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Random Amplified Polymorphic DNA Genotyping of *Mycobacterium malmoense*

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The suitability of random amplified polymorphic DNA-PCR for the detection of differences between *Mycobacterium malmoense* strains was evaluated. With two of the 32 tested primers seven fingerprint patterns which proved excellent in distinguishing intraspecies variations of *M. malmoense* were obtained. The combination of the results obtained with the two primers permitted a clear separation of the strains. This technique is useful for analyzing species whose DNA sequences are not known. It can easily be adapted to test any mycobacterial species.

Mycobacterium malmoense is a nontuberculous mycobacterial species which is being isolated with increasing frequency from humans in northern and northwestern Europe. After the *Mycobacterium avium-M. intracellulare* complex, it is the second most common species of slowly growing mycobacteria found in clinical specimens in Finland. The clinical manifestations of infections caused by *M. malmoense* are indistinguishable from those of tuberculosis (2, 5, 7, 14). The epidemiology and the natural habitats of *M. malmoense* are poorly known. The environment has been suggested as the source of human infections, as is the case with other nontuberculosus mycobacteria. Accurate identification of bacterial strains is essential for following the routes of infection. However, a lack of adequate methods of strain identification has been one of the fundamental problems limiting epidemiological studies with these mycobacteria.

Typing techniques based on DNA analysis have been introduced in recent years for mycobacteria. The most commonly used DNA technique is restriction fragment length polymorphism (RFLP) analysis (4, 9, 10, 16), which can be coupled with PCR to increase the sensitivity of the technique (12, 13). RFLP analysis is suitable for species whose genomic DNA sequence is at least partly known, particularly if the known sequence is repeated several times in the genome.

Random amplified polymorphic DNA (RAPD)-PCR is a method applied successfully in genotyping several species of bacteria other than mycobacteria (1, 3, 17). In RAPD-PCR, the amplification of the genomic DNA is attained by using one randomly selected oligonucleotide primer. At a low annealing temperature, the primer with an arbitrary sequence will hybridize to both strands of the DNA often enough to define a set of templates for amplification, provided that other conditions have also been optimized. In cases where the distance between the primers on opposite strands is appropriate, amplification with DNA polymerase will produce a fragment of DNA visible as a band in a subsequent gel electrophoresis. The number of bands of the same size has been shown to correlate with the degree of relationship between samples (15). The more common bands that are seen, the closer relatives the strains can be considered to be. Substitutions of single nucleotides in the bacterial DNA may affect the annealing of the primers and consequently lead to variations in the banding pattern. We have adapted RAPD-PCR for typing of mycobacteria by applying this technique to test a group of *M. malmoense* strains. Our results show that RAPD-PCR offers a new and useful tool for epidemiological study of nontuberculous mycobacteria.

Forty-five *M. malmoense* strains, including a reference strain (S-816; I. Juhlin, Malmö, Sweden), were examined. Six strains were obtained from P. A. Jenkins (Mycobacterium Reference Unit, Public Health Laboratory Service, Cardiff, United Kingdom). The rest were isolates from Finnish patients (6). The identity of the strains was confirmed by biochemical tests, gas-liquid chromatography analysis of cellular fatty acids, and thin-layer chromatography of mycolic acid and glycolipid composition (6). The strains were also analyzed by RFLP analysis using ribosomal RNA (rRNA) gene fragments as probes (8).

DNA was isolated from pure cultures of the strains by a modification of the method described by McFadden et al. (11), as we have reported earlier (8). In brief, mycobacteria were digested by Nagarse protease and were treated with lysozyme. After redigestion with Nagarse in the presence of sodium dodecyl sulfate, the lysates were extracted by phenol and chloroform. RNA was digested by ribonuclease A. DNA was precipitated by ethanol precipitation.

The primers were selected randomly from the primer pool used for different research purposes at our institute. They were 10 to 25 nucleotides in length; their G+C content ranged from 44 to 81%. Commercial OP primers (10-mers) (Operon Technologies, Inc., Alameda, Calif.) were also used.

