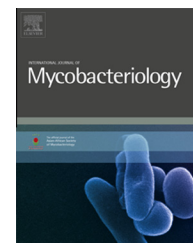


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## Occurrence of nontuberculous mycobacteria in aquatic sources of Sri Lanka

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### ABSTRACT

Nontuberculous mycobacteria (NTM) have been reported to cause opportunistic infections with increasing frequency, especially in immunocompromised patients. Water plays a major role in the epidemiology of nontuberculous mycobacterial infection in humans, as it is one of the natural sources for transmission of this group of organisms. The current study focused on determining the occurrence of NTM in different aquatic sources of Sri Lanka by using phenotypic tests and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of the *rpoB* gene. Of 290 water samples, 45 (15%) were positive for NTM on culture. The percentage of mycobacteria identified at species level by phenotypic tests and PCR–RFLP analysis were 44% (20/45) and 73% (33/45), respectively. The frequency of isolation of mycobacteria from aquarium water, surface water, ground water and chlorinated water were 29% (20/70), 26% (20/76), 5% (4/76) and 1% (1/68), respectively. Eleven different NTM species were identified by PCR–RFLP analysis. *M. fortuitum* type I was the most frequently isolated species from all the four water sources. The current study suggests that water is an environmental source harboring NTM, a potential public health hazard especially for those with immunodeficiency.

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### Introduction

Nontuberculous mycobacteria (NTM) is a collective term for different species of the genus *Mycobacterium* that do not belong to the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. pinnipedii*, and *M. tuberculosis*), *M. leprae*, and *M. lepraemurium*, which are generally not found in the environment [12,20]. NTM are widely distributed in the environment with high isolation

rates worldwide [6]. When the last American Thoracic Society (ATS) statement about NTM was prepared in 1997, there were approximately 50 NTM species that had been identified. Currently, more than 125 NTM species have been cataloged [10]. There has been a dramatic recent increase not only in the total number of mycobacterial species but also in the number of clinically significant species. Organisms can be found in soil and water, including both natural and treated water sources [10].

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NTM normally exist as saprophytes in the natural environment while some are opportunistic pathogens of humans, animals, poultry and fish [9,7]. There is no evidence of animal-to-human or human-to-human transmission of this group of organisms [6]. Human disease is suspected to be acquired from environmental exposures, although the specific source of infection usually cannot be identified [21]. NTM may cause both asymptomatic infection and symptomatic disease in humans. According to Griffith et al. [10] the current unprecedented high level of interest in NTM disease is the result of two major recent trends: the association of NTM infection with Acquired Immune Deficiency Syndrome (AIDS) and recognition that NTM lung disease is encountered with increasing frequency in the non-AIDS population. Furthermore, NTM infections are emerging in previously unrecognized settings, with new clinical manifestations. Another major factor contributing to increased awareness of the importance of NTM as human pathogens is improvement in mycobacteriology laboratory methodology, resulting in enhanced, rapid and more accurate identification of NTM from clinical specimens [10].

In particular, NTM species can survive in numerous water sources, including waste water, surface water, recreational water, ground water and chlorinated water with a wide range of pH and temperature conditions [2,5,3,13,14]. Hence, water is considered a significant vehicle for the transmission of NTM. Therefore, examination of the degree of mycobacterial contamination in different aquatic sources is important, in order to implement appropriate measures to control the degree of contamination. The current study focused on determining the occurrence of NTM in different aquatic sources of Sri Lanka. Surface, ground, aquarium and chlorinated water samples collected from all the 25 districts of Sri Lanka were decontaminated and cultured on Lowenstein–Jensen (LJ) medium and the resulting colonies were identified based on their phenotypic characteristics and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of *rpoB* gene.

## Materials and methods

A total of 302 water samples were collected from all 25 districts of Sri Lanka, from four different types of sources namely; surface water, ground water, aquarium water and chlorinated water, from different sites representing each source. From each site one or two samples were collected according to standard methods in 250 ml sterile sample bottles. Samples were transferred to the laboratory and were kept at 4 °C until analysis. All the samples were analyzed within 48 h of collection.

