Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 954 - 959

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

OCCURRENCE OF NON -TUBERCULOUS MYCOBACTERIA IN ENVIRONMENTAL SAMPLES AT PILGRIMAGE SITES

B.S.M.RONALD*[1] AND P.KAVITHA RANI[1]

1Vaccine Research Centre – bacterial vaccines, Centre for Animal Health Studies, Tamilnadu Veterinary and Animal Sciences University, Chennai – 600 051, Tamilnadu, India.

ABSTRACT

Non-tuberculous mycobacteria (NTM) inhabit a wide variety of environmental including different water bodies, soil, aerosol, animals and humans. NTM are also known as environmental mycobacteria which can cause mycobacteriosis .Humans, animals and fresh water fauna are exposed to mycobacteria in water. HIV seronegative and immunocomprised patients are most vulnerable to acquire this opportunistic infection. The objective of this study was to identify and characterize the non tuberculous mycobacteria from soil & water bodies near pilgrimage spots where humans have increased access. The mycobacterial isolates were identified using phenotypic methods and 16S rRNA PCR and sequencing of hyper variable region which was proved to be less time consuming and cost effective method. In conclusion, the present study revealed the presence of NTM (32%) viz. *M.intracellularae, M.shimoidei, M.terrae, M.aichense M.woliniski and M.fortuitum* in the water samples collected from pilgrimage sites which can be potential pathogens.

KEYWORDS:Environmental mycobacteria; Non-tuberculous mycobacteria (NTM); phenotypic methods; 16S rRNA PCR, Sequencing.

B.S.M.RONALD
Vaccine Research Centre – bacterial vaccines, Centre for Animal Health Studies, Tamilnadu Veterinary and Animal Sciences University, Chennai – 600 051, Tamilnadu,

***Corresponding author**

INTRODUCTION

Non-tuberculous Mycobacteria (NTM), also known as environmental mycobacteria was recognized as facultative pathogens, potential pathogens and saprophytes in the environment. They may infect human beings either by ingestion or inhalation.The NTM includes those *Mycobacterium* species that are not members of the *Mycobacterium tuberculosis* complex. It has two-fold effect, It may affect the efficacy of BCG [1] and depending on the nature of the NTM, their exposure can enhance, mask, or interfere with the efficacy of subsequent BCG vaccination [2] and NTM can cause mycobacteriosis in immunocompromised patients especially those with AIDS and present as disseminated disease [3,4] may serve as a most common cause of death. In recent years, there have been numerous reports showing that NTM can survive, persist, grow, and colonize in drinking water supply systems [5,6,7,8]. *M. avium* complex (MAC) and other NTM have been isolated from numerous environmental sources, including water, aerosols, soil, and plants. Water is likely the primary source of MAC infection in humans [9] though not the only source. There are a variety of situations where human and mycobacterial geographic and environmental distributions can overlap and lead to exposure of humans as well as impacting mycobacterial ecology. A major overlap occurs with water. Humans are exposed to mycobacteria in water through drinking, swimming, and bathing. The mycobacterial isolates were identified using phenotypic methods and 16S rRNA PCR followed by sequencing of hyper variable region A which was proved to be less time consuming and cost effective method. Hence, the present study was performed to identify and characterize the NTM from soil & water bodies near pilgrimage spots where humans have increased access.

MATERIALS AND METHODS

Collection of samples

A total of 50 samples (25 soil samples and 25 water samples) were collected from different pilgrimage spots suspected to have high human exposure, in a sterile container and transported to the laboratory and were kept at 4°C until analysis. All samples were analyzed within 48 h of collection.

Processing of samples

Wet soil samples of approximately 5g were collected from a depth of 3cm. Soil (5g) was suspended in 20 ml of double-distilled autoclaved water (D/W) in polycarbonate centrifuge tubes. After shaking manually for 60 sec, the suspension was centrifuged at 600× *g* for 5 min at room temperature to pellet the soil particles. The turbid supernatant (10 ml) was transferred into other sterile centrifuge tubes and centrifuged at 8,000× *g* for 15 min at RT. Water samples, 50ml each, were collected from water sources such as rivers and ponds. Water samples (50ml) were centrifuged at 8,000× *g* for 15 min at RT and the supernatant was discarded. Pellets from the soil and water samples were re-suspended separately in 100µl of 0.7% hexa decyl pyridium chloride and incubated at room temperature for 1 hour. After incubation the suspensions were centrifuged at 8,000× *g* for 15 min at RT, and the supernatants were again decanted. The pellet was washed twice with sterile PBS solution by centrifuging at 8,000× *g* for 5 min and the pellet was resuspended in 200µl of PBS.

