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Large-Restriction-Fragment Polymorphism Analysis of *Mycobacterium chelonae* and *Mycobacterium terrae* Isolates

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***Mycobacterium chelonae* and *Mycobacterium terrae* were reported to be frequently present in the environment of the *Mycobacterium bovis* BCG trial area in south India. Six isolates of *M. chelonae* and four isolates of *M. terrae* obtained from different sources in this area were analyzed by pulsed-field gel electrophoresis (PFGE) to examine large-restriction-fragment (LRF) polymorphism using the chromosomal DNA digested with *Dra*I and *Xba*I restriction enzymes. With the exception of one isolate of *M. terrae*, DNA from all other isolates could be digested with *Dra*I and *Xba*I and resulted in separable fragments. Visual comparison of the LRFs showed a unique pattern for each of the isolates tested. A computer-assisted dendrogram of the percent similarity demonstrated a high degree of genetic diversity in this group of isolates. This study demonstrates that species of nontuberculous mycobacteria, particularly *M. chelonae* and *M. terrae*, can be successfully typed by their LRF pattern using PFGE, which does not require species-specific DNA probes.**

Assessment of genetic diversity is important in epidemiological studies of nontuberculous mycobacteria (NTM), as data from these studies could be used to monitor trends in the occurrence of new strains, identify possible sources of infection, and differentiate individual strains (17). In addition, polymorphism studies may have value in providing comparative information for the basis of human colonization, infectivity, and virulence (5). The phenotypic techniques, such as antibiotic or heavy metal susceptibility testing, serotyping, and multilocus enzyme electrophoresis, used to characterize mycobacteria are labor-intensive and have inherent limitations. The molecular techniques, such as restriction fragment length polymorphism, random amplified polymorphic DNA, and pulsed-field gel electrophoresis (PFGE), are simple to perform; of these techniques, PFGE has been used with most bacterial species (16). It has now gained acceptance as a reliable epidemiological tool for the analysis of strain relatedness of both slow- and fast-growing mycobacteria (1, 2, 6, 20).

An earlier study on the characterization of *Mycobacterium avium* complex (MAC) isolates, the most commonly found NTM in the *Mycobacterium bovis* BCG trial area in south India, using techniques such as the AccuProbe test (Gen Probe Inc., San Diego, Calif.), PCR with DT1 and DT6 probes (in-house; Institut Pasteur, Paris, France), PCR restriction analysis of the *hsp65* gene, and 16S rRNA gene sequencing, showed that there was a high degree of heterogeneity in the isolates studied (3). *Mycobacterium chelonae* was reported to be the second most commonly found NTM in the environment of the south Indian BCG trial area (9) and the most frequent NTM associated with nosocomial disease, such as skin and soft tissue infections after outbreaks due to contaminated prosthetic valves, bronchoscopes, peritoneal dialysis equipments, injection vials, etc. (19). *Mycobacterium terrae* was shown to be the second most common organism, accounting for 12.5% of the NTM isolated from sputum samples from symptomatic subjects residing in the south Indian BCG trial area (13). Contamination of clinical samples with *M. terrae* was also reported (12). In recent years, increasing numbers of infections caused by these organisms have been diagnosed, particularly as opportunistic infections in AIDS patients (19). Characterization of these organisms is clinically important, and PFGE will be a useful technique, since it could distinguish the organism at the level of individual isolates (17). In the present study, the genetic relationships among several *M. chelonae* and *M. terrae* isolates obtained from different geographical sources was examined for large-restriction-fragment (LRF) polymorphism by PFGE.

Six isolates of *M. chelonae* (two each from soil, water, and sputum samples) and four isolates of *M. terrae* complex (one from water and three from sputum samples) obtained from the south Indian BCG trial area and maintained on Lowenstein-Jensen slopes were used for this study. All isolates were identified to the species and subspecies levels by routine biochemical methods (9). Species identification of *M. terrae* complex isolates was confirmed by mycolic acid analysis by high-performance liquid chromatography (18). *M. chelonae* strain TMC 1542 was used to optimize the extraction of genomic DNA and PFGE conditions, and this strain was tested in every batch of electrophoresis for quality assurance.

