Genotype analysis of human *Mycobacterium avium* isolates from India

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*Background & objectives:* *Mycobacterium avium* has emerged as a major opportunistic pathogen, infecting nearly 50 per cent of HIV/AIDS patients in the western world. There is no report from India regarding the typing profile of *M. avium*, a potential pathogen, the present study was undertaken to assess the genotypic diversity of Indian *M. avium* isolates of human origin.

*Methods:* A total of 65 biochemically identified *M. avium* isolates from sputum samples of patients with chronic pulmonary illness were subjected to IS1245 based restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) typing.

*Results:* IS1245 insertion sequence based RFLP demonstrated polymorphism in 84.6 per cent isolates, while 15.4 per cent isolates did not hybridize on Southern blot and therefore were RFLP negative. Among the 55 RFLP positive isolates, 8 showed 1-3 bands, 19 had bands ranging between 4-9, and 28 isolates had >10 bands each. Although the isolates could be clubbed on the basis of number of bands, the banding profile was highly polymorphic. Among the 55 isolates typeable by RFLP, four clusters and 40 unique types of polymorphism were observed. Application of IS1245 based PCR typing on the same isolates showed that 87.7 per cent isolates were typeable. Interestingly the 10 isolates that were not typeable by IS1245 RFLP were typeable by IS1245 based PCR typing. Among the 57 PCR typed isolates a cluster of 14 isolates with identical 3-banded pattern was observed. Notably, 5 of the ten IS1245 RFLP negative isolates were within this cluster.

*Interpretation & conclusion:* Our results demonstrated that *M. avium* isolates from India were highly polymorphic with remarkable genetic diversity and heterogeneous RFLP profile. We observed that 47 per cent (n=27) isolates had RFLP profile suggestive of bird and animal origin indicating a strong association with the environment. By applying two typing methods based on IS1245 on the isolates 100 per cent typeability could be achieved.

**Key words** Genotyping - IS 1245 - *M. avium* - polymerase chain reaction - restriction fragment length polymorphism
Mycobacterium avium complex (MAC) comprises of M. avium, M. intracellulare and probably other less well-defined genospecies. These mycobacterial pathogens primarily cause pulmonary mycobacteriosis in adult patients with chronic lung disease and lymphadenitis in children. However, a close association has been observed between HIV/AIDS and M. avium infection in many countries. M. avium organisms have been isolated from birds, pigs and environmental sources such as soil and water.

Genetic tools such as pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) based methods have been extensively applied for molecular fingerprinting of the pathogenic bacteria to study the molecular epidemiology and to monitor the dissemination of a disease within a community and population. Several of these methods have been applied to study M. avium genotypes also. Since results generated by different methods were difficult to compare, in 1998, a consensus method was proposed for RFLP typing of M. avium strains using IS1245. The application of this method has emphasized the remarkable diversity of genotypes within M. avium isolated from different geographic regions of the world.

Investigations conducted in our laboratory revealed that pulmonary disease due to non-tuberculous mycobacteria (NTM) were not uncommon among Indians and infection due to MAC was observed to be 27.7 per cent among a defined group of patients. Since not much information regarding the molecular fingerprint of the M. avium isolates was available from India, the present study was designed to investigate various genotypes of M. avium isolates of human origin.

Material & Methods

Isolates: The study included 65 MAC isolates obtained from sputum specimens received from the same number of patients with chronic pulmonary illness, attending outpatients department at V.P. Chest Institute, Delhi, India, between March 1998 and February 2001. The HIV status of the patients was not known. M. avium reference strain IWGMT49 (kindly provided by Dick van Soolingen, National Institute of Public Health and the Environment, RIVM, Bilthoven, The Netherlands) was included in all gels used for RFLP analysis.

Isolates were identified as MAC by applying conventional biochemical tests, amplification of M. avium specific insertion sequence IS1245, and PCR amplification of M. avium specific mig gene (macrophage induced gene).

Preparation of genomic DNA: Bacterial DNA was prepared for PCR by suspending loopful of organism in 100µl of sterile water containing 1 per cent w/v Triton-X 100 and heating at 100°C for 30 min. The bacterial lysate was centrifuged at 7227 g for 10 min to spin down the debris. The crude lysate was directly used as the source of DNA template.