Isolation

Lowenstein Jensen slants and Herrold's egg yolk medium were prepared and each tube was inoculated with 100µl of sample and incubated at 37^{0}C. The culture tubes were examined periodically for the presence of characteristic colonies. The conventional characterization methods consisted of microscopy after Ziehl-Neelsen staining, evaluation of growth on Lowenstein-Jensen slants and the selection of NTM colonies was based on the description of colonial morphologies [10].

Biochemical tests

Following phenotypic analysis, tests were performed in the laboratory for identification of temperature preference (ºC), growth at 45ºC, growth at 37ºC and 24°C, growth rate, pigment

production were carried out. The Mycobacterial isolates were subjected to various biochemical tests viz., Nitrate Reduction, Pyraminidase, Catalase, Niacin and Tween 80 hydrolysis.

Molecular Characterization

DNA was extracted from the isolates grown on LJ slants by using bacterial genomic DNA extraction kit (Real biotech Corp., India. Cat.No.YGB100) as per the protocol. Specific amplification of mycobacterial 16s rRNA fragment using the primers: 264(R) (5'TGC ACA CAG GCC ACA AGG GA 3') and 285 (F) (5' GAG AGT TTG ATC CTG GCT CAG 3') [11] which allows amplification of hyper variable region. Mycobacterial DNA (50 ηg) was amplified in a 25µl reaction mixture for the amplification of 16s rRNA gene. The cycling conditions used for PCR were: 94ºC for 3 minutes, 94ºC for 1 minute, 55ºC for 1 minute, and 72 º C for 1 minute. A total of 35 cycles were carried followed by a final extension at 72ºC for 7 minutes. The PCR products were resolved on 1% agarose gel. The PCR amplified products were purified using PCR DNA extraction kit (Real Biotech Corp., India,Cat.no.YDF100).The purified PCR product was sequenced to determine the species- specific nucleic acid sequence of hyper variable region of 16s rRNA.

RESULTS

Occurrence of NTM

NTM were isolated from 8 of the 25 water samples analyzed (32 %) and there is no NTM isolated from soil samples. All of the water samples collected were from pilgrimage site where there is a high level contact exists between water and human beings.

Identification of Isolates

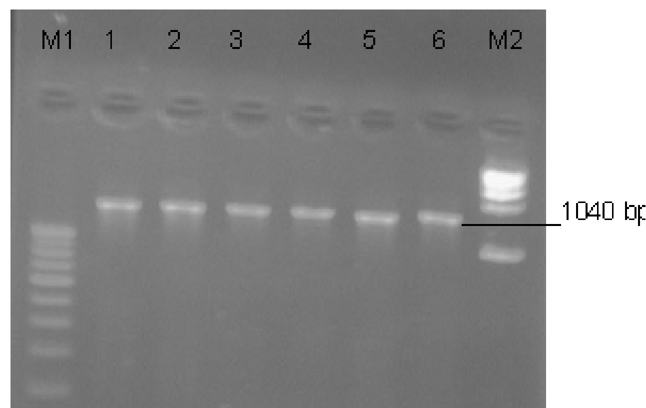
Five isolates out of eight were found to be slow growers taking 10-15 days and the other three isolates were rapid growers taking a week to form colonies. Only one isolate was found to be scotochromogenic. The colony morphology, biochemical tests viz. Nitrate Reduction, Pyraminidase, Catalase, Niacin and Tween 80 hydrolysis results were tabulated in table 1.With regard to the isolation rate of NTM in various water sources, we observed the highest incidence of NTM in water bodies The PCR method showed excellent sensitivity and specificity. The PCR products were resolved on 1% agarose gel and the isolates showed the band of 1040 bp size (Fig.1). The purified PCR product was sequenced to determine the species- specific nucleic acid sequence of hyper variable region of 16s rRNA. The sequence information of the isolates was submitted for BLAST analysis to the RIDOM (Ribosomal Databases for Mycobacterium) for assessing the sequence homology with referral sequences available with gene bank.Based on the sequencing of the 16S ribosomal gene, the isolation and identification of *M.intracellularae, M.shimoidei and M.fortuitum* in water were recognised as facultative pathogens to human beings. *M.terrae* was identified to be potential pathogen to human beings. *M.aichense* and *M.woliniski* are recognized as saprophytes.