The samples were filtered to discard impurities, decontaminated with 4% NaOH and inoculated on LJ medium in triplicates to culture Mycobacteria. The cultures were incubated at 25, 37 and 42 °C under aerobic conditions and examined for growth weekly up to eight weeks and the culture positive isolates were tentatively identified as mycobacteria based on their acid fast properties by Ziehl–Neelsen staining. Acid fast colonies were sub cultured and their phenotypic characteristics were analyzed; growth rate, photoreactivity, growth temperature, nitrate reduction, tween 80 hydrolysis,

5% NaCl tolerance, iron uptake, growth on MacConkey without crystal violet, catalase and urease production.

The DNA was extracted from the *Mycobacterium* cultures grown on LJ medium using a commercial kit (QIAmp<sup>®</sup> DNA mini kit) and quantified using Qubit<sup>®</sup> 2.0 Fluorometer. PCR was carried out in a final volume of 50 µl consisting of 1 µl of template DNA, 40 pmol each of primers RPO5' (5'-TCAAGGA-GAAGCGCTACGA-3') and RPO3' (5'-GGATGTTGATCAGG-GTCTGC-3'), 5× PCR reaction buffer, 4 µl of MgCl<sub>2</sub>, 200 mM deoxyribonucleoside triphosphate and 1U of Taq DNA polymerase (PROMEGA, USA). The thermocycling parameters consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min with a final extension for 7 min. After confirmation of the 360-bp amplicons by agarose gel electrophoresis, the PCR products were digested with the restriction enzymes *MspI* and *HaeIII*. The restricted fragments were separated on high resolution agarose gel (3%) followed by visualization under UV illumination and the RFLP patterns were interpreted according to an algorithm described by Lee et al. [15]. Limit of sensitivity of PCR assay was determined by serial dilutions of the extracted *M. fortuitum* DNA. PCR was run for each dilution from 10<sup>-1</sup> to 10<sup>-7</sup> according to the protocol given above. The PCR products were subjected to gel electrophoresis with a 100 bp DNA size marker (Fermentas, UK) and visualized under UV illumination.

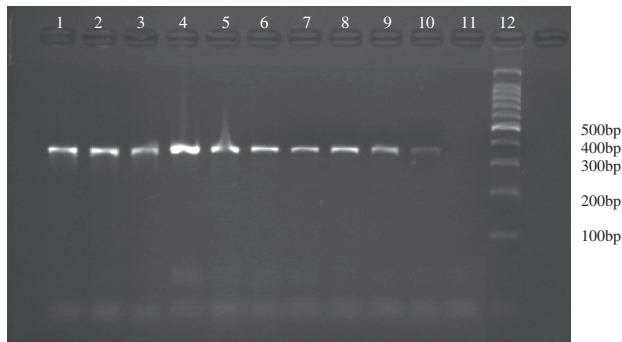
## Results

Of 302 water samples analyzed, mycobacteria were not detectable in 12 surface water samples owing to the overgrowth of background organisms. In all, 45 (15%) were positive for NTM on culture from the 290 water samples.

The number of samples collected from the districts varied from 4 to 19. Water samples collected from 19 districts yielded positive results for NTM, namely Kurunegalla (*n* = 8), Colombo (*n* = 3), Kandy (*n* = 3), Kegalle (*n* = 3), Matale (*n* = 3), Matara (*n* = 3), Puttalam (*n* = 3), Badulla (*n* = 2), Batticaloa (*n* = 2), Galle (*n* = 2), Hambanthota (*n* = 2), Mannar (*n* = 2), Monaragala (*n* = 2), Rathnapura (*n* = 2), Ampara (*n* = 1), Anuradhapura (*n* = 1), Gampaha (*n* = 1), Trincomalee (*n* = 1) and Vavuniya (*n* = 1). NTM were not present in the water samples collected from Jaffna, Killinochchi, Kalutara, Mullativu, Nuwara-Eliya and Polonnaruwa. Frequency of isolation of NTM varied with the water sources tested and the values for aquarium water, surface water, ground water and chlorinated water were 29% (20/70), 26% (20/76), 5% (4/76) and 1% (1/68), respectively.

Forty-five isolates belonging to the genus *Mycobacterium* were isolated from the collected water samples. Twenty of the isolates were identified up to the species level by phenotypic tests, namely *M. fortuitum*, *M. phlei*, *M. gordonae*, *M. scrofulaceum*, *M. chelonae*, *M. marinum*, and *M. parafortuitum*. Furthermore, 25 *Mycobacterium* isolates remained unidentified since their phenotypic characteristics could not be matched with the existing schemes.

