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Identification of *Mycobacterium* Species by Using Amplified Ribosomal DNA Restriction Analysis

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A rapid procedure for the identification of cultured *Mycobacterium* isolates, based on the combination of enzymatic amplification and restriction analysis, is described. The 16S rRNA genes (rDNA) of 99 strains belonging to 18 different species of the genus *Mycobacterium* were enzymatically amplified. Amplified rDNA restriction analysis with the enzymes *Cfo*I, *Mbo*I, and *Rsa*I was carried out. The combination of the amplified rDNA restriction analysis patterns obtained after restriction with *Cfo*I and *Mbo*I enabled differentiation between *Mycobacterium asiaticum* (number of strains = 4), *M. avium* (*n* = 22), *M. chelonae* (*n* = 5), *M. flavescens* (*n* = 1), *M. fortuitum* (*n* = 6), *M. gordonae* (*n* = 6), *M. intracellulare* (*n* = 13), *M. marinum* (*n* = 7), *M. nonchromogenicum* (*n* = 1), *M. simiae* (*n* = 5), *M. terrae* (*n* = 5), the *M. tuberculosis* complex (*n* = 11), and 2 of 4 strains of *M. xenopi*. Further restriction with *Rsa*I was necessary to differentiate between the species *M. kansasii* (*n* = 5), *M. scrofulaceum* (*n* = 4), and the 2 other *M. xenopi* strains. The *M. avium-M. intracellulare* complex was characterized by a specific *Mbo*I pattern, and *M. avium* and *M. intracellulare* strains could further be differentiated by restriction with *Cfo*I. The whole procedure, including sample preparation prior to the polymerase chain reaction, can be carried out within 8 h, starting from a pure culture.

Recently the clinical importance of several mycobacterial species has increased, especially since the spread of the human immunodeficiency virus pandemic (22). The antibiotic susceptibility of some of these species may be extremely low. Therefore, there is a growing need to identify most mycobacteria to the species level. The advent of polymerase chain reaction (PCR) technology not only has enhanced the sensitivity of the hybridization methods which are used for molecular identification but also has led to the development of new strategies to detect specific sequences. e.g., restriction analysis of the enzymatically amplified 65-kDa heat shock protein-encoding gene (*hsp65*) has been used thus far for the identification of mycobacterial species (15, 17, 18). rRNA gene (rDNA) sequence information has been used previously to identify mycobacteria (4). It has recently been shown that species-specific information enclosed in the rDNA can also be accessed by restriction analysis of the amplified gene and that this technique, which we have previously called amplified rDNA restriction analysis (ARDRA) (20), is suitable for the identification of closely related species (3, 7, 9, 20). In this study we evaluate the suitability of ARDRA for the identification of *Mycobacterium* species.

MATERIALS AND METHODS

Table 1 lists the strains used.

The identification method used has been described previously (20). Briefly, strains were grown on Ogawa egg yolk medium at 30°C. A 1-μl loopful of colony growth was suspended in 500 μl of distilled water, and the suspension was boiled for 10 min. Forty-five-microliter aliquots of PCR mix, containing 1.25 U of *Tth* polymerase, 200 μM (each) deoxynucleotide triphosphates, and 0.02 μM (each) primers in reaction buffer (1.5 mM $MgCl_2$ and 50 mM KCl in 10 mM Tris HCl, pH 9.0), were overlaid with 60 μl of mineral oil. Five-microliter aliquots of the sample preparations were added with filter-protected tips. After initial denaturation at 95°C for 5 min, the reaction mixture was run through 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. Finally, a 10-min extension period at 72°C was carried out. The presence and yield of specific PCR products were controlled by agarose (1% [wt/vol])-ethidium bromide (50 ng/ml) gel electrophoresis for 30 min at 7 V/cm. The length of the amplicon was about 1,500 bp. Oligonucleotide primers were derived from conserved regions present at the edges of the 16S rDNA and synthesized as described previously (19). The sequences of the primers were 5′ TGGCTCAGATTGAACGCTGGCGGC (forward) and 5′ TACCTTGTTACGACTTCACCCCA (reverse).

