Indian J Med Res 126, December 2007, pp 575-579

Identification & differentiation of *Mycobacterium avium* & *M. intracellulare* by PCR- RFLP assay using the *groES* gene

V. Aravindhan, S. Sulochana, Sujatha Narayanan, C.N. Paramasivam & P.R. Narayanan

Tuberculosis Research Centre (ICMR), Chennai, India

Received March 28, 2006

***Background & objectives*: We report a new polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) assay using mycobacterial *groES* as a target to identify *Mycobacterium avium* and *M. intracellulare* in clinical samples.**

***Methods*: The assay was standardized using *M. avium* and *M. intracellulare* standard strains obtained from ATCC and was tested with 45 *M. avium-M. intracellulare* complex (MAC) clinical isolates (Of which 31 were from HIV$^+$ individuals). The standard and clinical strains were typed with HPLC based mycolic acid fingerprinting.**

***Results*: Three polymorphisms (*BamHI, BstNI* and *HgaI*) were identified for inter-species differentiation among standard strains; of which, only *HgaI* was found to be useful in clinical isolates. Of the 45 isolates, 25 were *M. avium* and 20 were *M. intracelluare*. MAC isolates, which could not be differentiated by HPLC analysis, were also typed by this method.**

***Interpretation & conclusions*: The use of mycobacterial *groES* as a PCR-RFLP target for *M. avium* and *M. intracellulare* is a simple and rapid method that can complement HPLC in their differentiation.**

Key words *groES* - HPLC - MAC - *Mycobacterium avium* - *M. intracellulare* - PCR-RFLP

Mycobacterium avium – M. intracellulare complex (MAC) comprises two genetically distinct but difficult to discriminate species namely *M. avium*, which predominates (87-98%) in AIDS patients with no anti-retroviral therapy and *M. intracellulare*, which is more frequent among non-AIDS patients[1]. Identifying clinical isolates as *M. avium* and *M. intracellulare* would have both clinical and epidemiological implications. MAC strains occasionally cause human disease that is indistinguishable from tuberculosis. The organisms infect lung, lymph nodes, skin, bones, gastrointestinal and genitourinary tracts. Conventional techniques fail to discriminate *M. avium* and *M. intracellulare*. The assays used to identify these two species include both genotypic methods like polymerase chain reaction (PCR) (*mig*), PCR with restriction fragment length polymorphism (PCR-RFLP) (*hsp65*), probe hybridization and sequencing (16S rRNA) and phenotypic methods like high performance liquid chromatography (HPLC) and serotyping[2]. In general, genotypic assays have proved far superior to conventional methods in terms of specificity, sensitivity

and rapidity[3,4]. Of the several genotypic assays, PCR-RFLP analysis offers an easy, rapid and an inexpensive way to identify several mycobacterial species[5].

The *groESL* operon that encodes 10 kDa (GroES) and 60 kDa (GroEL) heat shock proteins is ubiquitous and evolutionarily conserved among bacteria[6]. The *Hsp65* (*GroEL2*) gene of mycobacteria is well documented for its utility in species identification[7,8]. But the *Hsp10* (*groES*) gene (Rv3418c), though well conserved like the *Hsp65* gene, has not been utilized for species identification, probably due to its small size[9]. Despite its highly conserved amino acid sequence, immunodominant species-specific T- and B-cell epitopes have been identified[10-12] and have been utilized for immunodiagnosis[13]. In the present study, we attempted *M. avium* and *M. intracellulare* species differentiation using *groES*-based PCR-RFLP assay. This assay was also validated using MAC clinical isolates obtained from HIV positive individuals.

Material & Methods

Bacterial strains: All the reference strains of mycobacteria (*M. avium*- ATCC no. 25291, 43015 and *M. intracellulare* – ATCC no. 23434, 23435 and 23436) were from American Tissue Culture Collection (ATCC), USA. The MAC clinical isolates from HIV patients and from non HIV patients (n=45) were collected from the Department of Bacteriology, Tuberculosis Research Centre (ICMR), Chennai. Species identification by high performance liquid chromatography (HPLC) and HIV testing were also carried out at the same centre. Of the 45 MAC clinical isolates, 32 were from HIV positive subjects. The study was conducted during 2002-2005 and most of the samples were collected during this period and a few were from the archival collection.