The sample DNA was quantified using Qubit<sup>®</sup> 2.0 Fluorometer, and the reported DNA concentration was 5.2 ng/µl. The PCR assay was capable of detecting up to 10<sup>-6</sup> dilution of *Mycobacterium* DNA (Fig. 1). Therefore the detection limit was calculated to be 5.2/1,000,000 i.e. 0.0000052 ng.3 µl = 0.0156 pg.

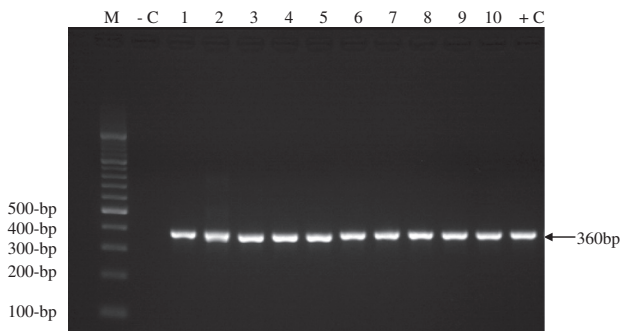


**Fig. 1** – Agarose gel electrophoresis of PCR products of DNA dilutions. Lane 1: *M. fortuitum*; Lane 2: *M. phlei*; Lane 3: *M. marinum*; Lane 4: 15.6 ng/μl; Lane 5: 1.56 ng/μl; Lane 6: 0.156 ng/μl; Lane 7: 0.0156 ng/μl; Lane 8: 0.00156 ng/μl; Lane 9: 0.156 pg/μl; Lane 10: 0.0156 pg/μl; Lane 11: 0.00156 pg/μl; and Lane 12: 100 bp marker.

When subjected to PCR, all 45 isolates yielded the expected 360-bp fragment (Fig. 2) and 73% (33/45) of the isolates provided comparable RFLP patterns to the known species. Eleven species of NTM were identified by PCR-RFLP, namely *M. fortuitum* type I ( $n = 8$ ), *M. fortuitum* type II ( $n = 4$ ), *M. phlei* ( $n = 6$ ), *M. marinum* ( $n = 3$ ), *M. gordonae* type I ( $n = 2$ ), *M. gordonae* type II ( $n = 3$ ), *M. malmoense* ( $n = 2$ ), *M. terrae* ( $n = 2$ ), *M. avium* ( $n = 1$ ), *M. smegmatis* ( $n = 1$ ), and *M. celatum* type II ( $n = 1$ ). The RFLP profiles of 12 NTM isolates did not match any known mycobacterium species.

Of the identified positive isolates, potentially human pathogenic mycobacteria (*M. fortuitum*, *M. marinum*, *M. malmoense*, *M. avium* and *M. celatum*) accounted for 57% (19/33) while the non-pathogenic species (*M. phlei*, *M. gordonae*, *M. smegmatis* and *M. terrae*) accounted for 42% (14/33). *M. fortuitum* type I was the most frequently isolated organism from all four water sources (8/45, 18%) and the predominant NTM isolate of aquarium water (8/17, 47%). *M. phlei* represented 36% of the isolates from surface water. *M. smegmatis* was the only NTM species recovered from chlorinated water samples.

Fig. 3a shows the *MspI* digestions of 10 strains and Fig. 3b shows the *HaeIII* digestions of the same strains run on a 3% high resolution agarose gel. Lane 1: DNA size markers (50 bp) Lane 2: Not assigned, 3: *M. phlei*, 4: *M. fortuitum* type



**Fig. 2** – PCR products of 360-bp DNA amplified from the *rpoB* gene of nontuberculous mycobacteria. Lanes: M: DNA size marker (100 bp); – C: Negative control; 1–10: Samples; + C: Positive control.