Restriction was carried out for 1 h at 37°C in 20-μl volumes of commercially supplied incubation buffer containing 5 U of restriction enzyme *Cfo*I (GCGC), *Mbo*I (GATC), or *Rsa*I (GTAC) and 5 to 15 μl of PCR product. The volume of amplicon used in the restriction mixture was adjusted arbitrarily, on the basis of the visually observed fluorescence intensity of the amplified rDNA fragment in the control gel. Restriction was stopped by the addition of 5 μl of 5× sample buffer (25% [wt/vol] glycerol, 0.5% [wt/vol] sodium dodecyl sulfate, 50 mM EDTA, 0.05% bromophenol blue). Restriction fragment patterns were analyzed by gel electrophoresis of 10 μl of each restriction mixture at 7 V/cm for 2 h in 3% (wt/vol) agarose in 89 mM Tris–89 mM boric acid–2 mM EDTA electrophoresis buffer, pH 8.0, containing 50 ng of ethidium bromide per ml.

Gels were photographed, patterns were observed and compared visually, and pattern labels were assigned arbitrarily. Fragments smaller than 300, 150, and 350 bp were not considered for the interpretation of restriction patterns with *Mbo*I, *Cfo*I, and *Rsa*I, respectively, because of poor visibility and reproducibility.

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TABLE 1. *Mycobacterium* species and strains used for the development of the identification scheme

Species	Strain(s)[a]
M. africanum	VUB B207
M. asiaticum	ITG 8181, ITG 8182, ITG 8183, ITG 8184
M. avium	ATCC 15679, ITG 5647, ITG 5887 (sv1), ITG 5872 (sv2), ITG 5878 (sv2), ITG 5893 (sv3), ITG 5874 (sv 4), ITG 5903 (sv5), ITG 5882 (sv8), ITG 5884 (sv8), ITG 5927 (sv9), ITG 5984 (sv10), ITG 5897 (sv11), ITG 7708, ITG 7849, ITG 7886, VUB B005, VUB B181, VUB B192, VUB B237, VUB D104, VUB D110
M. bovis BCG	VUB B206, VUB B238
M. bovis	VUB B114, VUB B185, VUB B198, VUB B204, VUB B235
M. chelonae	
A	ATCC 19977, VUB A007, ITG 7701
B	ITG 7794, ITG 7971
M. flavescens	ATCC 14474
M. fortuitum	
A	VUB A046, ITG 7907, ITG 8020
B	ITG 4166
C	ITG 4306, ITG 6158
M. gordonae	ITG 7703, ITG 7704, ITG 7836, ITG 7838, ITG 8059, VUB D045
M. intracellulare	ITG 5913 (sv13), ITG 5915 (sv12), ITG 5917 (sv14), ITG 5918 (sv14), ITG 5920 (sv18), ITG 5921 (sv17), ITG 5922 (sv19), ITG 5924 (sv20), ITG 5929 (sv15), ITG 5880 (sv16), VUB B004, VUB B186, VUB B240
M. kansasii	ITG 7727, ITG 8201, ITG 8238, ITG 8242, VUB E008
M. marinum	CIPT 140120001, VUB C006, ITG 1727, ITG 1728, ITG 3836, ITG 4621, ITG 7223
M. nonchromogenicum	ATCC 19530
M. scrofulaceum	ATCC 19981, VUB D083, VUB D087, VUB D097
M. simiae	ITG 4485, ITG 4821, ITG 4826, ITG 4833, ITG 4844
M. terrae	
A	ATCC 15755, ITG 7369, ITG 7414
B	CIPT 140320001
C	ITG 7415
M. tuberculosis	VUB B225, VUB B227, VUB B228
M. xenopi	
A	NCTC 10042, ITG 7828
B	ITG 6082/2, ITG 6147

[a] Abbreviations: VUB, Department of Microbiology, Academic Hospital of the Free University of Brussels, Brussels, Belgium; ITG, Department of Microbiology, Institute for Tropical Medicine, Antwerp, Belgium; ATCC, American Type Culture Collection, Rockville, Md.; sv, serovar (according to reference 11); CIPT, Collection Institut Pasteur, Paris, France; NCTC, National Collection of Type Cultures, London, United Kingdom.

