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Novel Allelic Variants of Mycobacteria Isolated in Brazil as Determined by PCR-Restriction Enzyme Analysis of *hsp65*

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Human isolates of *Mycobacterium* collected in 16 different states of Brazil were submitted to PCR-restriction analysis (PRA) of a 439-bp fragment of the *hsp65* gene with *Hae*III and *Bst*EII. Fourteen allelic variants not described in clinical isolates so far were observed among 36 (10%) of 356 Brazilian strains, including a new pattern for *Mycobacterium scrofulaceum*, *M. intracellulare*, and *M. flavescens*, two new patterns for *M. fortuitum*, three new patterns each for *M. gordonae* and *M. terrae*, and one new pattern for *M. avium* complex-like strains. Two unidentified strains each also presented a new pattern, strongly suggesting that *Mycobacterium* genotypes are distributed biogeographically. The PRA procedure was also performed with 43 reference isolates belonging to 34 species, adding a further six new patterns to the identification algorithm. A database containing the normalized restriction patterns of both enzymes was constructed. Patterns available on the Internet can be introduced into this database, which will make possible the comparison of genotypes from isolates from different parts of the world.

Identification of mycobacteria to the species level is relevant for patient management and is traditionally performed by evaluation of the morphological and biochemical characteristics of the organism. This is, however, a time-consuming and laborious process, and accurate identification is often not possible due to the increasing number of *Mycobacterium* species with overlapping phenotypic characteristics (20). During the last decade, nucleic acid sequence-based identification procedures have been developed, and commercially available systems such as Accuprobe (Gen-Probe, San Diego, Calif.) and INNO LiPA Mycobacterium (Innogenetics NV, Ghent, Belgium) are important new acquisitions for the diagnostic laboratory but characterize a limited number of species (13, 19).

Restriction enzyme analysis of *Mycobacterium*-specific PCR products generates mostly species-specific DNA patterns, and one approach, analysis of part of the *hsp65* gene by PCR-restriction analysis (PRA) (23), has been used for diagnosis (6, 23), taxonomic evaluations (15), and characterization of isolates with novel characteristics (12, 14). Originally, a PRA identification algorithm for 24 different *Mycobacterium* species was developed (23), and this was very recently expanded to 54 different species (2). In earlier studies with *Mycobacterium* strains isolated in Brazil, five undescribed *hsp65* patterns—three for *Mycobacterium avium* (11, 14), one for *M. fortuitum*, and one for *M. gordonae* (4)—were observed. Because those studies mainly concentrated on strains from Rio de Janeiro and São Paulo and geography-related variability in genotypes has been reported (5, 12), we analyzed a large number of strains from different states of Brazil.

Due to the growing number of *hsp65* alleles being described in the literature, visual interpretation is becoming more cumbersome and the use of computer-assisted pattern recognition for identification of a limited number of slowly growing *Mycobacterium* species had already been suggested in 1992 (16). At that time, however, no sophisticated software for pattern recognition was available, and although more recently a PRA database was presented for clinically significant actinomycetes, the genotypes of only nonpigmented rapidly growing mycobacteria were included (25). We therefore constructed a database with the *Bst*EII and *Hae*III patterns obtained for clinical and reference isolates with GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium).

