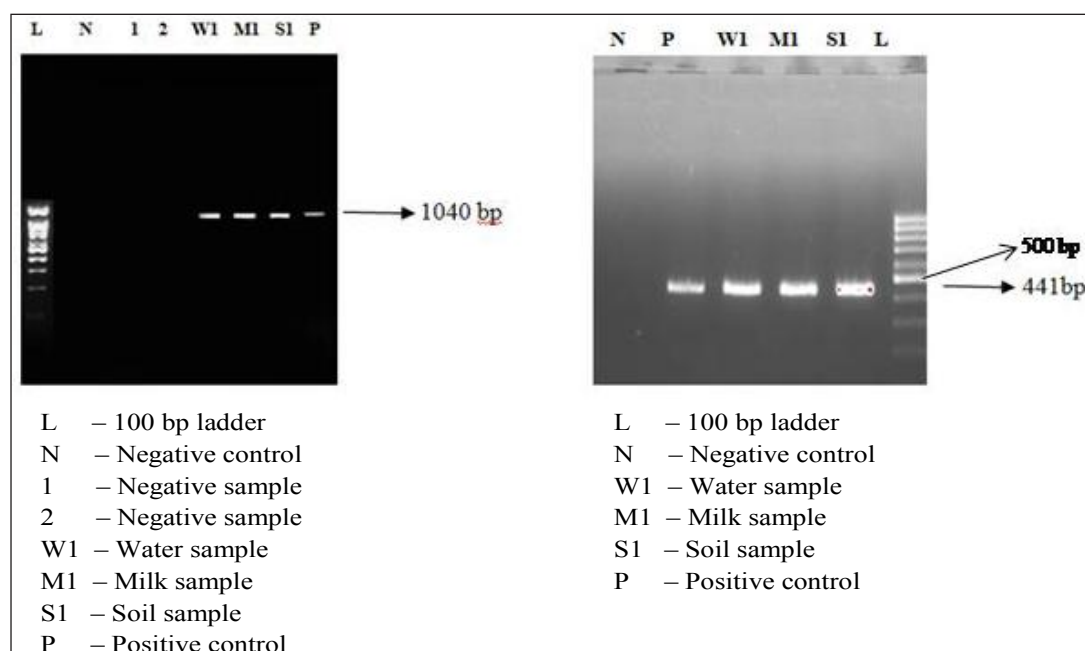
In this study, decontamination protocols were done with all the 218 samples by utilizing both 5% Oxalic Acid – 4% NaOH method and 1% CPC. Of the two methods, growth was noticed only in samples decontaminated by CPC whereas no growth was noticed in samples decontaminated by Oxalic acid – NaOH. Our study has clearly indicated that CPC is an efficient decontaminant in the process of culturing NTM species along with VAN

supplement. Our results are in accordance with the results of Pardini *et al.* (2005) who have also found that 0.5 % CPC decontaminated samples can be preserved for longer time and it reduced higher number of contaminants with positive cultures than 1% NaCl with NALC (1% NaOH).

After decontamination, all the samples were screened for Acid Fast Bacilli by Acid Fast Staining. Of the 218 samples only 6 samples (2 water samples, 1 milk sample, 1 soil sample, 2 faecal or dung sample) were found to be harbouring Acid Fast Bacilli. All the 6 samples were further propagated in Herrold's egg yolk media and Middlebrook 7H9 broth. In this study, we could notice no growth with Herrold's egg yolk media and growth was noticed only in Middlebrook 7H9 broth and 4 samples were positive which were named M1, S1, W1 and W2. In Middlebrook 7H9 broth, variation in growth pattern, incubation period and pigmentation was noticed with the isolates. Turbidity without pigmentation was visualized within 5 days in milk, soil and one of the water samples whereas in another water sample, turbidity was noticed with pigmentation within 14 days of incubation. Our result has documented that isolates obtained in the present study *viz.* water sample (W1), milk sample (M1) and soil sample (S1) were showing turbidity, growth within 5 days and there was absence of pigmentation. Based on this criterion, we could classify our isolates as non chromogenic NTM species which falls under RGM (Bhalla *et al.*, 2018). On the other hand, water sample (W2) showed growth, turbidity and reddish orange color pigmentation over an incubation period of 14 days. Based on this parameter, we could classify our isolate as chromogenic NTM species which falls under SGM.

Samples which showed growth in liquid media were subcultured in MB 7H10 agar. Growth of white buffy colonies in the samples W1, M1 and S1 was indicative of non chromogenic RGM and the lemon yellow colonies in the sample W2 was of chromogenic SGM based on the incubation period. Subculturing in MB 7H10 agar also suggested that the species might be of *M. fortuitum*, *M. abscessus* and *M. chelonae* in RGM and some chromogenic SGM. The present study correlates with the study of Ahmed *et al.* (2013) in which both RGM and SGM were isolated subculturing on MB 7H10 agar.

Biochemical characterization with the isolates obtained in the study yielded varying results. All the 4 isolates were subjected to various biochemical test parameters and the



**Fig. 1:** PCR assay targeting *16SrRNA* gene

**Fig. 2:** PCR assay targeting *hsp65* gene

results were correlated with Bhalla *et al.* (2018) who have described the various methods for distinguishing RGM and SGM species based on biochemical test. With regard to NTM species identification by biochemical tests, it has been reported that it cannot be reliable as it leads to inconclusive results and some of the isolates could not be identified by biochemical tests (Narang *et al.*, 2015). Based on the conventional tests it was noticed that the occurrence of NTM in water, milk and soil were recorded as 4%, 1.8% and 2% respectively.

Molecular confirmation was carried out by PCR assay targeting 16SrRNA and *hsp 65* which revealed that all the 4 isolates were confirmed as *Mycobacterium* spp. with an amplicon of 1040 bp for 16SrRNA (Fig. 1) and 441 bp for *hsp 65* (Fig. 2). Results of our PCR assay targeting 16SrRNA and *hsp65* confirmed the presence of *Mycobacterium* sp in water, milk and soil with a percent positivity of 4%, 1.8% and 2.0% respectively. Results of our PCR assay confirmed all the isolates as positive for *Mycobacterium* spp. which has already shown growth and tested for biochemical tests is an indication of high sensitivity of the employed PCR assay and it clearly indicated that the assay could be used for routine screening which will aid in the identification of NTM organisms. The PCR products obtained by targeting 16SrRNA were sequenced, assembled, annotated and

BLAST analysed at NCBI. The milk and soil isolate was identified as *M. chelonae* and *M. abscessus* respectively and the two water samples were identified as uncultured bacteria which is a common phenomena in environmental samples. Our study was in agreement with Sonia *et al.* (2014) in which they isolated *M. Chelonae* in milk and with the results of Thomson *et al.* (2013) and Moore *et al.* (2020) in which they isolated *M. abscessus* from water samples. Antimicrobial study by broth microdilution method revealed all the isolates were sensitive to all the first line antimycobacterial drugs. Disc diffusion method showed that *M. chelonae* was resistant to iminepam and intermediately resistant to azithromycin but sensitive to all the other drugs. *M. abscessus* was resistant to amikacin, arithromycin and intermittently resistant to imipenam and ciprofloxacin but sensitive to all the other drugs.

## CONCLUSION

This study identified the occurrence of NTM species like *M. chelonae* and *M. abscessus* from milk and soil samples is an indication of presence of these organisms in farm settings. Considering the potential of this pathogen in relation to human and animal infections there is a need for devising appropriate control strategies to prevent the

transmission and occurrence at farm level and to safeguard the health of farm workers, general public with special concern to immuno compromised individuals.

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