Original Article

Predominance of *Mycobacterium fortuitum-chelonae* Complex in Ghatampur Field Area, Endemic for Leprosy

M Lavania, K Katoch**, D Parashar***, P Sharma*, R Das*, DS Chauhan*, VD Sharma*, VM Katoch*****

Abstract

Non-tuberculous mycobacteria (NTM) are commonly found in the environment. As exposure to environmental mycobacteria has been reported to immunomodulatory in this study, the presence of environmental mycobacteria was investigated in soil, drinking water and drainage sample in Ghatampur, India, which is known for high endemicity for leprosy. Soil, drinking water from the hand pumps/wells and also drainage water collected in pools was collected in clean containers and cultured for environmental mycobacteria. Samples were processed according to the protocol established earlier. 69 soil, 62 drinking water and 31 drainage water samples were analysed from soil and water collected from 48 villages of this field area. After decontamination, cultures were set upon Lowenstein Jensen (LJ) medium. Mycobacteria were identified using biochemical tests and molecular techniques such as PCR-RFLP targeting *hsp65* kD and *rpoB* region as well as 16S ribosomal sequencing in case of isolates showing variable biochemical features. NTM (non-tubercular mycobacteria) were isolated from 47.82% of soil samples, 20.69% of drinking water samples and 19.35% of the drainage water samples, overall mycobacteria could be isolated 52/162 of samples (32.09%). Among these mycobacteria, *M. fortuitum-chelonae* complex was predominant in this area; other species isolated were *M.phlei, M.vaccae, M.terrae* and *M.flavescens.* Relevance of exposure to these mycobacteria on endemicity needs to be studied by immunological and epidemiological parameters.

Key words : Environmental mycobacteria, Identification, Characterization, PCR-RFLP

* M Lavania, MSc, Research Scholar, Department of Microbiology and Molecular Biology
P Sharma, MSc, Research Scholar, Department of Microbiology and Molecular Biology
R Das, PhD, Research Assistant, Department of Microbiology and Molecular Biology
DS Chauhan, PhD, Scientist C, Department of Microbiology and Molecular Biology
VD Sharma, PhD, Scientist E, Department of Microbiology and Molecular Biology

** K Katoch, MD, Scientist F and Head, Medical Unit-I; Model Rural Health Research Unit, Ghatampur, Kanpur, India.
National JALMA Institute for Leprosy and Other Mycobaceterial Diseases (ICMR), Dr M Miyazaki Marg, Tajganj, Agra- 28200 I, India.

*** D Parashar, PhD, Scientist B, National Institute of Virology, Pune, India.

**** VM Katoch, MD, Secretary, Department of Health Research, Ministry of Health and Family Welfare, Govt of India and Director-General, Indian Council of Medical Research, Ansari Nagar, New Delhi-110029, India.
Correspondence to : Dr VM Katoch, Email : vishwamohan_katoch@yahoo.co.in

Introduction

Genus mycobacterium comprises of more than 133 species (Euzbey 2009). Genus mycobacterium forms a heterogenous group in terms of their occurrence in clinical or environmental materials, complex phenotypic and genotypic features and disease association (Roth et al 2000). Nearly one third of the known species are associated with human diseases (Katoch 2004, Katoch et al 2007).

The effect of environmental mycobacterial interactions with humans resulting in increased or decreased immunity to infections are not yet well understood, much less with other pathogens or vaccinations. Thus mycobactcria may have far greater effects on humans than the clinically diagnosed mycobacterial infections (Primm et al 2004).

