Identification of a group of nontuberculous mycobacteria isolated from the South Indian BCG trial area by HPLC

J. Daisy Vanitha*, Chandra Immanuel*, Bogumila Szponar‡, Lennart Larsson‡ and C. N. Paramasivan*,†

*Tuberculosis Research Centre (Indian Council of Medical Research), Chennai 600 031, India
‡Department of Medical Microbiology, Dermatology and Infection, University of Lund, Sweden

Twenty-five isolates of nontuberculous mycobacteria isolated from the South Indian BCG trial area were analysed by high performance liquid chromatography (HPLC) for mycolic acid pattern. The chromatograms differentiated the isolates into four species, namely *M. terrae* complex, *M. intracellulare*, *M. parafortuitum* and *M. fortuitum*. Three strains were unidentified, one of which did not show any mycolic acid peaks. All isolates had been identified as *M. diernhoferi* by biochemical methods in a previous study. Nineteen of the isolates were analysed by gas chromatography-mass spectrometry (GC-MS) for the presence of tuberculostearic acid, 2-eicosanol and mycolic acid cleavage products, and were classified as nonchromogens or rapidly growing mycobacteria. The results show that HPLC can discriminate the described mycobacterial species better than biochemical methods and GC-MS.

IDENTIFICATION of nontuberculous mycobacteria (NTM) is important for epidemiological studies and a better understanding of the pathogenesis of the diseases they cause[1,2]. The fact that NTM may cause opportunistic infections among HIV patients necessitates rapid species identification for appropriate management of this increasingly important group of patients[3,4]. Identification of mycobacteria has traditionally been based on growth characteristics and a battery of time-consuming biochemical tests. Unfortunately, the specificity and sensitivity of these tests in recognizing new species are low[5]. Only two techniques seem to have the potential to recognize new species of mycobacteria, namely, genetic and chromatographic analysis[6]. Reverse-phase high performance liquid chromatography (HPLC) of the high molecular weight mycolic acids is rapid and, unlike genetic probes, not limited to the identification of only a few species[7,8]. The present retrospective study was performed in order to evaluate whether mycolic acid analysis by HPLC could be used as a substitute for biochemical tests, and also to characterize NTM by gas chromatography-mass spectrometry (GC-MS) lipid profile. A library of HPLC chromatograms was constructed using reference strains of mycobacteria since differences in instrumentation and analytical conditions could significantly affect the chromatographic profiles. The chromatograms were visually matched with the pattern recognition manual of Centers for Disease Control and Prevention (CDC)[9].

Twenty-five isolates of NTM obtained from the environment and sputum samples from the South Indian BCG trial area were tested by HPLC. These isolates were identified by growth characteristics and biochemical tests done in a previous study as *M. diernhoferi*[10]. Seven of the isolates were sent to CDC for pattern confirmation and 19 of them to Lund University for GC-MS lipid profile. *Staphylococcus aureus* ATCC 29213 was used as a negative control.

Mycolic acid analysis was performed according to the method of Butler *et al.*[9]. In brief, 2 to 3 loopfuls of approximately 3-week-old culture on L–J medium were saponified with methanolic potassium hydroxide at 121°C for one hour. The mycolic acids were extracted into chloroform in an acidic environment. After neutralization, mycolic acids were derivatized with 2,4′-dibromoacetophenone and dicyclohexyl-18-crown-6-ether in acetonitrile and converted to UV absorbing *p*-bromo phenacyl esters. The samples were clarified with acidic methanol and evaporated. The dried samples were reconstituted in methylene chloride and analysed using a reverse-phase C18 column (4.6 mm × 250 mm) packed with 5 µm silica particles (Spinco Biotech, India) with the UV detector set at 254 nm. A solvent gradient system described by Hagen was followed[11]. Briefly, after injection, the initial solvent mixture (methanol : methylene chloride 70 : 30) was maintained for one minute at a constant flow rate of 1.5 ml/min. Over the next 20 min, the solvent composition was changed to 45 : 55. During the next 0.5 min the solvent composition was changed back to the initial composition and the solvent mixture was held to equilibrate the column for five minutes. The total elution time was 26.5 min. Peaks eluted between 6 and 25 min were used for visual comparison. For GC-MS analysis, mycobacteria were heated in 1 M methanolic hydrogen chloride and extracted using *n*-heptane. The extracts were introduced and separated on a fused silica capillary column by using a nonpolar stationary phase. Peak identification was performed by using MS in the electron impact mode[12].