RESULTS

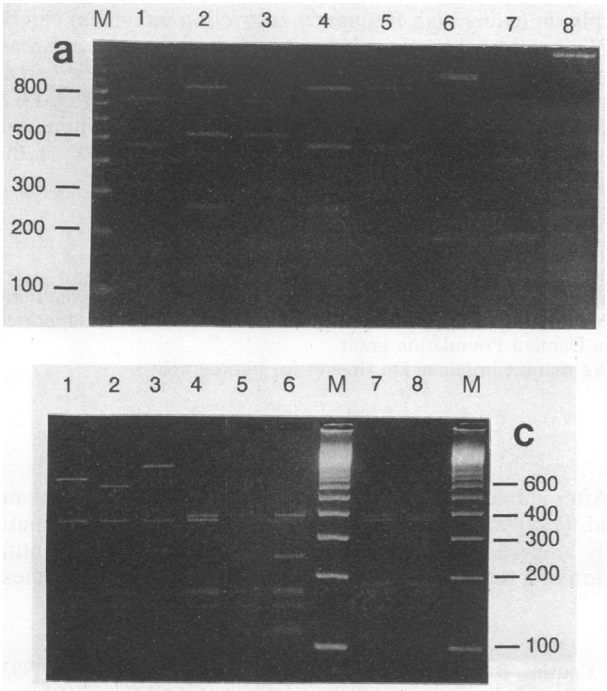
Figure 1 represents the different ARDRA patterns after restriction with *Mbo*I (Fig. 1a), *Cfo*I (Fig. 1b), or *Rsa*I (Fig. 1c). Figure 2 represents a more detailed illustration of some closely resembling ARDRA patterns after restriction with *Cfo*I. Table 2 represents an identification scheme based on the combinations of patterns obtained after restriction with *Cfo*I, *Mbo*I, and *Rsa*I. ARDRA patterns after restriction with *Rsa*I are indicated only for the three species for which this enzyme was needed to reach a final identification. Some species could be subdivided according to the different combinations of ARDRA patterns observed after *Mbo*I and *Cfo*I restriction. These pattern combinations were labelled arbitrarily A, B, or C. Restriction with *Mbo*I yielded a total of seven patterns. After restriction with *Cfo*I a total of 14 patterns was observed. Restriction with *Rsa*I was carried out for strains of 11 species only, and a total of five patterns was established.

Restriction with single enzymes yielded species-specific patterns for only a few species. Restriction with *Mbo*I yielded a characteristic pattern for *Mycobacterium simiae* strains (Fig. 1a, lane 8) and strains of the *M. avium-M. intracellulare* complex (Fig. 1a, lane 2). *M. gordonae* strains (Fig. 1b, lane 8) and the *M. flavescens* strain (Fig. 1b, lane 10) were readily identifiable after restriction with *Cfo*I. Restriction with *Cfo*I alone also enabled identification of *M. fortuitum* and *M. terrae*, since for both species a total of six patterns was found and these patterns were not encountered for strains belonging to other species (Fig. 1b, lanes 4, 6, and 7 and lanes 11, 12, and 13, respectively).

The combination of the patterns obtained after restriction with *Mbo*I and *Cfo*I was sufficient to achieve a final identification for most species. All *M. avium* and *M. intracellulare* strains could, as mentioned above, be separated clearly from other species by their characteristic *Mbo*I pattern (Fig. 1a, lane 2) and were further differentiated from each other by their different *Cfo*I patterns (Fig. 1b, lanes 1 and 2). The *M. tuberculosis* complex could be differentiated from other species by its unique combination of *Mbo*I and *Cfo*I restriction patterns (arbitrarily labeled *Mbo*I 1 and *Cfo*I 1).

Three species shared common *Mbo*I and *Cfo*I patterns and could be differentiated from each other only after restriction was carried out with a third enzyme. Restriction with *Rsa*I yielded different patterns for *M. kansasii*, *M. scrofulaceum*, and 2 strains of *M. xenopi* (Fig. 1c, lanes 1 to 3).

Most of the ARDRA patterns are confirmed by computer analysis, i.e., computer-assisted restriction analysis (DNAsis; Hitachi, Brisbane, Calif.) performed on sequence entries in the EMBL and GenBank data bases. e.g., the characteristic high-molecular-weight band observed after the amplified 16S rDNA of *M. simiae* was restricted with *Mbo*I (Fig. 1a, lane 8) was generated by computer restriction analysis and calculated to be 1,214 bp. Equally, the specific high-molecular-weight fragment observed when the 16S rDNA of *M. gordonae* was restricted with *Cfo*I (Fig. 1b, lane 8) was calculated to measure 682 bp.