Several observations have indicated that environmental mycobacteria have important effects in leprosy and tuberculosis diseases. Routine culture of sputum specimens from suspected tuberculosis patients has revealed that the sputum commonly contains environmental mycobacteria (Fine et al 2001). Skin test and interferon-gamma response surveys have provided evidence that there is widespread but differential exposure to and sensitization by a variety of environmental mycobacterial antigens (Fine et al 2001, Black et al 2003) and have suggested that exposure to certain antigens of fast-growing environmental mycobacteria is associated with a reduced risk of leprosy and tuberculosis (Fine et al 2001). Experimental studies done in mice (Brandt et al 2002) and epidemiological studies done in humans (Stanford et al 1981, Fine et al 2001) suggest that exposure to various slow/rapid growing mycobacteria alters immune response to BCG. Exposures of environmental mycobacteria is, thus, considered to be an important factor for the variable protection rate against TB (0-80%), by BCG vaccination in different regions of the world (Fine 1995). Chilima et al (2006) reported preponderance of *M.fortuitum* and *M.chelonae* in the environment of Malawi which was once endemic for leprosy.

The aim of our study was to isolate and to characterize the non-tubercular mycobacteria (NTM) from the environment of Ghatampur field area, also endemic for leprosy like Malawi.

Materials and Methods

A total of 69 soil, 62 drinking water and 31 drainage samples were collected from different pockets of varying endemicity of leprosy in Ghatampur region of Kanpur, India from February-October, 2004. Wet soil samples of approximately 5 g were collected from a depth of 3 cm and 50 ml water samples were collected from ditches, ponds in this region throughout the year in a sterile container (Himedia).

Processing of samples : Samples were processed by procedure optimized earlier by Parashar et al (2004). Soil was suspended in 20 ml of double-distilled autoclaved water in centrifuge tubes. After being shaken manually for 60s, the suspension was centrifuged at 600 x g for 5 min at 4°C to pellet the soil particles. The turbid supernatant was transferred in another sterile centrifuge tube and centrifuged at 8,000 x g for 15 min at 4°C. Water samples were centrifuged at 8,000 x g for 15 min at 4°C. Pellets from the soil supernatant and water samples were resuspended in 20 ml of treatment solution (3% sodium dodecyl sulfate [SDS] plus 4% NaOH) and then divided into two parts: A and B. Part A was incubated at room temperature (RT) for 15 min to obtain the growth of rapid growers and part B was incubated at RT for 30 min to obtain the growth of slow growers. After incubation, both the suspensions were centrifuged at

8,000 x g for 15 min at 4°C and then the supernatants were decanted. Sediments were processed for 2% cetrimide treatment. Part A was treated with cetrimide at RT for 5 min to obtain the growth of rapid growers and part B was treated at RT for 15 min to obtain the growth of slow-growing mycobacteria. Longer treatment with cetrimide was found to be inhibitory for RGMs. A 0.1 ml sample of the suspension was inoculated on Lowenstein Jensen (LJ) slants in duplicate and incubated at 30°C and 37°C.

Organisms : A total of 52 environmental isolates of mycobacteria from soil, drinking water and drainage were obtained. These isolates were identified by biochemical tests (Vestal 1977) and PCR-RFLP for *hsp65* (Telenti et al 1993) and *rpoB* (Kim et al 2001) genes. Reference control strains of *M.fortuitum* N-2, *M. chelonae* J-31, *M. phlei* N-14 and *M. vaccae* J-28 were used as controls for PCR-RFLP analysis.

Identification by phenotypic methods : Phenotypic characterization was carried out by determining growth rate and pigment production for each isolate. Each isolate was incubated at 37°C. Biochemical tests such as nitrate reduction, catalase production at 68°C, iron uptake, 5% NaCl tolerance, Tween hydrolysis, aryl sulfatase at 3rd and 14th day and MacConkey agar were performed for identification of these isolates (Vestal 1977).

Identification by molecular methods :

Isolation of DNA : DNA was extracted by physiochemical procedure using lysozyme and proteinase K (van Soolingen et al 1991) followed by chloroform isoamyl alcohol (24:1) extraction. After a brief centrifugation at 8,000 x g for 5 min, the upper phase was collected. DNA was then precipitated with 0.6 volume of isopropanol, washed with chilled ethanol and resuspended in 25 µl of Tris-EDTA buffer (pH-8.0) before being used for PCR.