MATERIALS AND METHODS

Culture of *Mycobacterium* strains and DNA extraction. The *Mycobacterium* reference strains used in this study are listed in Table 1. Clinical isolates from humans were obtained from the National Reference Center for Tuberculosis or from the Hospital Clementin Fraga Filho, Federal University of Rio de Janeiro. As demonstrated in Fig. 1, the 358 isolates were obtained from 16 different states from Brazil's five main regions (the number of strains isolated and the state populations as a proportion of the national population are given in parentheses), as follows: from the northern region, Amazonas (1 strain; 1.6% of the national population) and Pará (2 strains; 3.6%); from the central-east region, Distrito Federal (19; 1.2%), Goiás (3 strains; 2.9%), and Mato Grosso do Sul (3 strains; 1.2%); from the northeast region, Alagoas (2 strains; 1.6%), Bahia (13 strains; 7.6%), Ceará (2 strains; 4.4%), Pernambuco (3 strains; 4.6%), and Rio Grande do Norte (2 strains; 1.6%); from the southeast region, Espírito Santo (5 strains; 1.8%), Minas Gerais (6 strains; 10.5%), Rio de Janeiro (223 strains; 8.5%), and São Paulo (36 strains; 21.8%); and from the southern region, Rio Grande do Sul (11 strains; 5.9%) and Santa Catarina (5 strains; 3.2%); 21 strains were of unknown origin.

Identification of mycobacterial isolates by conventional or commercially avail-

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FIG. 1. Political map of Brazil showing the states where mycobacterial strains were isolated. Northern region, Amazonas (AM) and Pará (PA); central-east region, Distrito Federal (DF), Goiás (GO), and Mato Grosso do Sul (MS); northeast region, Alagoas (AL), Bahia (BA), Ceará (CE), Pernambuco (PE), and Rio Grande do Norte (RN); southeast region, Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), and São Paulo (SP); southern region, Rio Grande do Sul (RS) and Santa Catarina (SC).

able methods. All clinical isolates had been submitted to conventional identification procedures as described by Kent and Kubica (9). Some *Mycobacterium* samples that could not be identified by conventional testing were sent to Specialty Laboratories (Santa Monica, Calif.) for analysis of mycolic acids with a Beckman System Gold high-pressure liquid chromatography instrument and pattern recognition with Infometrix Pirouette software. A small number of strains were identified by the INNO LiPA Mycobacterium assay (Innogenetics NV, Zwijnaarde, Belgium) or were submitted to sequencing of the 16S-23S internal transcribed spacer region.

DNA extraction, PCR, and restriction enzyme analysis. High-quality DNA from *Mycobacterium* reference cultures was prepared as described earlier (18), while the extraction of nucleic acid from clinical isolates was performed by submitting 50 μl of a liquid culture or a loopful of a *Mycobacterium* mass from solid cultures to three cycles of freezing-boiling (5 min at −70°C and 10 min at 100°C) in 0.5 ml of 10 mM Tris-HCl–1 mM EDTA–1% Triton X-100. PCR-restriction fragment length polymorphism analysis was performed as described earlier (4). In summary, 10 ng of purified mycobacterium DNA or 2 μl of frozen-boiled material was added to a 50-μl PCR mixture containing 50 mM KCl (pH 8.3), 1.5 mM $MgCl_2$, 10% glycerol, 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer Tb11 and Tb12 (24), and 1.25 U of *Taq* polymerase. Amplification consisted of 45 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by a final extension step at 72°C for 7 min. Fifteen microliters of amplified product was digested with 10 U of *Hae*III or 6 U of *Bst*EII under mineral oil. Analysis of the restriction enzyme products was done by gel electrophoresis on a 5% agarose with a 50- or 25-bp DNA ladder (Gibco BRL, Gaithersburg, Md.) as a molecular size marker.