The method of Leblond et al. (10) for in situ DNA extraction from *Streptomyces lividans* was adapted with minor modifications for mycobacterial DNA extraction. Briefly, the cultures were grown in Middlebrook 7H9 (Difco, Detroit, Mich.) medium supplemented with 10% albumin-dextrose complex, 0.2% glycerol, and 0.1% Tween 80. *M. chelonae* cultures were incubated for 1 week, and *M. terrae* cultures were incubated for up to 3 weeks. After the purity of the cultures was checked by Ziehl-Neelsen staining, the cells were harvested and washed twice in TE buffer (10 mM Tris and 1 mM EDTA [pH 8.0]), and the concentration of the cells was adjusted to an optical

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density at 600 nm of 2.0 using sucrose TE buffer (0.3 M sucrose, 25 mM Tris-HCl, 25 mM EDTA). The cells were mixed with an equal volume of 1.5% low-melting-point agarose (Sigma), and 100-μl portions of the cell suspension were poured into agarose molds. The solidified plugs were collected in a 50-ml Falcon tube and incubated at 37°C overnight in 10 ml of sucrose TE buffer containing 2 mg of lysozyme per ml. The solution was replaced with 5 ml of 1% sodium lauryl sarcosine (Sigma, St. Louis, Mo.) in 0.5 M EDTA (pH 8.0) containing 1 mg of proteinase K per ml and incubated at 60°C for 48 h with a change of solution after 24 h. The plugs were washed three times in TE buffer, rinsed twice with TE buffer containing 40 μg of phenylmethylsulfonyl fluoride per ml at 4°C for 4 h to remove proteinase K, and washed thoroughly in TE buffer before storing at 4°C in 20 ml of TE buffer. The restriction enzymes *Dra*I and *Xba*I (Pharmacia Biotech) were selected on the basis of published reports (11, 15). The agarose plugs (10 mm wide) were cut into 1-mm-thick pieces with a sterile scalpel and immersed in restriction buffer containing 100 U of *Dra*I or *Xba*I and incubated overnight at 37°C. The agarose plugs were mounted on the teeth of the electrophoretic comb, and 1% pulsed-field-grade agarose (Amresco) in 0.5× TBE was poured around the comb and allowed to solidify.

PFGE was performed with contour-clamped homogeneous electric field mapper system XA (Bio-Rad, Richmond, Calif.). *Dra*I-digested DNA samples were electrophoresed for 24 h at 14°C at 6 V/cm, with a linear switch time of 0.47 s to 1 min 13.58 s. To separate *Xba*I-digested samples, the program was as described above except that the switch time was linearly ramped from 0.47 to 21.79 s for 20.18 h. *Saccharomyces cerevisiae* whole chromosomes (in-house preparation) and bacteriophage λ DNA concatemer (Pharmacia Biotech) were used as DNA standards. After electrophoresis, the gel was stained with 1 μg of ethidium bromide per ml and photographed using the gel documentation system (Ultra-Violet Products Ltd.), and the image of each gel was stored electronically for analysis.

PFGE pattern analysis was done by visual comparison of the number and similarity of bands. As visual analysis of PFGE profiles was not sufficient to compare highly banded patterns obtained by *Xba*I digestion, computer-assisted analysis was performed. The LRFs of isolates were scored manually for the presence (scored as 1) or absence (scored as 0) of all the bands, and the data were entered into Phylip35 software to generate a dendrogram. The identity of a visually scored fragment was ascertained by comparing the restriction pattern of a standard strain in each gel and calculating the molecular weight based on the standards which were also run in parallel. The results were interpreted according to the criteria of Tenover et al. (16).

