

Identification of environmental mycobacteria isolated from Agra, north India by conventional & molecular approaches

Deepti Parashar, Ram Das, D.S. Chauhan, V.D. Sharma, Mallika Lavania, V.S. Yadav, S.V.S. Chauhan* & V.M. Katoch⁺

*National JALMA Institute for Leprosy & Other Mycobacterial Diseases (ICMR) & *School of Life Sciences Dr B.R. Ambedkar University, Agra, India*

Received June 7, 2007

Background & objectives: Several environmental mycobacteria have been shown to be important human pathogens linked to immunomodulation especially in relation to effect on vaccination. Hence identification of mycobacteria to the species level is not only relevant to patient management but also to understand epidemiology of mycobacterial diseases and effect on vaccination. We undertook this study to assess the usefulness of various conventional and molecular methods in identification of environmental mycobacterial species from Agra, north India.

Methods: One hundred nineteen isolates of environmental mycobacteria were grown from 291 (116 soil and 175 water) samples. These isolates were identified by standard biochemical tests, and a simple, rapid and cost-effective in-house developed gene amplification restriction analysis targeting 16S-23S rRNA spacer and flanking region and 16S rRNA sequencing.

Results: Biochemical tests could clearly identify only 68.1 per cent (81/119) of isolates to species level. An in-house developed gene amplification - restriction analysis method could confirm the identity of 102 of 119 (85.7%) isolates and the remaining 17 isolates (14.3%) were confirmed by 16S rRNA sequencing also. These 119 environmental mycobacterial isolates, included several potentially pathogenic species such as *M. fortuitum*, *M. chelonae*, *M. avium*, *M. marinum*, *M. manitobense*, *M. kansasii* and others belonged to nonpathogenic species, *M. terrae*, *M. smegmatis* and *M. flavescens*. *M. chelonae* was isolated from water samples only whereas *M. fortuitum* was isolated from both water as well as soil samples.

Interpretation & conclusion: The in-house developed gene amplification restriction analysis method though failed to accurately identify 14.3 per cent of isolates, facilitated rapid differentiation of most of environmental mycobacteria including potential pathogens from this area and thus would have diagnostic potential in cases with NTM infections. This combination strategy using PCR-RFLP and 16S rRNA sequencing may be useful for characterization of mycobacteria from similar environmental settings from other parts of world.

Key words Environmental mycobacteria - gene amplification restriction analysis

⁺Present address: Secretary, Department of Health Research, Ministry of Health & Family Welfare & Director-General, Indian Council of Medical Research, Ansari Nagar, New Delhi 110 029, India

Environmental mycobacteria also referred to as atypical mycobacteria or non tuberculous mycobacteria (NTM) are common saprophytes in all natural ecosystems, such as water, soil, food and dust¹⁻³. Many of these environmental mycobacteria have been shown to be important human pathogens^{1,4-6}. In western countries, NTMs have emerged as a major cause of opportunistic infections in patients with and without AIDS. Exact magnitude of infections due to NTM is not known in India, however there are several reports of their isolation from clinical specimens⁷⁻¹². The correct diagnosis differs from that of diseases caused by other non tuberculous mycobacteria, which are often resistant to drugs and dosages used for treating tuberculosis. Further, exposure to these mycobacteria has been thought to be important in influencing the immune response, particularly in relation to vaccines like BCG¹³⁻¹⁷. It is important to study these NTMs as these might have contributed to varying protective efficacy of vaccines such as BCG¹³⁻¹⁷ and *M.w*^{18,19}. Vaccine trials conducted in India have also not analysed the protection with respect to possible effect of exposure to environmental mycobacteria²⁰. While a profile of environmental mycobacteria from south India has been reported¹⁷, scanty information about these mycobacteria is available from north India¹⁹ where the climatic conditions are different²⁰. It has been reported earlier that mycobacterial flora in the environment is influenced by different physiochemical conditions²¹.