PCR amplification: DNA extraction was carried out following the standard Cetyl trimethyl ammonium bromide (CTAB)-NaCl method[14, 15]. Mycobacterial *groES* specific primers (MAF- 5' C C T T G A G T A C T A G C A C T C T C A T G 3'; MAR- 5' A T C A G C T T G C T C A T C A G G C T C C 3') (Invitrogen, USA) were used for PCR amplification[16]. PCR reaction was carried out in a final volume of 25µl that contains 5-10ng DNA, 20pM each of forward and reverse primers, 1.5mM $MgCl_2$; 125µM dNTPs, 2.5U Taq polymerase (Amersham, Singapore), 10mM Tris-HCl (*p*H 9.0) and 50mM KCl. The PCR conditions were: 5 min initial denaturation at 95°C followed by 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C, 1 min extension at 72°C which is followed by a final extension at 72°C for 5 min. PCR products were visualized on 2 per cent agarose gel.

PCR-RFLP: Equal quantity of PCR products were digested for 3h with *BamHI*, *BstNI* or *HgaI* (New England Biolabs, USA) at 37°C for *BamHI* and *HgaI* and 60°C for *BstNI* following manufactures instruction. The digested products were visualized on a 2 per cent agarose gel.

HPLC analysis: The use of HPLC to separate the bromophenacyl esters of mycobacterial mycolic acids was as described by Butler *et al*[15]. HPLC was conducted on a Beckman model 330 liquid chromatograph (Beckman Instruments, Inc., Berkeley, CA) equipped with an Altex/Hitachi model 155 UV detector for measuring A254, two Beckman model 110A solvent pumps, and a model 420 pump controller. The mycolic acid p-bromophenacyl esters (5-p.l samples) were applied to a 5-ri. particle size, C18 reverse-phase column (4.6 mm by 25 cm) equilibrated in 91 per cent methanol-9 per cent chloroform. After injection of the sample, the gradient was changed linearly to 30 per cent methanol-70 per cent chloroform over a period of 65 min at a total flow rate of 2 ml/min.

Results & Discussion

PCR was attempted using conserved primers for *groES*, as reported by Cobb and Forthinham[16] on two *M. avium* and three *M. intracellulare* standard strains obtained from ATCC. This yielded a single band of 600bp in both species. The PCR products were gel purified and sequenced (data not shown). Sequence analysis revealed the presence of *BamHI*, *BstNI* (both sites present in *M. avium* and absent in *M. intracellulare*) and *HgaI* (absent in *M. avium* and present in *M. intracellulare*) polymorphisms which could be used for PCR-RFLP analysis. The PCR products were digested with *BamHI*, *BstNI* or *HgaI*. *BamHI* digestion yielded a 400bp and 200bp doublet in *M. avium* and an undigested band in *M. intracellulare*. Several other non tuberculous mycobacteria (NTM) species yielded the same doublet pattern obtained for *M. intracellulare* and thus this polymorphism was not found to be useful for species identification (results not shown). Upon *BstNI* digestion, *M. avium* yielded two distinct bands (500 and 100bp) while the 600bp fragment of *M. intracellulare* remained intact due to

the absence of this restriction site. Upon *HgaI* digestion, *M. avium* fragment remained undigested due to the lack of the *HgaI* site (600bp) while *M. intracellulare* yielded a doublet of 300bp. Thus, out of *BamHI*, *BstNI* and *HgaI*, only *Hga*I and *BstNI* were found to be useful for species differentiation.

To validate this assay on clinical specimens, 45 MAC clinical isolates were subjected to PCR-RFLP assay. These isolates were selected based upon their HPLC pattern. HPLC analysis showed three distinct chromatographic patterns for these isolates: those