All the reference strains analysed gave HPLC chromatograms, which when compared visually, resembled the patterns reported by the CDC. The information on growth rate already available makes visual comparison easy and accurate. The results of 25 test isolates are summarized in Table 1. By visual comparison with CDC manual, 12 were identified as *M. terrae* complex, 7 as *M. intracellulare*, two as *M. parafortuitum* and one as *M. fortuitum*. Two strains could not be identified be-

†For correspondence. (e-mail: trcicmr@md3.vsnl.net.in(bact3))

Table 1. Summary of results obtained by HPLC and GC-MS

				GC-MS				
						MACP		
Isolate	Source	HPLC-TRC	HPLC-CDC	TBSA	2-eicosanol	C 22:0	C 24:0	C 26:0
B92/1	Water	*M. terrae* complex		+	+	+	+	+
B151/2	Water	*M. terrae* complex		+	+	+	+	+
TS09865	Sputum	*M. terrae* complex		+	–	+	+	–
TS10088	Sputum	*M. terrae* complex	*M. terrae* complex	+	+	+	+	+
TS10408	Sputum	*M. terrae* complex	*M. terrae* complex	–	+	+	+	–
TS11431	Sputum	*M. terrae* complex	*M. terrae* complex	+	+	+	+	+
TS11531	Sputum	*M. terrae* complex		+	+	+	+	+
TS11589	Sputum	*M. terrae* complex		+	+	+	+	+
TS11601	Sputum	*M. terrae* complex		+	+	–	–	–
TS16563	Sputum	*M. terrae* complex	*M. terrae* complex	+	+	+	+	–
TS19592	Sputum	*M. terrae* complex	*M. terrae* complex	+	+	+	+	–
TS16040	Sputum	*M. terrae* complex		…	…	…	…	…
B132/124	Water	*M. intracellulare*		+	+	+	+	–
TS10983	Sputum	*M. intracellulare*		+	+	+	+	–
TS11343	Sputum	*M. intracellulare*		+	+	+	+	+
TS11392	Sputum	*M. intracellulare*		+	+	+	+	–
TS10962	Sputum	*M. intracellulare*		…	…	…	…	…
TS16202	Sputum	*M. intracellulare*		…	…	…	…	…
TS17657	Sputum	*M. intracellulare*		…	…	…	…	…
A145/3	Soil	*Mycobacterium* sp.	NCP	+	+	+	+	–
TS10064	Sputum	*Mycobacterium* sp.	NCP	+	+	+	+	+
TS11378	Sputum	*M. fortuitum*		+	–	+	+	+
TS10186	Sputum	*M. parafortuitum*		…	…	…	…	…
TS17197	Sputum	*M. parafortuitum*		…	…	…	…	…
TS15727	Sputum	No peaks		–	–	–	–	–

TRC, Tuberculosis Research Centre; CDC, Centers for Disease Control and Prevention; TBSA, Tuberculostearic acid; C22:0, Docosanoic acid; C24:0, Tetracosanoic acid; C26:0, Hexacosanoic acid; NCP, No common pattern, …, Not done.

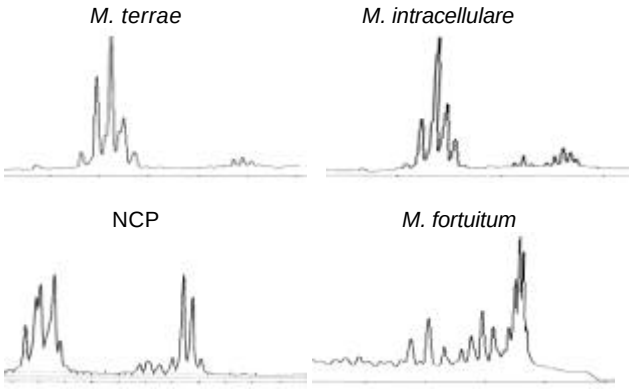

Figure 1. Representative chromatograms.