Studies on mycobacteria depend heavily upon the discrimination value of identification methods. Conventional identification tests are time consuming and at times may be inefficient in characterizing and distinguishing various mycobacteria²². Available data show that molecular methods have greatly improved the taxonomic knowledge, which allows better differentiation of different mycobacterial organisms²³⁻²⁹. The PCR using primers targeting the internal transcribed spacer (ITS) region can be utilized to differentiate the mycobacterial isolate as a rapid or slow grower based on the variations in the amplification product size, within 4 h of isolation, when compared to the conventional methods which require 4-6 wk time.

In the present study, an attempt has been made for adopting a strategy of using conventional biochemical and molecular approaches - an in-house developed gene amplification - restriction analysis of spacer and flanking region of rRNA gene region and 16S rRNA sequencing to identify mycobacteria isolated from soil

and water samples from Agra, north India with a hot dry climate for most of the year.

Material & Methods

Soil (n=116) and water samples (n=175) were collected from different habitats of Agra every month from December 1998 to December 2000. These included tap water (8), industrially polluted water (23), sewage (80), drainage (90) and garbage (90). The samples were processed by our earlier optimized procedure for isolation of mycobacteria from soil and water samples²¹.

Identification by conventional methods: Identification of these mycobacterial isolates at species level was done by growth rate, morphology of colonies and standard biochemical tests like catalase, nitrate reduction, tween 80 hydrolysis, arylsulphatase, growth on Lowenstein Jenson (L-J) medium containing 5 per cent NaCl, MacConkey agar and pigment production tests as per Centres for Disease Control and Prevention, Atlanta Manual³⁰.

Identification by molecular methods:

Extraction of nucleic acid - DNAs from the growth harvested from L-J slants were extracted by procedure established earlier using lysozyme (SIGMA, USA) and proteinase K (Bangalore Genei, India)²⁷.

Gene amplification- restriction analysis - Method described earlier was employed for amplification of the 16S - 23S rRNA spacer and flanking region (1.8 kb fragment) using primers P-1 (5'>GATTGACGGTAGGTGGAGAAGAAG<3') and P-2 (5'>CACGGGCCCCGCTGCTACTCG<3') (Patent filed: Indian Application number: 2418/DEL/2006)³¹. Fragment sizes were estimated by comparison with appropriate controls run in parallel type with reference strains of *M. fortuitum*, *M. chelonae*, *M. smegmatis*, *M. flavescens*, *M. avium*, *M. kansasii*, *M. marinum*, *M. terrae* and molecular weight marker in gel documentation system using Quantity One software (Bio-Rad, USA).

Sequencing of the 16S rRNA gene - PCR amplification of the 16S rRNA gene was performed for selected isolates by using the method of Edward *et al*³². The BLAST program (version 2.0, European Molecular Biology Laboratory) (<http://dove.Embl-Heidelberg.De/Blast2>) was used to compare the sequence of the study strains with those in the databases.

Statistical analysis: Data were analysed using Chi square test with the help of SPSS software (SPSS Incorporation, Chicago, USA).

Results

Out of the 291 environmental samples (175 water samples and 116 soil samples), 119 mycobacterial isolates were obtained. These included 69 isolates from water and 50 from soil. Of the 69 water isolates, 43 (66.15%) were rapid growers and 26 (37.68%) were slow growers whereas out of 50 isolates from soil 27 (54%) were rapid growers and remaining 23 (46%) were slow growers (Table I).

Of the 119 isolates, 93 (78.2%) were potentially pathogenic mycobacterial species *M. fortuitum* (29), *M. chelonae* (30), *M. avium* (29), *M. marinum* (3), *M. manitobense* (1), *M. kansasii* (1) whereas 22 (18.5%) were of mycobacterial species which are usually nonpathogens *i.e.*, *M. terrae* (15), *M. smegmatis* (6) and *M. flavescens* (1) (Table II). The remaining four isolates could not be amplified by PCR.