Figure 1 shows the LRF patterns of chromosomal DNA from representative isolates of *M. chelonae* and *M. terrae*. *Dra*I digestion of chromosomal DNA generated well-separated DNA fragments ranging from 64 to 1,000 kb in size. Bands between 97.5 and 800 kb in size were used for visual comparison. Lanes 1 through 6 contain *Dra*I-digested *M. chelonae* DNA, and lanes 7 and 8 contain *Dra*I-digested *M. terrae* DNA. The LRF pattern of the two isolates of *M. chelonae* from soil differed by six fragments, the major ones being a 450-kb fragment in the first isolate and a 750-kb fragment in the second isolate. The two isolates of *M. chelonae* from water differed by

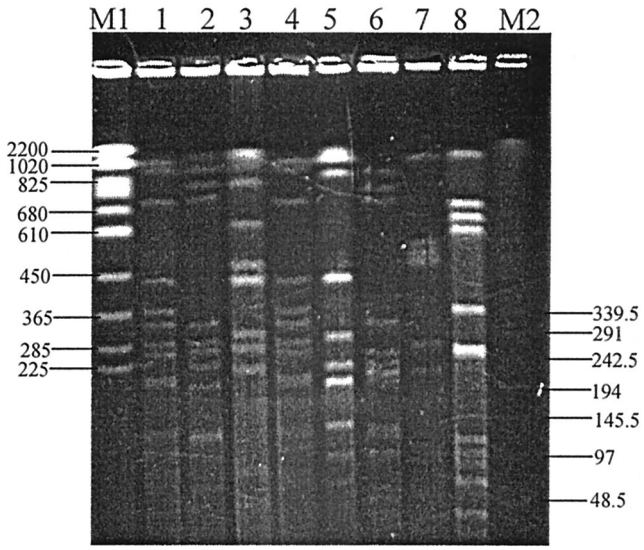

FIG. 1. PFGE analysis of mycobacterial genomic DNA digested with *Dra*I. Pulse time increased linearly from 0.47 to 1 min 13.58 s. Lane M1, yeast chromosomal DNA markers; lanes 1 and 2, *M. chelonae* isolates from soil; lanes 3 and 4, *M. chelonae* isolates from water; lane 5, *M. chelonae* isolate from sputum; lane 6, *M. chelonae* TMC 1542; lane 7, *M. terrae* isolate from water; lane 8, *M. terrae* isolate from sputum; lane M2, λ DNA PFGE markers. Molecular sizes (in kilobases) of the DNA standards are given at the sides of the gel.

seven fragments, and the prominent fragments were the 365- and 680-kb fragments in the second isolate. The two isolates of *M. chelonae* from sputum differed by more than six fragments. Genomic DNA from the three *M. terrae* isolates differed by more than six fragments. One of the *M. terrae* isolates did not give any pattern. Overall, *Dra*I digestion produced a unique LRF pattern for each of the *M. chelonae* and *M. terrae* isolates studied. *Dra*I digestion represents the entire genome of the organism. Hence, the approximate genomic DNA size of each of the isolates could be calculated. The sizes are given in Table 1. The genome size of the majority of the isolates was approximately 4.0 Mb.

The ability to detect LRF polymorphism varies with the restriction enzyme used (21). *Dra*I is shown to be a suitable enzyme for analysis of mycobacterial genomes (15). However, it has been reported that minor changes in genomic DNA of mycobacteria could be detected only with *Xba*I (21). Hence, this enzyme was used to confirm the results obtained with *Dra*I.

Figure 2 shows *M. chelonae* isolates digested with *Xba*I, which generated approximately 15 to 24 smaller fragments ranging from 25 to 450 kb. DNA fragments of 48.5 to 388 kb were used for comparison. Since there were several doublets in almost all the lanes, a second electrophoresis with an extended run time (26 h) was performed to separate the fragments. The two isolates from soil differed in the presence of a 339-kb DNA fragment in the second isolate. The two isolates from water differed in the presence of two DNA fragments of approximately 291 kb in the first isolate. The two isolates from sputum samples differed by more than six bands. Figure 3 shows the *Xba*I LRF profile of *M. terrae* isolates and the patterns of three *M. terrae* isolates, which differed by more than eight fragments. The strain that failed to give a separable digest with *Dra*I also

TABLE 1. Summary of PFGE results for *M. chelonae* and *M. terrae* isolates[a]