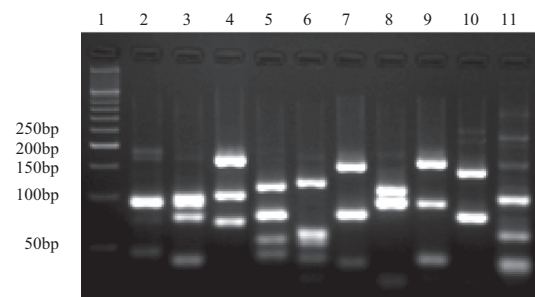
I, 5: *M. malmoense*, 6: *M. terrae*, 7: *M. gordonae* type II, 8: *M. avium*, 9: *M. gordonae* type I, 10: Not assigned and 11: *M. marinum*.

## Discussion

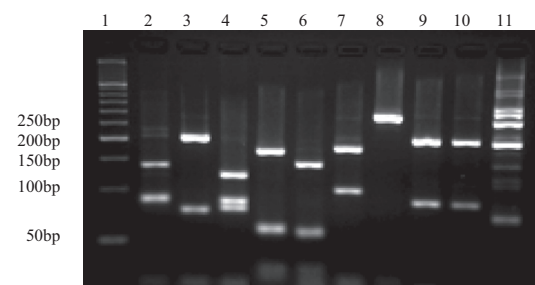
Nontuberculous mycobacteria are common saprophytes in all ecosystems including diverse waters [14]. Since these mycobacteria have recently been reported to be implicated in variety of human diseases [3], their occurrence and species identification in waters have been a major concern for public health. However, little information is available for their occurrence in waters owing to their slow growth and lack of appropriate detection methods. In this study, phenotypic and molecular methods were combined to accurately detect and identify NTM in different water sources of Sri Lanka.

In the present study, out of 302 water samples collected from various geographical locations in Sri Lanka, 45 (15%) samples were positive for NTM on culture and eleven species namely, *M. fortuitum* type I ( $n = 8$ ), *M. fortuitum* type II ( $n = 4$ ), *M. phlei* ( $n = 6$ ), *M. marinum* ( $n = 3$ ), *M. gordonae* type I ( $n = 2$ ), *M. gordonae* type II ( $n = 3$ ), *M. malmoense* ( $n = 2$ ), *M. terrae* ( $n = 2$ ), *M. avium* ( $n = 1$ ), *M. smegmatis* ( $n = 1$ ), and *M. celatum* type II ( $n = 1$ ) were identified indicating that the NTM are common in water sources of Sri Lanka.

No selective medium is available for mycobacteria, and the associated flora consisting of bacteria and fungi, has to be eliminated by decontaminating procedures involving alkali or acid treatment. Mycobacteria are more resistant to these treatments than bacteria and fungi, but they are not fully resistant. Decontamination procedure using 4% NaOH was performed to remove or reduce the growth inhibition by other microorganisms present in the water samples. Although 4% NaOH is widely used to decontaminate water samples,



a.: *MspI* digestion



b.: *HaeIII* digestion

**Fig. 3** – PCR-RFLP pattern of *rpoB* gene of nontuberculous mycobacteria with *MspI* (a) and *HaeIII* (b) digestion; an example of typical results of RFLP analysis.

several authors have shown that only a small percentage of NTM survive pretreatment with higher concentrations (>1%) of NaOH [8,18,16]. Therefore, it is safe to assume that, the water samples which had low mycobacterial concentration than the limit of sensitivity of the isolation method, probably gave a negative result.

It was apparent that the occurrence of NTM in aquarium water (29%) and surface water (26%) was considerably high. This may be attributed to higher levels of organic matter, feces and soil in aquarium and surface waters contributing to the mycobacterial flora [2]. The most frequently occurring isolate was *M. fortuitum*. The low mycobacterial load in chlorinated water observed in the study is probably related to the lethal effect of chlorine on mycobacteria. In a recent study carried out in Sri Lanka, several NTM species including *M. fortuitum* has been isolated from various organs of clinically affected and apparently healthy guppies [17]. Therefore, there is a possibility of introducing these species to water through excreta or by death and decomposition of infected fish. The studies carried out in other countries also reported the higher occurrence of *M. fortuitum* in surface water. Covert et al. [2] reported the isolation of *M. fortuitum* from surface water, ice and ice machine treatment cartridges. Similarly, Bland et al. [1] reported the isolation of over 20 different species of NTM from the Rio Grande, United States and the most commonly isolated species was *M. fortuitum*.