Table 1
Biochemical identification of Mycobacterial isolates

Isolates	Growth			Pigmentation	Colony Morphology	Catalase		Tween 80 hydrolysis	Nitrate reduction test	Pyraminidase reduction test	Niacin W/syringe test
	45ºC	37ºC	24ºC			Heat stable 68ºC	Semi quanti tative				
1.	-	S	-	Sc	s	+	+	+	-	-	-
2.	R	R	R	N	s	+	+	-	+	-	-
3.	V	S	S	N	r	+	-	-	-	+	-
4.	-	S	S	N	ir	+	+	+	+	V	-
5.	-	R	R	N	r	+	+	+	+	+	-
6.	-	S	S	N	r	+	-	-	-	+	-
7.	-	R	R	N	R	+	+	+	+	+	-
8.	-	S	S	N	ir	+	+	+	+	V	-

+ = present, - =absent, V=variable, S=slow, R=rapid, r=rough, s=smooth, ir =intermediate in roughness, N=non photo chromogenic, Sc= scotochromogenic

Figure 1



16s r RNA PCR AMPLIFICATION
M1= 100 bp DNA ladder,
M2=1Kb DNA ladder,
Lane1-6 = isolates

DISCUSSION

Environmental opportunistic mycobacteria are distinguished from the members of the *M. tuberculosis* complex (and *M. leprae*) by the fact that they are not obligate pathogens but are the true inhabitants of the environment. They can be found as saprophytes, commensals, and symbionts [12]. Environmental mycobacteria are normal inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, protozoans, animals, and humans. There is no evidence of human-to-human or animal-to-human transmission [13]. NTM infections mostly contracted through water and soil. MAC and *M.fortuitum* are most frequently isolated from water and swimming pools in developing and developed countries.. In India, most pilgrimage sites have water bodies, lake or pond, where a devotee can take a purifying dip because it is believed that water washes physical dirt. Hindus gives it symbolic value as a cleanser of souls. This ritual has led to the overlapping of human beings with the mycobacterial ecology leading to exposure. In

the present study, NTM was isolated from 32% of the samples which was found to be very high compared to other prevalence reports of NTM. The isolates include *M.intracellularae, M.shimoidei, M.terrae, M.aichense M.woliniski and M.fortuitum* There has been previous studies examining drinking water supplies in the United States for NTM. Generally, these studies have focused on the occurrence of MAC organisms (*M. avium* and *M. intracellulare*) only and examined samples from a limited geographical area. The International Union against Tuberculosis and Lung Diseases (IUATLD) reviewed data from 14 countries and found that the *M. avium* complex (MAC) was the most frequently isolated species in all these countries, which included China, India, and Korea. While *M. fortuitum* was the most frequently encountered species in Belgium (2.1%), the Czech Republic (17.5%), Denmark (5.3%), Finland (6.7%), France (6.5%), Germany (12.2%), Italy (2.5%), Portugal (16.5%), Spain (10.8%), Switzerland (17.5%), Turkey (33.9%), and the United Kingdom (6.0%), undoubtedly, environment is the main reservoir of NTM.The conventional phenotypic characterization by biochemical method, pigment production, growth characteristics and colony morphology were found to be a slow process, labour intensive, hazardous and not always reproducible. Biochemically unreactive or inert mycobacteria can be formidable opponents in identification. Interspecies homogeneity, intra species variability and the existence of undescribed species often lead to phenotypic misidentification [13].To overcome the shortcoming of conventional methods, in recent years molecular techniques have grown increasingly popular as they are rapid, highly sensitive, and specific that can be used on a large number of samples. In this trend, 16srRNA gene analysis is the most promising molecular method and found to be cost effective method in identifying NTM [3]. In contrast to phenotypic and biochemical features, the 16s rRNA sequence of a species is a stable property which is specific for a micro organism at the species level. The nucleic acid sequence determination of the rRNA allows proper identification of the isolates to already established taxa, as well as rapid recognition of unrecognised species. In the present study, *M.intracellularae, M.shimoidei and M.fortuitum* were isolated from water bodies which were recognised as facultative pathogens and *M.terrae* was identified to be potential pathogen to human beings[14]. For instance, the strongest association of NTM pulmonary infections has been reported with structural lung disease, such as COPD, bronchiectasis, cystic fibrosis, pneumoconiosis, pulmonary alveolar proteinosis, and oesophageal motility disorders [12]. Though disseminated NTM infections typically occur only during severe immune suppression, in HIV-negative patients disseminated NTM infections are rare and have been associated with specific genetic syndromes. Under certain circumstances these mycobacteria can cause a variety of symptoms, including serious morbidity and mortality .In conclusion, the present study revealed the presence of NTM (32%) in the water samples collected from pilgrimage sites which can be potential pathogens detrimentally affecting human, animals and fresh water fauna especially in immuno compromised individuals. There is a pressing need for improvement in methods to recover NTM from environmental samples. Further, in depth studies for gaining better understanding of the various mechanisms like drug resistance, efflux pump etc., are required which may help in developing better management and treatment of NTM infections.