TABLE 1. Primers used in the pilot study to type eight *M. malmoense* strains

Primer	Sequence	Length (nt)[a]	G+C content (%)
VGH3	TGAGCTCGCAGACATGCGGTGGGCC	25	68
SP26	GTGGCCGCCCTGCTGC	16	81
OPA2	TGCCGAGCTG	10	70
OPA7	GAAACGGGTG	10	60
OPA18	AGGTGACCGT	10	60
OPA20	GTTGCGATCC	10	60

[a] nt, nucleotides.

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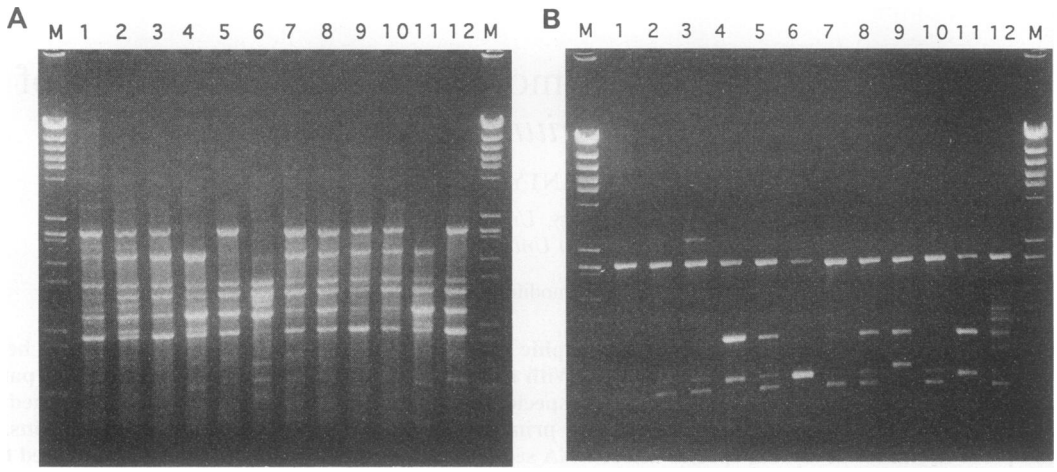

FIG. 1. Examples of fingerprints generated in genotyping of *M. malmoense* strains (lanes 1 to 12) by RAPD-PCR using (A) OPA2 and (B) OPA7 as the primers. Lane 1 is the *M. malmoense* reference strain. Lanes 4, 6, 7, and 11 represent the British strains. Lanes M are *Hin*dIII- and *Eco*RI-digested lambda DNA used as a molecular weight marker.

Twenty-five microliters of PCR mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 40 ng of primer, 240 μM (each) dATP, dCTP, dGTP, and dTTP (Promega), 20 ng of genomic DNA, and 1 U of DynaZyme DNA polymerase (Finnzymes Oy). The amplification program (DNA Thermal Cycler; Perkin Elmer Cetus) included 40 cycles consisting of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. After the PCR was complete, the samples were electrophoresed in 1.5% agarose gel containing 0.5 μg of ethidium bromide per ml and 1× Tris-acetic acid-EDTA running buffer. The bands were visualized by UV light.

As the first step, primers suitable for RAPD fingerprinting of the template DNA of *M. malmoense* had to be selected. To find primers which generate an optimal number, i.e., 3 to 20 bands after electrophoresis of the PCR products, 32 randomly selected primers were tested with the *M. malmoense* reference strain. Sixteen of the 32 primers were 10-mers, and the rest were 16- to 25-mers. The 10-mers generally produced better fingerprints of the *M. malmoense* reference strain than did the 16- to 25-mer primers, some of which generated no PCR products at all (results not shown). Six primers produced 4 to 12 easily discernible bands, and they were selected for further studies (Table 1). Eight strains, including the reference strain, were used in the next step to test the six primers. The results proved that two primers, OPA2 and OPA7, were superior for distinguishing the intraspecies variations of *M. malmoense*.