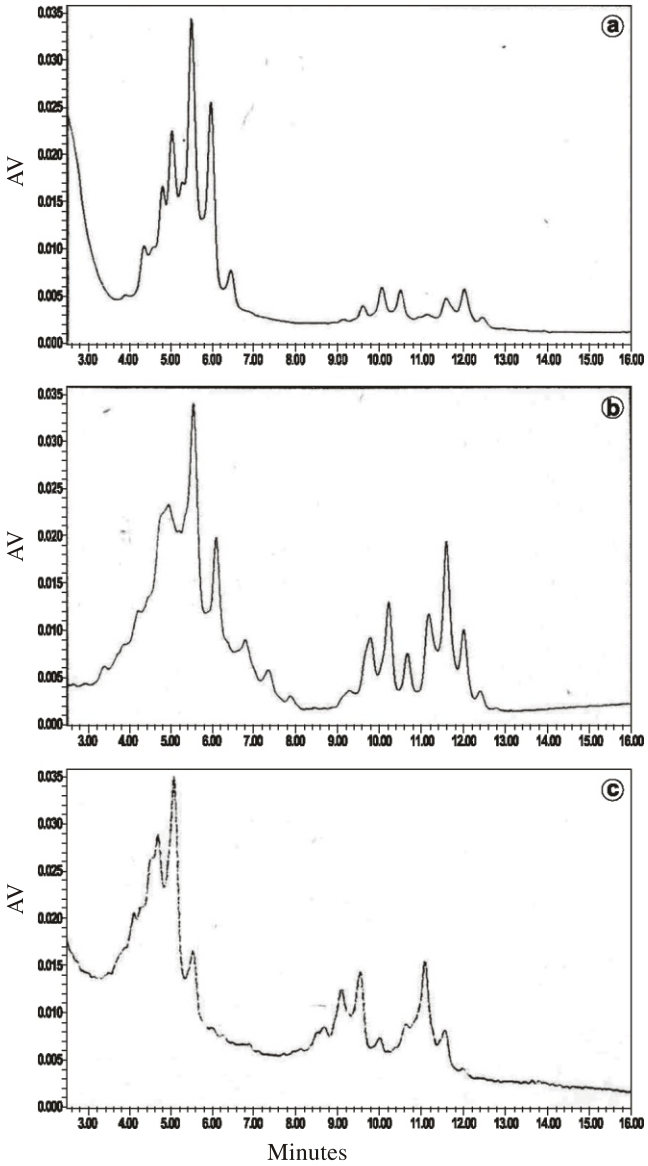

Fig. 1. Representative mycolic acid profile for clinical isolates of (**a**) *M. avium,* (**b**) *M. intracellulare* and (**c**) *M. avium-intracellulare* complex (MAC).

Table. Summary of comparison between the newly developed PCR-RFLP assay and the well established HPLC based mycolic acid analysis. MAC clinical isolates (n=45) were used to validate the *groES* based PCR-RFLP assay. These isolates were also subjected to HPLC analysis for species identification

		HPLC analysis		
		M. avium (n=14)	*M. intracellulare* (n=18)	MAC (n=13)
PCR-RFLP assay	*M. avium* (n=25)	12	4	9
	M. intracellulare (n=20)	2	14	4
	Total		14+18+13= 45	

MAC refers to strains showing an intermediate HPLC profile between *M. avium* and *M. intracellulare*. This group was discriminated by the PCR-RFLP assay

resembling *M. avium*, *M. intracellulare* and an intermediate pattern (Fig. 1a, b and c) indicating significant phenotypic heterogeneity. Based on HPLC results, 14 *M. avium*, 18 *M. intracellulare* and 13 MAC isolates were subjected to PCR-RFLP assay. Upon *BstNI* and *BamHI* digestion all the 45 MAC clinical isolates gave identical band pattern resembling *M. intracellulare* (Fig. 2a, data not shown for *BamHI* digestion) irrespective of their HPLC based phenotype. This indicates the inability of these polymorphisms in permitting species identification among clinical isolates. This also shows that not all polymorphisms seen in standard laboratory strains would be useful for diagnosis. When the same samples were subjected to *HgaI* digestion, clear species discrimination was seen (Fig. 2b). Twelve out of 14 (85.7%) and 14 out of 18 (77.7%) isolates were correctly identified as *M. avium* and *M. intracellulare* respectively by both HPLC and PCR-RFLP (Table). Two were *M. avium* by HPLC and *M. intracellulare* by PCR-RFLP. Similarly 4 were *M. intracellulare* by HPLC and *M. avium* by PCR-RFLP. More importantly, 13 isolates which could not be typed by HPLC, were identified as *M. avium* (9 isolates) and *M. intracellulare* (4 isolates). Thus, of the 45 isolates, 25 were found to be *M. avium* and 20 were *M. intracellulare* by the PCR-RFLP assay. Of the 25 *M. avium* and 20 *M. intracellulare* isolates, 22 and 10 isolates were from HIV-positive subjects respectively. These results were in good accordance with previous reports showing the preponderance of *M. avium* infection among HIV-positive subjects[17,18].