Identification of mycobacterial isolates: Of the 119 mycobacterial isolates, 81 (68.1%) could be confirmed by biochemical tests. Using primers targeting 16S-23S rRNA spacer and flanking gene region and restriction analysis using several restriction enzymes *i.e.*, *Hha* I (Figure shows the profiles of *M. marinum*, *M. smegmatis*, *M. terrae* and *M. fortuitum*), *Hinf* I and *Rsa* I confirmed the identity of 102 (85.7%) isolates (Tables II, III). Remaining 17 isolates were subjected to 16S rRNA sequencing for final confirmation and were identified as *M. fortuitum*, *M. avium*, *M. terrae* and *M. manitobense*. Three isolates namely *M. fortuitum*, *M. avium* and *M. terrae* showing

Table I. Type of mycobacteria isolated from environmental samples from Agra

Samples	No. of samples	No. of isolates obtained (%) [*]	Rapid growing mycobacteria No. (%) ^{**}	Slow growing mycobacteria No. (%) ^{**}
Water	175	69 (39.42)	43 (62.3)	26 (37.68)
Soil	116	50 (43.1)	27 (54)	23 (46)

^{*}No. of isolates obtained/no. of water or soil samples

^{**}No. of rapid or slow growers/no. of mycobacterial isolates obtained

biochemical variable features were identified by gene-amplification restriction analysis targeting 16S-23S rRNA, 16S rRNA of these isolates were sequenced and were also confirmed as the same species as indicated by gene-amplification restriction analysis. Thus 16S rRNA sequencing could identify remaining 14.3 per cent of the isolates up to species level.

Recovery of NTM species from water and soil samples: All *M. chelonae* as well as all the *M. marinum* isolates were obtained from water samples only whereas nearly two third of *M. fortuitum* isolates were obtained from soil samples. *M. avium* was isolated almost with equal frequency from water and soil samples. *M. manitobense* was recovered from soil samples only and *M. kansasii* was recovered from water sample only. Non pathogenic mycobacterial species *M. terrae* and *M. smegmatis* were recovered from both water and soil samples and *M. flavescens* was recovered from water sample only (Table IV).

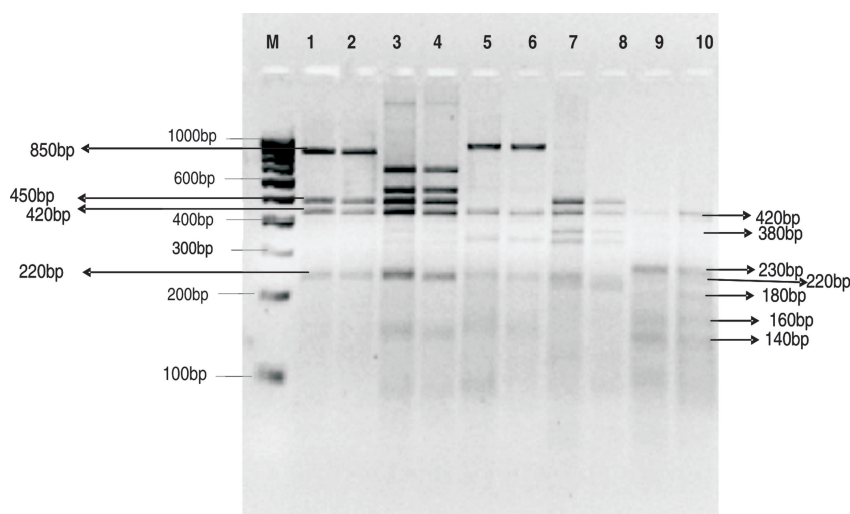


Fig. Gene amplification restriction analysis of environmental mycobacterial isolates belonging to *M. terrae*, *M. avium*, *M. vaccae*, *M. chelonae* and *M. fortuitum* using with *Hha* I for amplicon digestion. Lane-M, Marker, Lane-1 *M. terrae*, Lane 2 *M. terrae* (Standard strain), Lane-3 *M. avium*, Lane-4 *M. avium* (Standard strain), Lane-5 *M. vaccae*, Lane-6 *M. vaccae* (Standard strain), Lane-7 *M. chelonae*, Lane-8 *M. chelonae* (Standard strain), Lane-9 *M. fortuitum*, Lane-10 *M. fortuitum* (Standard strain).