IS1245 based restriction fragment length polymorphism (RFLP): Isolates were grown in complete Middlebrook 7H9 liquid medium (DIFCO). Genomic DNA was isolated by cetyltrimethylammonium bromide (CTAB) method. Approximately 2 µg of genomic DNA was digested with enzyme PvuII (MBI, Fermentas) (15U) for 1 h at 37°C. The DNA fragments generated were electrophoretically separated on a 25 cm long 0.8 per cent agarose gel. DNA was blotted on to nylon membrane (Sigma, USA) using vacuum blotter (Bio-Rad, USA) and probed with DIG labeled 427 bp probe generated by PCR from IS1245. Membranes were washed and the detection was performed using the DIG nucleic acid detection kit according to the manufacturers recommendations (Roche Applied Science, Germany). Membranes were scanned and the banding patterns obtained were used for analysis. Fingerprints were analyzed visually and considered identical when all bands shared the same molecular weight position. Each membrane had standard strain (IWGMT49) in one slot.

The probe was prepared by the method described previously. The target DNA used was the plasmid pMA12 (kindly provided by D. van Soolingen, RIVM, Netherlands). Probe was labeled using PCR DIG probe synthesis kit according to the
manufacturers recommendations (Roche Applied Science, Germany). The amplified product was checked on a gel and stored at -20°C. The amplified product was used directly as probe without further treatment.

**PCR typing:** The utility of the PCR typing method for the typing of Indian MAC isolates was also investigated. The PCR typing method used in this study was a previously reported procedure. Briefly, the amplification reaction mixture in a 50 µl volume consisted of 5 µl of template DNA, 5 µl 10X buffer, 2U Taq DNA polymerase (MBI Fermentas), 0.2 mM deoxynucleoside triphosphate (dNTP), 100pM (each) of primers PA (5’-CAGAGCCTCACGCGGA) and PB (5’-CAGAGCCTCACGCGGA). Amplification was performed in a thermocycler (Techne, UK) with an initial denaturation cycle at 95°C for 5 min, and 35 consecutive cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C, with a single final extension step of 10 min at 72°C. Negative control (mix without template) was included in each amplification reaction. PCR products were electrophoretically separated on a 2 per cent agarose gel and detected by ethidium bromide staining and photographed on a UV transilluminator (Fotodyne, UK). It may be noted that though the PCR products are from the DNA sequence lying between IS1245 and IS1311, the method is conventionally known as IS1245 typing. IS1311 sequences were not included in the analysis.

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**Fig. 1.** Representative photograph of IS1245 based RFLP. S- standard strain IWGMT49, M- Molecular wt marker (λ-DNA, cleaved with Eco RI and Hind III), Lanes 1-18 clinical isolates of MAC, Lanes 2, 8 and 9 negative isolates for RFLP, rest positive for RFLP.

**Fig. 2.** Representative photograph showing the banding patterns of clinical isolates obtained on PCR typing. M- 50 bp mol wt marker, P - positive control (IWGMT49), N- negative control.
Discussion

In developing countries like India, little is known about *M. avium* infection. In India, mycobacterial strains are not routinely identified to the species level and hence the true prevalence of *M. avium* is not known. Though a few reports are available from different parts of India stating the prevalence of NTM, in most of these studies the sample size was so small that no specific conclusion could be drawn. However, in the Chingleput BCG trial in south India, it was observed that the isolation of NTM was significant and MAC topped the list accounting for 22.6 per cent of the first thousand NTM isolates identified by a battery of 16 biochemical tests.

Along the same line, our laboratory reported isolation rate of MAC to be 27.69 per cent in a defined group of patients from the Delhi area.

Analysis of our results showed that 84.6 per cent of the isolates tested were typeable by IS1245 based RFLP where as 87.7 per cent were typeable by PCR typing technique.

The 55 (84.6%) isolates that were typeable by IS1245 RFLP showed multiple banding patterns, whereas 15 isolates could be placed in four clusters of identical banding patterns. Cluster I (two isolates) had 10 identical bands, cluster II (three isolates) had 14 bands, cluster III (five isolates) had 12 bands, and cluster IV (five isolates) had 16 bands. All the isolates were analyzed at least twice before reporting.