PCR-RFLP analysis targeting *hsp65* gene target : PCR reactions were performed in 50µl reaction mixtures consisting of 5µl of DNA template, 0.2m mol l^{-1} deoxynucleoside triphosphates, 0.5 µmol l^{-1} primers for *hsp65* kD and 1.5U Taq polymerase (Bangalore Genei, Bangalore, India). 439 bp fragment was amplified by using primers and procedure described by Telenti et al (1993). The forward primer for *hsp65* was Tb11, 5-'ACCAACGATGGTGTGTCCAT-3' and the reverse primer was Tb12, 5'-CTTGTCGAACCGCATACCCT-3'. The PCR conditions for *hsp65* gene were 95°C for 5 min followed by 45 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min and then a final extension at 72°C for 10 min.

PCR-RFLP analysis targeting *rpoB* gene target : PCR reactions were performed using the primers (20pmol) and experimental conditions were described by Kim et al (2001). Forward primer for *rpoB* gene was *rpoB1*, 5'-CGACCACTTCGGCAACCG-3' and the reverse primer was *rpoB2*, 5'-TCGATCGGGCACATCCGG-3' PCR conditions were 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec and then final extension at 72°C for 10 min.

PCR-RFLP : The PCR products were analyzed on 2% agarose gels, electrophoresed and photographed. The amplified products of *hsp65* and *rpoB* gene regions were digested with 5U of different restriction enzymes like *HaeIII* (*hsp65 and rpoB*) and *BstEII* (*hsp65*) according to the recommendations of the manufacturers and electrophoresed for 4 hrs at 1.2 V/cm. Fragment sizes were estimated visually by gel documentation system using Quantity One software (Bio-Rad).

Statistical analysis : Data obtained from experiments were analyzed to calculate the sensitivity of each method and also

concordance among different methods. Statistical analysis was performed using SPSS package; Q-test, Cochran's test was used to study significance ($p< 0.02$ and $p< 0.05$).

Nucleotide sequencing : Mycobacterial 16S rRNA gene (Edward et al 1989) was amplified from the isolates of environmental samples were recovered from agaroge gel after electrophoresis using a Qiagen Gel Extraction Kit (Qiagen). Determination of sequences was performed using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin Elmer).

Results

In this study, NTM were isolated from 52 of the 162 samples analyzed from Ghatampur region.

Distribution of non-tubercular mycobacteria (NTM) : The occurrence of NTM in soil samples was found more (47.82%) in comparison to drinking water (20.96%) and drainage (19.35%) (Table 1). 44 isolates out of 52 belonged to *M. fortuitum-chelonae* complex and was the most frequently isolated NTM from soil.

Table 1: Occurrence of NTM according to sources

Sample source (No. detected)	No. (%) of samples with mycobacteria
Soil (69)	33 (47.82%)
Drinking water (62)	13 (20.96%)
Drainage (31)	6 (19.35%)
Total (162)	**52 (32.09%)**

Characterization of isolates : These isolates were identified to the species level by biochemical and PCR-RFLP targeting 65 kD heat shock protein (*hsp65*) and *rpoB* gene followed by RFLP analysis using the restriction enzymes *HaeIII and BstEll*. 40/52 (78.84%) of isolates could be identified by biochemical tests. PCR-RFLP targeting *hsp65* gene region could identify 90.38%, assay targeting *rpoB* gene region could identify 78.84% isolates correctly whereas together these could identify 96.15% (Table 2, Figs 1 and 2). Only two isolates (3.8%) one *M. fortuitum* and one *M. chelonae* could not be identified by any of the systems and were confirmed by 16S rRNA sequencing.