Table II. Identification of mycobacterial isolates

Species	Isolates confirmed by biochemical tests		Isolates showing variation in biochemical tests			Total isolates confirmed by gene amplification restriction analysis	
	No. of isolates	Isolates confirmed by gene amplification restriction analysis	No. of isolates	Biochemical tests showing variation	Isolates confirmed by gene amplification restriction analysis	Isolates confirmed by 16S rRNA sequencing	Total isolates confirmed by gene amplification restriction analysis
<i>M. fortuitum</i> (n=29)	22	22/22	7	Nitrate reduction, MacConkey, 5% NaCl	3/11	4	25/29
<i>M. chelonae</i> (n=30)	19	19/19	11	Nitrate reduction, MacConkey, 5% NaCl	11/11	-	30/30
<i>M. avium</i> (n=29)	24	24/24	5	Catalase, Nitrate reduction	2/5	3	26/29
<i>M. mantobense</i> (n=1)	-	-	1	Nitrate reduction, 5% NaCl	-	1	0/1
<i>M. terrae</i> (n=15)	8	8/8	7	Catalase, Nitrate reduction	2/7	5	10/15
<i>M. smegmatis</i> (n=6)	3	3/3	3	Catalase, Nitrate reduction	3/3	-	6/6
<i>M. marinum</i> (n=3)	3	3/3	-				3/3
<i>M. kansasii</i> (n=1)	1	1/1	-				1/1
<i>M. flavescens</i> (n=1)	1	1/1	-				1/1
Total	81/119 (68.1%)	81/81 (100%)	34/119 (28.1%)		21/34 (61.7%)	13/34 (38.2%)	102/119 (85.1%)

*Four of the isolates were not amplified by PCR

Table III. Fragments sizes of rDNA generated by restriction of 1.8 kb segment with *Hha* I, *Hinf* I and *Rsa* I restriction enzymes from different species of mycobacteria

Species	Restriction fragment sizes (bp)		
	<i>Hha</i> I	<i>Hinf</i> I	<i>Rsa</i> I
<i>M. fortuitum</i> (n=29)	420,380,230,220,180,160,140	568,470,425,277	733,442,296,266
<i>M. chelonae</i> (n=30)	450,400,350,320,258	811,710,674,251	622,367,282,231,197
<i>M. smegmatis</i> (n=6)	517,425,387,341	805,670,281,253	622,369,233,205,189,170
<i>M. avium</i> (n=29)	500,450,400,250	715,673,259	551,379,273,240,208
<i>M. marinum</i> (n=3)	650,480,420,220	700,673,377,255	540,374,235,200,189,170
<i>M. flavescens</i> (n=1)	500,450,400,350	848,677,255	639,650,454,377
<i>M. terrae</i> (n=15)	850,450,420,220	682,470,364,255	643,379,238,202,193,168
<i>M. kansasii</i> (n=1)	785,503,445,260	714,677,255	536,372,235,197,182,172,160
<i>M. manitobense</i> (n=1)	420,240,200,150,100	560,460,440,240,190	770,700,550,420,220

Table IV. Recovery of mycobacteria in different seasons

Species	Summer		Winter		Rainy		Total		Grand total
	Water	Soil	Water	Soil	Water	Soil	Water	Soil	
<i>Pathogenic:</i>									
<i>M. fortuitum</i>	3	2	3	5	1	15	7	22	29
<i>M. chelonae</i>	4	-	13	-	13	-	30	-	30
<i>M. avium</i>	1	1	14	6	-	7	15	14	29
<i>M. marinum</i>	-	-	2	-	1	-	3	-	3
<i>M. manitobense</i>	-	-	-	1	-	-	-	1	1
<i>M. kansasii</i>	-	-	1	-	-	-	1	-	1
<i>Nonpathogenic:</i>									
<i>M. terrae</i>	2	1	3	5	1	3	6	9	15
<i>M. smegmatis</i>	2	-	-	3	-	1	2	4	6
<i>M. flavescens</i>	-	-	1	-	-	-	1	-	1
Total	12/80	4/58	37/55	20/26	16/40	26/32	65	50	115
Grand total	16/138 (11.5%)		57/81 (70.3%)		42/72 (58.3%)		115		