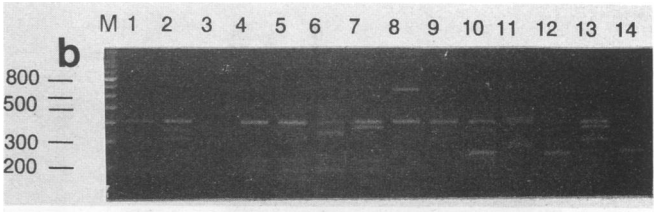


FIG. 1. Overview of ARDRA patterns observed on an ethidium bromide-stained 3% agarose gel (after 2 h of electrophoresis at 7 V/cm) after restriction of the enzymatically amplified 16S rDNA with *Mbo*I (a), *Cfo*I (b), or *Rsa*I (c). Lanes M, marker lanes. Molecular sizes are indicated in base pairs. (a) Only fragments of more than 300 bp are taken into account to differentiate the *Mbo*I patterns. Lanes: 1, *Mbo*I pattern 1, *M. bovis* VUB B114; 2, pattern 2, *M. avium* VUB B005; 3, pattern 3, *M. chelonae* A (ATCC 19977); 4, pattern 4, *M. gordonae* ITG 7703; 5, pattern 4, *M. kansasii* ITG 7727; 6, pattern 5, *M. fortuitum* B (ITG 4166); 7, pattern 6, *M. terrae* A (ITG 4977); 8, pattern 7, *M. simiae* ITG 4485. (b) Only fragments of more than 150 bp are taken into account to differentiate the *Cfo*I patterns. Lanes (corresponding with assigned *Cfo*I pattern numbers): 1, *M. bovis* BCG (VUB B206); 2, *M. avium* ATCC 15679; 3, *M. chelonae* A (ATCC 19977); 4, *M. fortuitum* A (ITG 7907); 5, *M. simiae* ITG 4485; 6, *M. fortuitum* C (ITG 4306); 7: *M. fortuitum* B (ITG 4166); 8, *M. gordonae* ITG 7703; 9, *M. marinum* ITG 1728; 10, *M. flavescens* ATCC 14474; 11, *M. terrae* A (ITG 7369); 12, *M. terrae* B (CIPT 140320001); 13, *M. terrae* C (ITG 7415); 14, *M. xenopi* B (ITG 6082/2). (c) Only fragments of more than 350 bp are taken into account to differentiate the *Rsa*I patterns. Lanes: 1, *Rsa*I pattern 1, *M. kansasii* ITG 8242; 2, pattern 2, *M. avium* ATCC 15679 (and also observed for *M. scrofulaceum* strains); 3, pattern 3, *M. xenopi* A (NCTC 10042); 4, pattern 4, *M. simiae* ITG 4833; 5 and 6, pattern 5, *M. chelonae* A (ATCC 19977 and ITG 7701); 7 and 8, pattern 4, *M. chelonae* B (ITG 7794 and ITG 7971).

DISCUSSION

The technique described here combines several important features of appropriate species identification methods. First, ARDRA is technically less demanding (PCR, restriction, and agarose gel electrophoresis) than other molecular biology approaches, like direct sequencing (4) or detection and identification using species-specific probes (e.g., see reference 5). Second, ARDRA allows identification within 1 day, starting from a pure culture, and thus its speed is comparable with that of other molecular approaches. Finally, ARDRA allows identification of every species (except for the species of the *M. tuberculosis* complex) studied here. Techniques which use specific amplification of fragments (1, 2, 12) or specific lengths of the amplica (12, 14, 21) as the identification strategy can be applied for a few species only. The amplified genome fragment restriction analysis techniques which have been described for *Mycobacterium* spp., all relying on the restriction of amplified *hsp65* gene fragments, have a low discrimination capacity (15, 18) or have to contend with high intraspecific variability (17). Hybridization with species-specific probes (2, 5, 8, 16) requires the development and the availability of probes for every species. Sequencing of parts of the mycobacterial 16S rDNA (4) yields the most taxonomic information of the molecular techniques compared here but is generally thought to be too laborious for use in the clinical microbiology laboratory.

The low number of patterns that is obtained after the 16S rDNA is restricted with a single enzyme is disadvantageous with respect to the discrimination capacity of the technique, since the same restriction profile may be found for different species. As a result, at least two, and for some species three, different enzymes have to be used to reach a final identification. However, this disadvantage may turn out to be an advantage. Using restriction of amplified protein-encoding genes may yield a large number of restriction profiles and result in frequent intraspecific differences. This problem was met in the study of Plikaytis et al. (17), in which a total of 10 patterns was observed for 31 *M. gordonae* strains studied. The six *M. gordonae* strains tested in this study yielded a single pattern whether *Cfo*I or *Mbo*I was used. This may

TABLE 2. Proposed identification scheme based on ARDRA patterns obtained after restriction with *Cfo*I, *Mbo*I, and *Rsa*I