Table 2 : Sensitivity of the three methods for species identification

Species	Reference	Biochemical tests		*PCR-RFLP*			
				hsp65 kD		*rpoB*	
	N	N	%	N	%	N	%
M. fortuitum	34	28*	82	30*	88	26*	76
M. chelonae	10	06#	60	09#	80	07#	70
M. phlei	02	02	100	02	100	02	100
M. vaeeae	04	04	100	04	100	04	100
M. terrae	01	0	0	01	100	01	100
M. flavescens	01	01	100	01	100	01	100

Q-Cochran test, * $p < 0.02$, # $p < 0.05$

N: Number of isolates

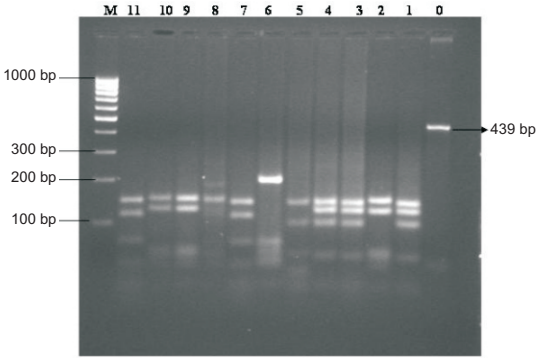

Fig 1 : PCR-RFLP targeting *hsp65* kD gene restricted with *HaeIII* enzyme

Lane 0: Uncut, Lane 1: *M. chelonae*, Lane 2: *M. fortuitum*, Lane 3-5: *M. chelonae*, Lane 6: *M. chelonae* by 16S rRNA sequencing, Lane 7: *M. chelonae*, Lane 8: *M. fortuitum*, Lane 9: *M. fortuitum*, Lane 10: *M. fortuitum* standard, Lane 11: *M. chelonae* standard, Lane 12: 100 bp ladder

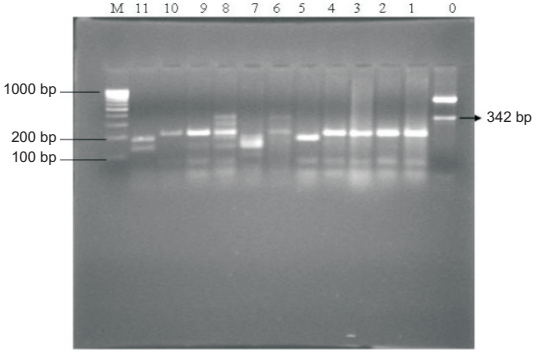

Fig 2 : PCR-RFLP targeting *rpoB* gene restricted with *HaeIII* enzyme

Lane 0: Uncut, Lane 1: *M. fortuitum*, Lane 2: *M. fortuitum*, Lane 3: *M. fortuitum*, Lane 4: *M. fortuitum*, Lane 5: *M. fortuitum*, Lane 6: *M. chelonae*, Lane 7: *M. chelonae*, Lane 8: *M. fortuitum*, Lane 9: *M. fortuitum*, Lane 10: *M. fortuitum* standard, Lane 11: *M. chelonae* standard, Lane 12: 100 bp ladder

PCR-RFLP targeting *hsp65* gene region was found more sensitive than *rpoB*. This technique identified 30 out of 34 *M. fortuitum* isolates while the *rpoB* and the biochemical tests only identified 26 and 28 isolates of *M. fortuitum* respectively. This was due to the observation of 4 unusual patterns. These could be subtypes within the same species.

Discussion

Environmental mycobacteria are common heterotrophic bacteria in soils and other natural reservoirs. Some species may cause infections similar to tuberculosis in humans and animals (Primm et al 2004). They pose risk of severe infections in individuals with immunocompromising conditions. The pathogenic rapidly growing mycobacteria (RGM), capable of producing disease in humans, consist primarily of the *M.abscessus, M.chelonae, M.fortuitum* and *M.smegmatis* group (Katoch et al 2007). In our study, we found that environmental mycobacteria are common in soil, drinking water and drainage in Ghatampur area of Kanpur. The complex effects of environmental mycobacterial