It is a well known fact that a number of climatic and geological factors can contribute to the growth and distribution of these organisms. Thus the distribution of different strains in different water sources can vary widely. Lee et al. [14] reported *M. gordonae* as the most frequently isolated species in surface water and *M. lentiflavum* in tap water samples. In 2002, Le Dantec et al. [13] reported the recovery of *M. gordonae* and *M. nonchromogenicum* from treated surface water and ground water respectively. Several studies [14,1,2,4] have reported the isolation of *M. avium* complex consisted of *M. avium* and *M. intracellulare* from freshwater rivers and surface water samples.

Previous studies have indicated that the conventional tests used for the identification of mycobacteria may frequently result in erroneous identification and underestimate the diversity within the genus *Mycobacterium* [19]. Differentiation of mycobacteria to the species level by evaluation of phenotypic characteristics is time-consuming because of the slow growth rate of mycobacteria. Phenotypic test results may vary depending on the growth conditions, sometimes leading to inaccurate results [2]. This problem is further aggravated since the results of the biochemical reactions are considerably dependent on the testing methodologies including medium composition and incubation conditions. In the present study, phenotypic differentiation of the isolates up to species level was accomplished using the criteria published in Bergey's Manual of Determinative Bacteriology [11]. Only 44% (20/45) of the presumptive *Mycobacterium* isolates were identified up to the species level through the phenotypic identification. However, due to the extensive phenotypic diversity, 56% (25/45) of isolates could not be placed in any of the known species. It was an obvious finding that many isolates differed from the given biochemical results only by one or two

reactions making them difficult in assigning to exact groups. Furthermore, the routine phenotypic tests were unable to differentiate the subtypes of *M. fortuitum* and *M. gordonae*.

In order to avoid the potential misidentification using phenotypic tests, all the isolates were characterized by PCR-RFLP analysis of the *rpoB* gene and as a result, some discrepancies between phenotypic and genetic identifications were discerned (Table 1).

Seventy-three percent (33/45) of the isolates provided comparable RFLP patterns to the known species, while the RFLP profiles of 12 NTM isolates did not match any known *Mycobacterium* species. These might represent mutants or hitherto undescribed NTM species. Eleven species of NTM were identified by PCR-RFLP, namely *M. fortuitum* type I, *M. fortuitum* type II, *M. phlei*, *M. marinum*, *M. gordonae* type I, *M. gordonae* type II, *M. malmoense*, *M. terrae*, *M. avium*, *M. smegmatis* and *M. celatum* type II. Furthermore, the PCR-RFLP of the *rpoB* gene proved to be simple, rapid and accurate in identifying NTM species, when compared to phenotypic tests. The results clearly indicate that the phenotypic identification schemes alone could not be used successfully to assign the isolates into the species level correctly and emphasize that they should be used in combination of the other identification methods, such as PCR-RFLP. A number of isolates could not be identified by PCR-RFLP of the *rpoB* gene, which necessitates the need to construct a more diverse database.

In conclusion, the findings of the present study suggest that water is an important environmental source harboring NTM. Further, the wide presence of NTM in the aquatic sources throughout Sri Lanka is a potential public health hazard especially for those with immunodeficiency. This is where the genetic identification using molecular techniques becomes useful since they can increase the specificity, sensitivity and the accuracy of the diagnosis.

**Table 1 – Comparison of results obtained from phenotypic tests and PCR-RFLP.**

Identification	Number of NTM species identified	
	Phenotypic tests	PCR-RFLP
<i>Mycobacterium fortuitum</i> type I	9	8
<i>Mycobacterium fortuitum</i> type II		4
<i>Mycobacterium phlei</i>	3	6
<i>Mycobacterium scrofulaceum</i>	2	0
<i>Mycobacterium marinum</i>	1	3
<i>Mycobacterium malmoense</i>	0	2
<i>Mycobacterium gordonae</i> type I	2	2
<i>Mycobacterium gordonae</i> type II		3
<i>Mycobacterium terrae</i>	0	2
<i>Mycobacterium chelonae</i>	2	0
<i>Mycobacterium parafortuitum</i>	1	0
<i>Mycobacterium avium</i>	0	1
<i>Mycobacterium smegmatis</i>	0	1
<i>Mycobacterium celatum</i> type II	0	1
Unidentified	25	12
Total	45	45



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## Conflict of interest

We have no conflict of interest to declare.

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