In the next phase, 45 *M. malmoense* strains were analyzed by using the OPA2 and OPA7 primers. Examples of the fingerprints obtained are presented in Fig. 1A and B. Differences between some strains were distinct, whereas the fingerprint patterns of others looked similar, except for some differences in band intensities, if only one of the primers was used (lanes 1, 3, and 7 to 10 in Fig. 1A).

Seven distinct fingerprint patterns could be obtained by the use of both primers, OPA2 and OPA7. Though some of the strains had similar fingerprint patterns when studied with one primer, they could be separated when the other primer was also used. RAPD-PCR distinguished *M. malmoense* strains better than the RFLP method that we have used previously (8). When we combined the typing results produced by amplification with two or more primers run in parallel, the ability to distinguish differences among strains was excellent. Hence, we recommend the use of at least two primers.

The reproducibility of the technique was analyzed by testing every DNA batch twice. No differences in repeat fingerprints could be detected. Only minor variations in the intensities of bands in parallel runs were evident; these were apparently caused by variations in the amounts of template DNA (Fig. 2).

Thin-layer chromatography of surface glycolipids present on mycobacteria has so far demonstrated only five chemotypes (6), and with RFLP of rRNA genes, only five different RFLP ribotypes could be obtained (8). However, in our earlier studies, the chemotypes and RFLP types did not correlate with each other. Since RAPD-PCR is based on random hybridization between the primers and mycobacterial DNA, it was not surprising that no connection between RAPD-PCR fingerprinting results and the chemotypes or the RFLP types could be observed.

Fragmentation of genomic DNA during storage was ob-

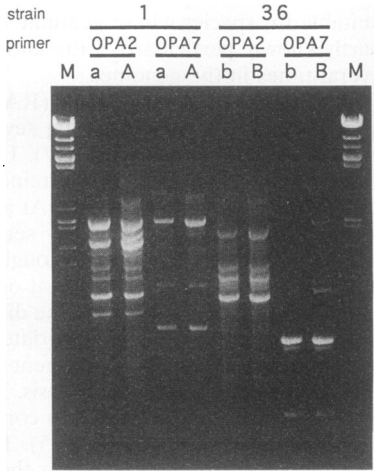

FIG. 2. Reproducibility of fingerprinting patterns in RAPD-PCR was confirmed by analyzing in parallel runs the amplicons obtained from different batches of four strains. Every batch was analyzed by primers OPA2 and OPA7. This figure shows the results of strain 1 (batches a and A) and strain 36 (batches b and B). Lanes M are *Hin*dIII- and *Eco*RI-digested lambda DNA used as molecular weight markers.

served to result in band patterns which were difficult to interpret. The use of freshly prepared DNA is therefore recommended. However, the effect of storage on the template DNA was not analyzed in detail. We visualized the RAPD-PCR products by agarose gel electrophoresis with ethidium bromide, although it is known that this allows only the strongest bands to be detected. Better detection of bands could probably be obtained by using polyacrylamide gel electrophoresis combined with silver staining for visualization of the PCR products (3). However, although this might produce more information, the increase in the number of the bands might cause an overall loss of accuracy.

Since prior knowledge of the genomic DNA sequence is not required in RAPD-PCR, in contrast to typing methods based on restriction polymorphism or sequencing, this method enabled us to study *M. malmoense* strains. Another advantage of this technique was the small amount of DNA needed; this is also the case with other PCR techniques. A requirement, however, is that a pure culture of the strain be used, because foreign DNA would yield extra bands in RAPD fingerprints. In further use of the method to examine other mycobacterial species, the selection of suitable primers for each species will be critical to produce an optimal number of discernible bands for sufficient resolution. In random amplifying methods, primers have usually been about 10 nucleotides in length, although divergent sizes (4 to 29 nucleotides) have also been used (1, 3, 17, 18). All in all, this technique is simple, rapid, and cheap to perform, but it demands extremely meticulous work practices to avoid contamination. At the moment, this appears to be the only method which offers a reliable tool for the distinct separation of *M. malmoense* strains.

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