Sample	Strain	Isolate source	Identification[b]	No. of large restriction fragments		Genome size[c]
				*Dra*I	*Xba*I	
1	A86/3	Soil	*M. chelonae*	10	22	4,050
2	D73/123	Soil	*M. chelonae*	11	20	4,410
3	B85/3ab	Water	*M. chelonae*	11	22	4,785
4	B86/12	Water	*M. chelonae*	11	20	4,160
5	TS09896	Sputum	*M. chelonae*	11	16	3,869
6	TS10108	Sputum	*M. chelonae*	10	21	4,090
7	TMC 1542		*M. chelonae*	10	15	4,166
8	B92/1	Water	*M. terrae* complex	8	15	3,510
9	TS10088	Sputum	*M. terrae* complex	11	21	4,295
10	TS11431	Sputum	*M. terrae* complex		NP[d]	
11	TS16563	Sputum	*M. terrae* complex	10	19	4,226

[a] *M. chelonae* strain TMC 1542 was used as a reference strain.
[b] Species identification by biochemical methods and high-performance liquid chromatography.
[c] Approximate genome size (in kilobases) based on *Dra*I digestion results.
[d] NP, no pattern.

did not give any pattern with *Xba*I (lane 4). *Xba*I-digested DNA was similar to *Dra*I-digested DNA and generated seven unique LRF patterns for the seven *M. chelonae* isolates and three unique patterns for the three *M. terrae* isolates. This study shows that the use of a single restriction enzyme is sufficient to type all isolates, as unique patterns were obtained with *Dra*I or *Xba*I. It was reported earlier that isolates of *Mycobacterium fortuitum* could be reliably distinguished by using only one restriction enzyme (7). The discriminatory power can be greatly enhanced by performing two-dimensional PFGE (14).

Figure 4 shows a schematic representation of different LRF patterns of *M. chelonae* and *M. terrae* generated by digestion of genomic DNA with *Dra*I and *Xba*I and a dendrogram generated by comparing bands from *Xba*I digestion. The dendrogram revealed that one *M. chelonae* isolate from water and one *M. chelonae* isolate from soil had 85% genetic similarity, and they were closely related to the *M. chelonae* reference strain. Another isolate from water was similar to one of the isolates from sputum. One isolate from soil and one isolate from sputum were genetically distinct. Two *M. terrae* isolates from sputum were closely related and exhibited 75% similarity with the isolate from soil. Earlier reports on polymorphic studies using PFGE patterns of clinical isolates of *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, and *Mycobacterium*

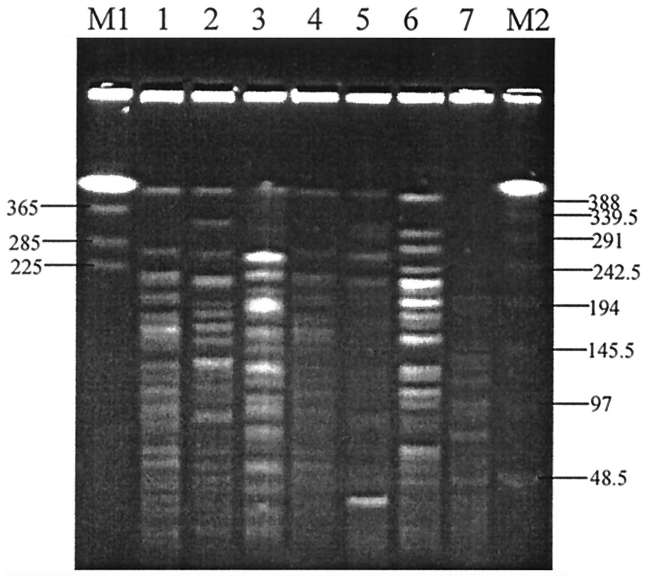

FIG. 2. PFGE analysis of genomic DNA of *M. chelonae* digested with *Xba*I. Total run time was 20.18 h. Pulse time ramped from 0.47 to 21.79 s. *M. chelonae* isolates from soil (lanes 1 and 2), water (lanes 3 and 4), and sputum (lanes 5 and 6) and strain TMC 1542 (lane 7) were used. Lane M1, yeast chromosomal DNA markers; lane M2, λ DNA PFGE markers. Molecular sizes (in kilobases) of the DNA standards are given at the sides of the gel.