**PCR typing patterns:** All the 65 isolates were analyzed by the PCR typing using primers directed at the conserved inverted repeats of IS1245 and IS1311. The PCR was designed to amplify DNA segments between multiple copies of these elements, resulting in a strain specific banding profile. Fifty seven isolates were typeable while eight did not produce any PCR product or banding pattern on repeated attempts. The PCR banding profiles were diverse. Among the 57 PCR typeable isolates, a cluster of 14 isolates with identical 3 banded pattern was observed. The positive and negative controls produced reproducible and consistent results in each PCR test.

### Table

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<tr>
<th>No. of fragments</th>
<th>No. (%) of isolates</th>
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<tbody>
<tr>
<td>No banding pattern (0 bands)</td>
<td>10 (15.4)</td>
</tr>
<tr>
<td>&lt;3</td>
<td>8 (12.3)</td>
</tr>
<tr>
<td>4-9</td>
<td>19 (29.2)</td>
</tr>
<tr>
<td>10-15</td>
<td>19 (29.2)</td>
</tr>
<tr>
<td>&gt;16</td>
<td>9 (13.8)</td>
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has also been reported in both human and porcine isolates\textsuperscript{4,25}. The second group had 19 isolates and bands produced were between 4-9. This is a pattern observed in human and porcine isolates in general\textsuperscript{27}. The fourth group had nine isolates with 16-18 bands each. It has been generally propagated that isolates with less than 10 bands are indicative of bird and animal origin whereas isolates with more than 10 bands represent human isolates. Among the Indian human isolates examined by us, 27 (47\%) had RFLP profile suggestive of bird and animal origin indicating a strong association with the environment. In addition, it was observed that the maximum number of bands produced by our isolates was 18 as compared to 27 bands observed in the western isolates\textsuperscript{9,27}.

An attempt was also made to look for the clustering of RFLP profile (identical banding pattern) among the isolates. Four clusters and 40 unique patterns could be observed. Clustering indicated a possibility that these isolates were acquired from a common source of infection. However, the sources of infection could not be determined in the present investigation. Since human-to-human transmission of \textit{M. avium} has not been reported so far, we suspect that the patients acquired the organisms from the environment.

Generally, the climatic conditions of India is diverse because of its vast land mass and geographical position hence, the rate of isolation of NTM from different parts of India would vary. It is a matter of relevance to the future of mycobacterial diseases in India, because with revised national tuberculosis control programme (RNTCP), there is possibility of reduced burden of TB in India, hypothetically and there could be emergence of non-tubercular mycobacterial infections.

As 10 of our isolates were negative for IS1245 RFLP we subjected all the 65 isolates to analysis by PCR typing. Fifty seven isolates gave multiple banding patterns while eight were untypable. The PCR amplification failure may be due to the absence of IS1311 in these isolates as one of the primers has been directed towards this IS. The presence of non specific PCR inhibitors was ruled out by positive amplification for other loci using the same DNA samples (\textit{mig}, IS1245).

It was interesting to note that all the 10 IS1245 RFLP negative isolates could be typed by PCR typing and 5 of the ten RFLP negative isolates were among the 14 isolates with 3-banded pattern. The RFLP profiles of the rest nine isolates were diverse. On analyzing the typing results, it was evident that all the 65 isolates could be typed by one of the two methods used in the study.

Our report confirms the earlier\textsuperscript{27} observation that IS1245 based RFLP is a useful tool for the epidemiological fingerprinting of the \textit{M. avium}. A considerable degree of polymorphism was observed among the isolates from the Indian subjects. In addition, the RFLP analysis indicated environment as the possible source of origin of many of these isolates. PCR typing was simple and easy to perform and could be applied for rapid screening of large number of isolates or to screen multiple colonies in case of suspected polyclonal infection.

In conclusion, our findings showed that by combining the IS1245 based RFLP and PCR methods 100 per cent of the isolates could be genotyped. \textit{M. avium} isolates of human origin from India were highly polymorphic with remarkable genetic diversity and heterogenous RFLP profile.

\textbf{Acknowledgment}

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\textbf{References}