Table 3 : Recovery of NTM species categorized by sample source

Species isolated	Soil	Drinking water	Drainage	Total
M. fortuitum	20	10	04	34
M. chelonae	05	03	02	10
M. phlei	02	-	-	02
M. vaccae	04	-	-	04
M. terrae	01	-	-	01
M. flavescens	01	-	-	01

interaction with humans resulting in the alteration in the immunity to tuberculosis and leprosy have been suggested but are not yet well understood (Stanford et al 1981, Primm et al 2004). Mycobacteria may have greater effects on humans than the clinically diagnosed mycobacterial infections. Identification of RGM isolates by the PCR-RFLP technique provided identification within 2 to 3 working days upon receiving the isolate which was significantly faster than conventional methods (which required 2-4 weeks). The time difference was largely accounted for the biochemical tests needed for the identification by conventional methods. After the completion of long and time consuming procedure, we found in 21% of the isolates, the results were ambiguous due to variation in enzymatic activity and phenotypic characteristics. In our study, these variations made the situation particularly difficult in the identification of species belonging to the *M.fortuitum* and *M.chelonae.* PCR-RFLP targeting both *hsp65* and *rpoB* gene region and 16S rRNA sequencing identified these isolates. Of the 52 isolates, 50 were identified by these methods. NTM were isolated from 32.09% of all samples. NTM were isolated from 47.82% from soil, 21% from drinking water and 19.36% from drainage samples. Only two NTM species *(M. flavescens* and *M. terrae)* were isolated only from soil samples whereas others were present in all the sources. The most frequently occurring species in this were *M. fortuitum* 34/52 (65.38%) followed by *M. chelonae* 10/52 (19.23%), *M. phlei* 2/52 (3.8%) and *M. vaccae* 2/52 (7.6%).

PCR-RFLP targeting *hsp65* gene region could achieve efficient identification of most of our mycobacterial isolates. Our data clearly indicate the superiority of the PCR-RFLP using *hsp65* (90.38%) in comparison to *rpoB* gene region (78.84%) for the identification of rapid growing mycobacteria compared to conventional techniques (78.84%) which has also been reported in other studies (Wong et al 2003, Kim et at 2005, Cheunoy et al 2005). As reported by Wong et al (2003) PCR-RFLP targeting *hsp65* gene region could identify 100% of *M. tuberculosis* isolates and 74.5% of nontuberculous mycobacteria (NTM). Cheunoy et al (2005) reported that the concordant percentage of results obtained from PCR-REA compared with biochemical method was 100%, 98.8%, and 83.3% for *M. tuberculosis* complex, rapidly growing and slowly growing mycobacteria respectively and the results of *hsp65* PCR-REA was in agreement with those obtained from *rpoB* PCR-REA. Therefore we can say that the characterization of environmental isolates were variable from area to area.

In our study, sequencing was required only for identification of limited number of isolates (3.8%). In addition, RFLP analysis and direct sequencing of the amplicon of NTM could be used for species identification. Though not relevant to this study, the faster turnaround time will enable clinicians to provide a more rapid diagnosis and enable them to administer the appropriate therapy.

As highlighted above, *M. fortuitum* and *M. chelonae* mycobacterial species were most prevalent in the environment of Ghatampur region and such findings correlate with a results of study done at Malawi which was once endemic for leprosy (Chilima et al 2006). Preponderance of *M. fortuitum* and *M. chelonae* in the environment of Ghatampur as well as Malawi points to possible common influences of these species on endemicity of leprosy. This, however, needs to be investigated by using appropriate immunological and epidemiological parameters.

Acknowledgements

We are thankful to ICMR Task Force Project (No CJIL/100/1/KK/ICMR-TASK FORCE) for the financial support and indebted to VS Yadav for assisting in statistical analysis. We are thankful to the Field Staff of Ghatampur for assisting in the sample collection, technical support from Hari Shanker and suggestions from seniors and colleagues in this study are greatly acknowledged .