REFERENCES

1. Stanford J.L, Shield M.J and Rook G.A, How environmental mycobacteria may predetermine the protective efficacy of BCG. Tubercle (62): 55-62, (1981).
2. Demangel C, Garnier T, Rosenkrands I and Cole S.T, Differential Effects of Prior Exposure to Environmental Mycobacteria on Vaccination with *Mycobacterium bovis* BCG or a Recombinant BCG Strain

Expressing RD1 Antigens. Infect Immun (73):2190-2196 (2005).
3. Cook V.J, Tureone C.Y and Wolle J, Conventional methods versus16s ribosomal DNA sequencing for identification of non-tuberculous mycobacteria: cost analysis. J. Clin. Microbiol.(41):1010-1015 (2003).
4. Macher A.M, Kovacs J.A, Gill V, Roberts G.D, Ames J and Park C.H, Bacteremia due to *Mycobacterium avium intracellulare* in the acquired immunodeficiency syndrome. Ann Intern Med; (99):782-785(1983).
5. Du Moulin G.C and Stottmeier K.D, Waterborne mycobacteria: an increasing threat to health. ASM News (52):525-529 (1986).
6. Falkinham J.O.III, Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. (9):177-215 (1996).
7. von Reyn C.F, Waddell R.D, Eaton T, Arbeit R, Maslow J.N, Barber T.W, Brindle R.J, Gilks C.F, Lumio J, Lahdevirta J.A, Ranki A, Dawson D and Falkinham III,J.O, Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire, and Kenya. J. Clin. Microbiol.(31):3227-3230 (1993).
8. von Reyn C. F, Maslow J.N, Barber T.W, Falkinham III J.O and Arbeit R.D, Persistent colonization of potable water as a source of Mycobacterium avium infection in AIDS. Lancet (343):1137-1141(1994).
9. Goslee1 S and Wolinsky E, Water as a source of potentially pathogenic mycobacteria. Am. Rev. Respir. Dis. (113):287-292(1976.
10. Glover N, Holtzman A, Aronson T, Froman S, Berlin O.G, Dominguez P, Kunkel K.A, Overturf G, Stelma G.N, Smith C and Yakrus M, The isolation and identification of *Mycobacterium avium* complex (MAC) recovered from Los Angeles potable water, a possible source of infection in AIDS patients. Int. J. Environ. Health Res. (4):63-72(1994).
11. Boddinghose B, Rogall T, Flohr T, Blocker H and Bottger E.C, Detection and identification of Mycobacteria by amplification of Rrna. J.Clin.Microbiol.(28):1751-1759(1990).
12. Todd P. Primm, Christie A. Lucero and Joseph O. Falkinham, Health Impacts of Environmental Mycobacteria, Clinical Microbiology Reviews, 17(1): 98-106(2004).
13. Griffith D.E, Aksamit T, Brown-Elliott B.A., Catanzaro A and Daley C, An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med (175): 367–416(2007).
14. Auxilia Hemamalini Tilak, Geetha Priya S and Raja K, Microbial Contamination in Dental unit water line. Int. J. Pharma Bio. Sci. 3(4): 1032 – 1044(2012).