M. tuberculosis and MAC species are the most important mycobacterial pathogens since they account

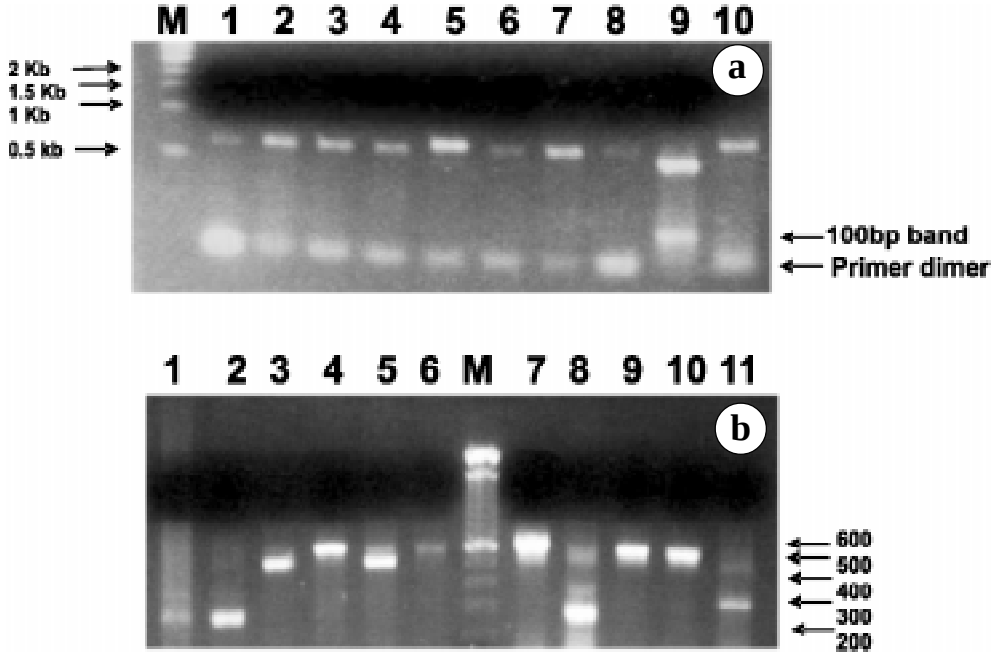

Fig.2. (**a**). RFLP pattern generated by *BstNI* digestion of *groES* gene from the MAC clinical isolates. Molecular size marker- 1kb ladder (NEB) (lane M), MAC clinical isolates (lanes 1-8), *M. avium* std strain (lane 9) and *M. intracellulare* std strain (lane 10). *M. avium* standard strain gave a 500+100bp while *M. intracellulare* remained undigested. The band at the bottom is the primer dimer. (**b**). RFLP pattern generated by *Hgal* digestion of *groES* gene from the MAC clinical isolates. Clinical isolates (lanes 1-6 and 7-9), Molecular size marker -100 bp ladder (BRL) (lane M), *M. avium* std strain (lane 10) and *M. intracellulare* std strain (lane 11). Samples in lanes 1, 2 and 8 were identified as *M. intracellulare* while those in lanes 3, 4, 5, 6, 7 and 9 were identified as *M. avium* based on the band pattern.

for more than 90 per cent of all the mycobacterial infections[11]. Among these three species, *M. avium* and *M. intracellulare* have overlapping phenotypic properties that make their identification difficult by conventional methods. But these two species differ markedly at the genome level as evidenced by unique polymorphisms in otherwise highly conserved genes[3,16,17]. Because of the scarceness of biochemical differences between *M. avium* and *M. intracellulare*, additional techniques such as HPLC, serotyping and genotypic assays have been attempted with the aim of discriminating them. HPLC is time-consuming and not readily available to most clinical laboratories. On the other hand, serotyping has several drawbacks such as producing inconsistent data among laboratories and inability to type all isolates. PCR-RFLP assay is a simple genotypic method which has been used worldwide for mycobacterial species identification.

Earlier Delvallois *et al*[19], have reported limited utility of *Hsp65* based PCR-RFLP in species identification among the south Indian isolates, while the same and other reports[7] have shown the usefulness of this assay among isolates from other countries. Thus, there is a need to use other mycobacterial genes as PCR-RFLP targets, since a single assay may be inadequate in discriminating *M. avium* and *M. intracellulare* strains from diverse geographical locations. Therefore, we have developed an indigenous *groES* based PCR-RFLP assay which was found to be useful for species identification among the south Indian clinical isolates. This assay was also validated using MAC clinical isolates from HIV-positive individuals.

In conclusion, our data showed that *HgaI* based *groES* PCR-RFLP is a simple and rapid method for the identification and differentiation of *M. avium* and *M. intracellulare* among strains that are refractory to HPLC based species identification. The use of DNA amplification based techniques for the diagnosis of MAC is essential for the timely onset of chemotherapy, which can lead to improvement of symptoms, clearing of bacilli and in some instances an increased rate of survival in patients with AIDS suffering from MAC disease.

Acknowledgment

The first author (VA) acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi, for senior research fellowship.