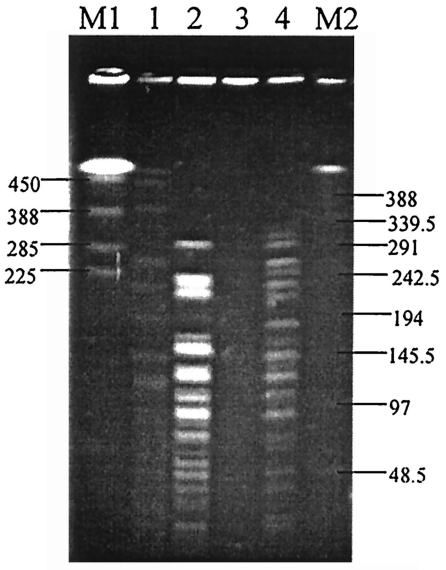

FIG. 3. PFGE analysis of *M. chelonae* digested with *Xba*I. Total run time was 24 h. Pulse time ramped from 0.47 to 21.79 s. *M. chelonae* isolates from water (lane 1) and sputum (lanes 2 to 4) were used. Lane M1, yeast chromosomal DNA markers; lane M2, λ DNA PFGE markers. Molecular sizes (in kilobases) of the DNA standards are given at the sides of the gel.

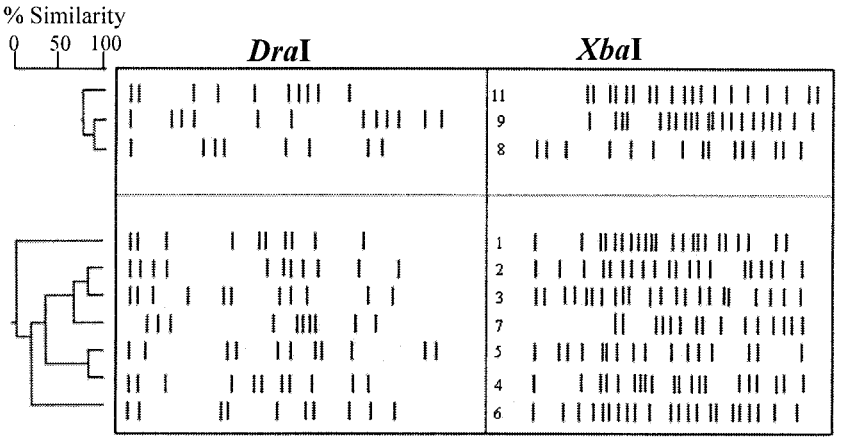

FIG. 4. Schematic diagram showing distinct LRF patterns obtained after cleavage of the genomic DNA from *M. chelonae* and *M. terrae* complex with restriction enzymes. A dendrogram of the LRF types obtained with *Xba*I is shown to the left. The lane numbers are the sample numbers shown in Table 1.

simiae using software programs, such as Biosystematica, Taxotron, and Dendron, have shown that there was genetic diversity among the isolates of one species (4, 8, 11).

In this study, *M. chelonae* and *M. terrae* isolates for which species-specific probes are not available commercially were successfully typed by PFGE. The isolates were tested on several occasions, and reproducible patterns were obtained. The unique pattern obtained for each of the isolates demonstrates that there is a high degree of genetic polymorphism between the isolates originating from the same geographical area. It seems likely that horizontal transfer of large segments did not occur. This also demonstrates that genetic variation may influence their antibiotic susceptibility profile and disease pathogenesis. A recent phylogenetic analysis of clinical and laboratory isolates of *M. tuberculosis* using large-sequence polymorphism and single-nucleotide polymorphism suggests that polymorphism among mycobacteria is more extensive than anticipated and that genetic variation may have an important role in disease pathogenesis and immunity (5). Animal pathogenicity studies of *M. chelonae* and *M. terrae* may help in determining their difference in colonization and pathogenicity.

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