cause of unusual patterns. One strain had no peaks. Representative chromatograms are shown in Figure 1. Five of the twelve *M. terrae* isolates and the two unidentified species were sent to CDC for mycolic acid analysis to confirm the results. CDC reported similar patterns and the unidentified species were referred as no common pattern (NCP). Of the 25 isolates, 11 of *M. terrae*, 4 of *M. intracellulare* and both the isolates of NCP and *M. fortuitum*, and the one with no peaks were analysed by GC-MS. The lipid markers analysed were tuberculostearic acid (TBSA, 10-methyl octadecanoic acid), 2-eicosanol (2-OH C20:0) and the three mycolic acid cleavage products (MACP)–docosanoic acid (C22:0), tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0). The presence of 2-eicosanol in all but three isolates demonstrated that the mycobacteria belong to species containing wax ester mycolates. The isolate of *M. fortuitum* and unexpectedly, one of the *M. terrae* strains did not contain any 2-eicosanol. The absence of TBSA in one of the isolates could not be explained; this isolate was identified as *M. terrae* by CDC. The isolate that did not have any mycolic acid showed absence of all the lipid markers studied.

It is reported that many species of mycobacteria cannot be identified by biochemical methods and precise identification is uncertain[13]. This type of discrepancy has been found between biochemical methods and Accuprobe or 16S rRNA gene sequencing[14]. Our observation confirmed this phenomenon since the test isolates were previously identified as *M. diernhoferi* by biochemical methods, whereas by using HPLC they were classified as belonging to four different species (*M. terrae* complex, *M. intracellulare*, *M. fortuitum* and *M. parafortuitum*). Among these, *M. intracellulare*, *M. for-*

tuitum and *M. parafortuitum* have three clusters of mycolic acid peaks. The majority of the test isolates were identified as *M. terrae*, which has only two clusters, similar to *M. diernhoferi*. The good agreement between our HPLC results and those from CDC shows that, in spite of the subjectivity in identification by visual recognition and differences in instrumentation and analytical conditions, the chromatographic patterns were consistent, clearly demonstrating inter-laboratory agreement. While the GC-MS data classified the test isolates into rapid growers or nonchromogens, HPLC profiles further differentiated the nonchromogens into three groups, namely *M. terrae, M. intracellulare* and NCP.

This is a report on the comparison of biochemical methods and HPLC for identification of mycobacteria conducted in India. It was found that mycolic acid analysis has high discriminating power and is rapid since it generates results within a day, in contrast to biochemical methods. The cost of HPLC is reported to be 60% less than that of the conventional biochemical tests and 89% less than that of isotopic probes[13]. However, for those species that cannot be identified by HPLC, use of a combination of identification methods is recommended[15].

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Changes in erythrocyte aggregation and deformability during human ageing*

Sanjay Jayavanth and Megha Singh†

Biomedical Engineering Division, Indian Institute of Technology Madras, Chennai 600 036, India

Blood flow through the cardiovascular system is associated with erythrocyte aggregation and deformability. This study is aimed at determining the changes in these parameters associated with human ageing. Blood samples of healthy subjects of different age groups (ranging from 20 to 65 years) were obtained by venepuncture in test tubes containing citrate phosphate dextrose as an anticoagulant. The aggregation of erythrocytes, a reversible process, was determined by the changes in laser-transmitted intensity during gravitational sedimentation of erythrocytes and their formed aggregates. The deformability of erythrocytes was measured by the filtration of erythrocyte suspension through cellulose membrane. The erythrocyte aggregation increases significantly ($P \leq 0.008$) in age groups III (41–50 years) and IV (51–65 years) compared to that of controls (20–30 years). In contrast to this, the erythrocyte deformability in these groups compared to that of the control group, is significantly ($P \leq 0.04$) reduced. These haemorheological changes may lead to disturbed blood flow, and thus could contribute to the increased incidence of circulatory disorders in the elderly.

HUMAN blood contains cells suspended in the plasma. The cellular constituents include erythrocytes, leucocytes and thrombocytes. Erythrocytes form the majority of the cellular constituents (up to 98%); hence any change in their flow behaviour affects the blood flow through the cardiovascular system. Erythrocyte aggregation is one of the most important factors which determines the blood flow at low flow-rates in the microcirculation. The growth process of the chain-like three-dimensional structure of the aggregates depends on the deformation of the shape of the outermost cell (concave or convex) available for attachment to individual erythrocytes or small-size aggregates[1]. Formation of these aggregates represents the balance of energies at the cell surface, contributed by the plasma proteins, erythrocyte membrane charge and its deformability[2]. Under stasis conditions large aggregates are formed, and with increasing flow rate these are broken into smaller aggregates and finally to individual flattened discs of cells aligned in the flow direction[2]. The major

*This paper is dedicated to Dr. R. Natarajan

†For correspondence. (e-mail: msingh@acer.iitm.ernet.in)