Highest number of mycobacterial isolates were obtained from sewage waste pollutants 60/80 (75%) followed by drainage 39/90 (43.3%), industrial wastes 7/23 (30.4%) and garbage and dead animal wastes 13/90 (14.4%). *M. chelonae* appears to be widely distributed in sewage wastes (8), drainage (8), garbage and dead animal wastes (5) and industrial wastes (5). On the other hand, *M. avium* was isolated from sewage wastes (11), drainage (13), *M. terrae* complex was isolated from sewage wastes (7), drainage (7) while *M. fortuitum* was isolated from sewage wastes (26) (Table V).

Recovery of mycobacteria in different seasons: The isolation of mycobacteria was more in winter season (57/81, 70.3%) compared to that in summer (16/138, 11.5%) and rainy season (42/72, 58.3%) (Table IV). There was marginal difference observed in isolation rate in the winter and rainy season compared to summer ($P < 0.1$). No significant difference in isolates

number was found in different seasons in the case of water samples whereas in soil samples the isolation rate for mycobacteria was found more in winter and rain comparative to summer season ($P < 0.005$).

M. fortuitum, *M. chelonae*, *M. avium*, *M. terrae* and *M. smegmatis* were recovered throughout the year while *M. manitobense*, *M. kansasii* and *M. flavescens* were recovered during winter season. *M. marinum* was recovered during winter and rainy season (Table IV).

Discussion

In TB endemic countries like India due to heavy burden of disease caused by *M. tuberculosis*, NTM disease has been considered less important. In our study more than three fourth of environmental isolates included potentially pathogenic mycobacterial species

Table V. Relationship of mycobacterial species isolated from specimens with different type of pollutants

Species	Sewage wastes	Drainage	Garbage & dead animal wastes	Industrial wastes
<i>M. fortuitum</i>	26	5	2	-
<i>M. chelonae</i>	8	8	5	5
<i>M. phlei</i>	2	-	-	-
<i>M. vaccae</i>	-	2	-	-
<i>M. smegmatis</i>	3	2	1	-
<i>M. flavescens</i>	1	-	-	-
<i>M. avium</i>	11	13	3	2
<i>M. kansasii</i>	-	1	-	-
<i>M. marinum</i>	2	1	-	-
<i>M. terrae</i> complex	7	7	1	-
<i>M. manitobense</i>	-	-	1	-
Total*	60/80 (75%)	39/90 (43.3%)	13/90 (14.4%)	7/23 (30.4%)

*A total of 8 samples from clean sources were included

such as *M. fortuitum*, *M. chelonae*, *M. avium*, *M. kansasii*, *M. marinum* and *M. manitobense*. Keeping in view the importance of nontuberculous mycobacteria in medical and veterinary medicine, identification of pathogenic nontuberculous mycobacteria from their reservoir in the environment is very important^{5,6}. The presence of NTM species may be influenced by levels of organic matter, faeces in soil and surface water contributing to the mycobacterial flora¹. In a somewhat similar study on isolation of environmental mycobacteria in the south Indian *M. bovis* BCG trial area, pathogenic isolates belonging to the *M. avium-intracellulare-scrofulaceum* complex organisms were found to be predominant in water, dust and sputum samples whereas *M. fortuitum* – *chelonae* complex organisms were predominant in soil samples¹⁷ while in our study *M. fortuitum* – *chelonae* complex were isolated apparently more from water sample instead of soil samples. Stanford *et al*¹⁴ hypothesized that exposure to *M. scrofulaceum*, *M. kansasii*, *M. nonchromogenicum* and *M. vaccae* species present in the environment may be important in predetermining the protective efficacy of BCG. Out of these species *M. kansasii* has been isolated in the present study but the proportion was very low (1 in 119). Two important rapid growing pathogens namely *M. fortuitum* and *M. chelonae* were present throughout the season in soil / water and these have been isolated from clinical specimens of north India^{8-11,21}. Potentially pathogenic *M. marinum* was recovered in the beginning of winter and also in rainy season from water samples because of its optimum temperature of growth being 33°C. In spite of collection of more samples in summer season recovery of mycobacteria was low as compared to winter and rainy seasons, which could be due to several factors more so due to temperature, which goes even up to 50°C in May and June. Apparent difference in the vaccine efficacy of *Mw* in north and south may be due to immunomodulation due to exposure to these mycobacteria.