ARDRA pattern after restriction with:			Species	No. of strains tested
*Mbo*I	*Cfo*I	*Rsa*I[a]		
1	2		*M. asiaticum*	4
	10		*M. flavescens*	1
	4		*M. fortuitum* A	3
	7		*M. fortuitum* C	2
	9		*M. marinum*	7
	12		*M. terrae* B	1
	1		*M. tuberculosis* complex	11
	14		*M. xenopi* B	2
2	2		*M. avium*	22
	1		*M. intracellulare*	13
3	3		*M. chelonae* A	3
4	5		*M. chelonae* B	2
	8		*M. gordonae*	6
	1	1	*M. kansasii*	5
	1	2	*M. scrofulaceum*	4
	1	3	*M. xenopi* A	2
	13		*M. terrae* C	1
5	6		*M. fortuitum* B	1
6	9		*M. nonchromogenicum*	1
	11		*M. terrae* A	3
7	5		*M. simiae*	5

[a] Results for *Rsa*I are indicated only for the three species which cannot be identified after restriction with *Mbo*I and *Cfo*I.

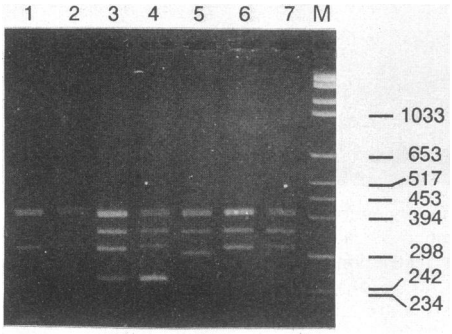


FIG. 2. Detailed illustration of some closely resembling ARDRA patterns observed on an ethidium bromide-stained 3% agarose gel (after 3 h of electrophoresis at 7 V/cm) after restriction with *Cfo*I of the enzymatically amplified 16S rDNA. Lanes M, marker lanes. Molecular masses are indicated in base pairs. Lanes: 1, *Cfo*I pattern 1, *M. bovis* BCG (VUB B206); 2, pattern 11, *M. terrae* A (ATCC 15755); 3, pattern 2, *M. asiaticum* ITG 8181; 4, pattern 12, *M. terrae* B (CIPT 140320001); 5, pattern 3, *M. chelonae* A (ATCC 19977); 6, pattern 9, *M. marinum* VUB C006; 7, pattern 9, *M. nonchromogenicum* ATCC 19530.

reflect the better-conserved nature of the 16S rDNA compared with that of protein-encoding genes.

On the other hand, the intraspecific differences observed in this study may correspond with true taxonomical entities, e.g., at the subspecies level. For the six *M. fortuitum* strains and the five *M. chelonae* strains, respectively, three and two pattern combinations were observed. Whether this subdivision of *M. fortuitum* corresponds to the three biovariants of *M. fortuitum* (10) remains to be elucidated. Apparently the cluster of three *M. chelonae* strains (ATCC 19977, VUB A007, and ITG 7701) named *M. chelonae* A in this study is characterized by unique ARDRA patterns whatever enzyme is used. The data presented here suggest that *M. chelonae* A strains belong to a separate species, even only distantly related to other *Mycobacterium* species, and confirm the elevation of *M. chelonae* subsp. *abscessus* to the species level (strain ATCC 19977 is the type strain of *M. abscessus* [13]).

When ARDRA is used to identify species, starting from a pure culture, both primers are preferably universal, to cover the range of all eubacteria. Because of the universality of the primers used, strains other than mycobacteria, by preference those which are laborious and/or difficult to identify with conventional methods, e.g., nonfermenting gram-negative rods, can be included in a single ARDRA test. Preliminary results (data not shown) indicate that an rDNA sequence (ACA AGC TTC GTC CCA ATC GCC GAT C) may be specific for the genus *Mycobacterium* and thus could be used for detection.

We prefer to use the name "ARDRA" for the technique described here. ARDRA has been named "restriction fragment length polymorphism analysis of 16S ribosomal DNA" by Jayarao et al. (9). Although this name is correct, it does not refer to the use of enzymatic amplification, and the use of this name can lead to confusion with ribotyping (6), which is sometimes—inappropriately—referred to as "rRNA gene restriction analysis" (6), although a more correct definition for ribotyping is "analysis of chromosomal restriction fragment length polymorphisms with rDNA."

In summary, we showed that enzymatic amplification of the 16S rDNA combined with restriction analysis of the amplicon (using high-frequency restriction enzymes) can be applied to identify several species of the genus *Mycobacterium*. The technique, as it is presented here, is conceived to enable rapid identification starting from pure cultures. PCR applied for this purpose is less prone to contamination problems than is PCR used for detection, because of the large number of target molecules initially present.

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ADDENDUM

After submission of the first version of this paper, Telenti et al. (19a) showed that restriction analysis of an enzymatically amplified fragment of the *hsp65* gene enabled identification of a total of 29 mycobacterial species and subspecies.

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