References

1. Black GF, Weir RE, Chaguluka SD et al (2003). Gamma interferon responses induced by a panel of recombinant and purified mycobacterial antigens in healthy, non-*Mycobuclerium bovis* BCG-vaccinated Malawian young adults. *Clin Diagn Lab lmmunol*. **10**: 602-611.
2. Brandt L, Cunha JF, Olsen AW et al (2002). Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect Immun*. **70**: 672-678.
3. Cheunoy W, Prammananan T, Chaiprasert A et al (2005). Comparative evaluation of polymerase chain reaction and restriction enzyme analysis : two amplified targets, hsp65 and rpoB, for identification of cultured mycobacteria. *Diagn Microbiol Infect Dis*. **51**: 165-171.
4. Chilima BZ, Clark IM, Floyd S et al (2006). Distribution of environmental mycobacteria in Kronga District northern Malawi. *Appl Environ Microbiol*. **72** : 2343-2350.
5. Edwards U, Rogall T, Blocker H et al (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res*. **17**: 7843-7853.
6. Euzbey JP (2009). List of bacterial names with standing in nomcnclature genus Mycobactcrium. 2009. *http://www.bactcrio.cict.fr/m/mycobacterium*. Assessed on 16 September, 2009.
7. Fine PE (1995). Variation in protection by BCG : implications of and for heterologous immunity. *Lancet*. **346**: 1339-1345.
8. Fine PE, Floyd S, Stanford JL et al (2001). Environmental mycobacteria in Northern Malawi: implications for the epidemiology of tuberculosis and Ieprosy. *Epidemiol Infect*. **126**: 379-387.
9. Katoch VM, Lavania Mallika, Chauhan DS et al (2007). Environmental Mycobacteria: Friends and Foes. *Environ Biol Conserv*. **12**: 87-100.
10. Kent PT and Kubica GP (1985). Public Health Mycobacteriology: A guide for the level III Laboratory. Atlanta, USA: Center for Disease Control, Department of Health and Human Service.
11. Kim BJ, Lee KH, Park BN et al (2001). Differentiation of mycobacterial species by PCR-restriction analysis of DNA (342 base pairs) of RNA polymerase gene (rpoB). *J Clin Microbiol*. **39**: 2102-2109.
12. Kim H, Kim SH, Shim TS et al (2005). PCR restriction fragment length polymorphism analysis (PRA)-algorithm targeting 644 bp Heat Shock Protein 65 (hsp65) gene for differentiation of Mycobacterium spp. *J Microbiol Methods*. **62**: 199-209.
13. Le Dantec C, Duguet JP, Montiel A et al (2002). Chlorine disinfection of atypical mycobacteria isolated from water distribution system. *Appl Environ Microbiol*. **68**: 1025-1032.
14. Parashar D, Chauhan DS, Sharma VD et al (2004). Optimization of procedures for isolation of environmental mycobacteria from soil and water samples obtained in northern India. *Appl Environ Microbiol*. **70**: 3751-3753.
15. Primm TP, Lucero CA and Falkinham JO 3rd (2004). Health impacts of environmental mycobacteria. *Clin Microbiol Rev*. **17**: 98-106.
16. Roth A, Reischl UDO, Streubel A et al (2000). Novel diagnostic algorithm for identification of mycobacteria using genus-specific

amplification of the 16S -23S rRNA gene spacer and restriction endonucleases. *J Clin Microbiol*. **38**: 1094-1104.

17. Stanford JL, Shield MJ and Rook GAW (1981). Hypothesis: How environmental mycobacteria may predetermine the protective efficacy of BCG. *Tubercle* **62**: 55-62.
18. Telenti A, Marchesi F, Balz M et al (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol*. **31**: 175-178.
19. van Soolingen D, Hermans PWM, de Haas PEW et al (1991). Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology or tuberculosis. *J Clin Microbiol*. **29**: 2578-2586.
20. Vestal AL (1977). Identification test techniques. In: Procedure for Isolation and Identification of Mycobacteria, US Department of Health, Education and Welfare Publication (CDC 77-8230), Atlanta, Georgia, pp 65-89.
21. Wong DA, Yip PC, Tse DL et al (2003). Routine use of a simple low-cost genotypic assay for the identification of mycobacteria in a high throughput laboratory. *Diagn Microbiol Infect Dis*. **47**: 421-426.