References

1. Falkinham JO, 3rd. Epidemiology of *Mycobacterium avium* infections in the pre- and post-HIV era. *Res Microbiol* 1994; *145* : 169-72.
2. Beggs ML, Stevanova R, Eisenach KD. Species identification of *Mycobacterium avium* complex isolates by a variety of molecular techniques. *J Clin Microbiol* 2000; *38* : 508-12.
3. Teng LJ, Hsueh PR, Wang YH, Lin HM, Luh KT, Ho SW. Determination of *Enterococcus faecalis groESL* full-length sequence and application for species identification. *J Clin Microbiol* 2001; *39* : 3326-31.
4. Yakrus MA, Hernandez SM, Floyd MM, Sikes D, Butler WR, Metchock B. Comparison of methods for identification of *Mycobacterium abscessus* and *M. chelonae* isolates. *J Clin Microbiol* 2001; *39* : 4103-10.
5. Plikaytis BB, Plikaytis BD, Yakrus MA, Butler W R, Woodley CL, Silcox VA *et al*. Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. *J Clin Microbiol* 1992; *30* : 1815-22.
6. Zeilstra-Ryalls J, Fayet O, Georgopoulos C. The universally conserved GroE (Hsp60) chaperonins. *Annu Rev Microbiol* 1991; *45* : 301-25.
7. Brunello F, Ligozzi M, Cristelli E, Bonora S, Tortoli E, Fontana R. Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. *J Clin Microbiol* 2001; *39* : 2799-2806.
8. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993; *31* : 175-8.
9. Baird PN, Hall LM, Coates AR. Cloning and sequence analysis of the 10 kDa antigen gene of *Mycobacterium tuberculosis. J Gen Microbiol* 1989; *135* (Pt 4) : 931-9.
10. Barnes PF, Mehra V, Rivoire B, Fong SJ, Brennan PJ, Voegtline MS *et al*. Immunoreactivity of a 10-kDa antigen of *Mycobacterium tuberculosis*. *J Immunol* 1992; *148* : 1835-40.
11. Chua-Intra B, Ivanyi J, Hills A, Thole J, Moreno C, Vordermeier HM. Predominant recognition of species-specific determinants of the GroES homologues from *Mycobacterium leprae* and *M. tuberculosis*. *Immunology* 1998; *93* : 64-72.
12. Deshpande RG, Khan MB, Navalkar RG. Immunological evaluation of a 12-kilodalton protein of *Mycobacterium tuberculosis* by enzyme-linked immunosorbent assay. *Tuber Lung Dis* 1993; *74* : 382-7.
13. Hussain R, Shahid F, Zafar S, Dojki M, Dockrell HM. Immune profiling of leprosy and tuberculosis patients to 15-mer peptides of *Mycobacterium leprae* and *M. tuberculosis GroES* in a BCG vaccinated area: implications for development of vaccine and diagnostic reagents. *Immunology* 2004; *111* : 462-71.
14. Hermans PWM, van Soolingen D, Dale JW, Schuitema ARJ, McAdam RA, Gatty D, *et al*. Insertion element IS 986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J Clin Microbiol* 1990; *28* : 2051-8.
15. Butler WR, Guthertz LS. Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species. *Clin Microbiol Rev* 2001; *14* : 704-26.
16. Cobb AJ, Frothingham R. The GroES antigens of *Mycobacterium avium* and *Mycobacterium paratuberculosis*. *Vet Microbiol* 1999; *67* : 31-35.
17. Krentz HB, Kliewer G, Gill MJ. Changing mortality rates and causes of death for HIV-infected individuals living in Southern Alberta, Canada from 1984 to 2003. *HIV Med* 2005; *6* : 99-106.
18. Lange CG, Woolley IJ, Brodt RH. Disseminated *Mycobacterium avium-intracellulare* complex (MAC) infection in the era of effective antiretroviral therapy: is prophylaxis still indicated? *Drugs* 2004; *64* : 679-92.
19. Devallois A, Picardeau M, Paramasivan CN, Vincent V, Rastogi N. Molecular characterization of *Mycobacterium avium* complex isolates giving discordant results in AccuProbe tests by PCR-restriction enzyme analysis, 16S rRNA gene sequencing, and DT1-DT6 PCR. *J Clin Microbiol* 1997; *35* : 2767-72.

Reprint requests: Dr P.R. Narayanan, Director, Tuberculosis Research Centre (ICMR), Mayor V.R. Ramanathan Road, Chetput, Chennai 600031, India
e-mail: prnarayanan@trcchennai.in