Identification of environmental mycobacteria by biochemical tests has been used with considerably success during the last 50 years. However, these techniques have their own limitations. Nearly 30 per cent of environmental isolates in the present series could not be identified by biochemical tests. These could be identified to species level by gene amplification restriction analysis using different restriction enzymes and 16S rRNA sequencing (100%, additional benefit of 14.3% above PCR-RFLP and 30% above biochemical

tests respectively). In the PCR-RFLP method *Rsa I* yielded the largest number of bands followed by *Hha I* and *Hinf I*. The pattern produced by *Hha I* (close in case of *M. avium* and *M. flavescens*) was found to be useful to distinguish all the species that could be identified by this method. These isolates were found to belong to *M. fortuitum*, *M. chelonae*, *M. avium*, *M. marinum*, *M. manitobense*, *M. kansasii*, *M. terrae*, *M. smegmatis* and *M. flavescens* species. A larger battery of biochemical tests, lipid / chemical markers may reduce the sensitivity gap but that would make the approach more labour intensive and impractical. PCR sequencing of variable stretches of 16S rRNA has been found to be very useful for identification of mycobacteria and has led to identification of new species, which could not be identified by conventional methods³² and this has been used as gold standard in the present study. It is well known that sequencing of 16S rRNA gene is a powerful technique of differentiating species, however it needs specialized settings and is thus difficult to implement for routine use in common clinical laboratories. This study showed that the sequencing of the 16S rRNA would be required for the identification of a small section of environmental mycobacteria.

Several gene regions have been used as molecular targets for the identification of mycobacterial species, these include *hsp65*^{29, 33,34}, *rpoB* gene³⁵, *dnaJ* gene³⁶, 32 kDa protein gene³⁷, 16S rRNA gene^{38,39}, 16S-23S rRNA gene spacer^{24-26,28,36}, promoter region of 16S rRNA³⁹ and *secA1*⁴⁰. Gene amplification restriction analysis holds promise to identify several mycobacteria simultaneously. We have developed an assay to characterize mycobacteria by amplifying 1.8 kb fragment targeting spacer and flanking region of 16S-23S rRNA gene³¹. This study confirms the usefulness of this in-house gene amplification restriction analysis procedure for accurate identification of different mycobacteria from environment. Successful identification of nearly 90 per cent of mycobacterial isolates rapidly by the PCR-RFLP assay can be considered reasonably good for clinical settings. As infections due to NTM will come from environment, this technique holds promise. This technique allowed better and easier differentiation on gels as it targets a larger region than internal transcribed spacer (ITS) sequences between the 16S–23S rRNA genes alone which has been used by other workers^{25,26} and the fragments generated from amplicons by this assay are bigger than generated by other techniques³⁴⁻⁴⁰ which can be

easily separated and analyzed. *HhaI*, *HinfI* and *RsaI* enzymes were found to be useful for identification of various environmental mycobacterial isolates. It would be interesting to compare the complimentary role of other gene amplification - restriction analysis systems to confirm the identity without having the necessity of 16S rRNA sequencing, so as to evolve a more effective composite scheme for identification of environmental mycobacteria.

Acknowledgment

This work was supported by grants from the Department of Biotechnology, Government of India (Grant No BT/PR/1253/MED/09/203/98) and LEPR, UK. Authors thank to Dr Anil Kumar, Scientist F for statistical analysis and Shriyuts Pradeep Dagur and Gaurav Pratap Singh for their help in scanning gel photograph and Harishankar for technical support.

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Reprint requests: Dr V.M. Katoch, Secretary, Department of Health Research, Ministry of Health & Family Welfare & Director-General Indian Council of Medical Research, Ansari Nagar, New Delhi 110 029, India
e-mail: vishwamohan_katoch@yahoo.co.in/ rohinik@sancharnet.in