Construction of PRA pattern database. For evaluation of the influence of the electrophoresis matrix on DNA fragment size determination, restriction fragments of *M. avium* and *M. tuberculosis* were separated in 8% polyacrylamide and 4% Nusieve agarose–1% agarose (4:1; FMC BioProducts, Rockland, Maine), 5% agarose (Gibco BRL), 4% MS12 agarose (Hispanagar S. A., Burgos, Spain), or 3 or 4% MS8 agarose (Hispanagar). Molecular sizes, calculated from the band positions, were compared after they were normalized against pUC18, PBR322, and a 25-, 50-, or 100-bp ladder (Gibco BRL). Electrophoresis conditions were standardized to ensure reproducibility within and between gels. The photographs were scanned at 300 dots per inch, and the images were introduced into GelCompar software (version 4.2; Applied Maths, Sint-Martens-Latem, Belgium). Conversion and normalization were performed as described in the manufacturer's manual by using 700 dots as a working frame on the *y* axis, and similarity between restriction patterns was defined by using the Dice similarity coefficient. The position tolerance applied for correct identification of identical patterns was determined separately for each restriction enzyme and on pairs of patterns containing bands in different molecular size ranges. The reproducibility of the whole procedure was evaluated by comparing the restriction patterns for *M. tuberculosis* and *M. avium* applied in different gels against the database. The patterns were also compared with those present on the Internet (http://www.hospvd.ch:8005), after the images were downloaded as TIF files and converted and normalized with GelCompar software.

RESULTS

Identification patterns of clinical isolates. Genetic patterns were obtained for 357 clinical isolates, including published and new patterns, and the pattern distribution is presented for each species in Table 1. The clinical samples containing *M. leprae* had the pattern published by Rastogi et al. (17), confirming the lack of genetic variability in this species. Isolates of the *M. tuberculosis* complex, *M. szulgai*, *M. triviale*, and *M. marinum*, had PRA pattern as described by Telenti et al. (24). Among the strains of *M. gordonae*, the recently described pattern *M. gordonae* VII (4) was observed in a single strain, while three patterns that have so far been undescribed were observed in eight strains (42%). The first new pattern is identical to pattern *M. gordonae* III except for the presence of an extra *Hae*III band of 60 bp, while the third new pattern is similar to pattern 8 present on the PRA website (http://www.hospvd.ch:8005) and to the pattern described by Brunello et al. (2), except that it lacks the bands of 70 and 55 bp. Among the strains identified as *M. chelonae* or *M. abscessus*, all were *M. abscessus* (6) and presented bands of 70 or 60 bp and 55 bp upon digestion with *Hae*III. The largest group consisted of isolates belonging to the *M. avium* complex (MAC), including 170 strains (89%) of *M. avium* and 22 strains (11%) of *M. intracellulare*. Among the strains of *M. avium*, pattern *M. avium* I was observed in 75% of the strains, while a smaller fraction had the recently described patterns *M. avium* II (31 strains; 18.1%) and *M. avium* III (11 strains; 6.4%). Among the strains of *M. intracellulare*, half had pattern *M. intracellulare* I, while the other half had a new pattern that lacked the 60-bp band upon digestion with *Hae*III. Among five strains of *M. scrofulaceum*, four (80%) had a new pattern. Among 10 strains that had initially been conventionally identified as MAC, 2 were characterized by PRA as having pattern *M. lentiflavum* I and 4 were identified as having pattern *M. simiae* I; 4 strains presented with unpublished patterns. When the conventional identification procedure was repeated for these strains, some characteristics were not consistent with those of MAC (positive Tween 80 test), and sequencing of the internal transcribed spacer of one of these strains demonstrated similarities of 96.8% with those of MAC-like strains, 94.3% with those of *M. scrofulaceum* strains, 90.4% with those of *M. avium* strains, and 89.9% with those of *M. intracellulare* strains (W. Mijs, personal communication); we therefore consider these strains MAC-like. Among the 11 strains of the *M. fortuitum-M. peregrinum* complex, 7 were *M. fortuitum*, including 3 strains with pattern *M. fortuitum* I (containing 60- and 55-bp fragments in the *Hae*III digest), 3 strains with Brazilian pattern *M. fortuitum* III, which was observed previously (4), and 1 strain of *M. fortuitum* with a new pattern. Among the four strains of *M. peregrinum*, one had pattern *M. peregrinum* I

TABLE 1. PRA patterns observed in 356 clinical mycobacterial isolates

Species	No. of strains	PRA pattern[a] (no. of strains)
M. tuberculosis	72	*M. tuberculosis* complex
M. avium	171	*M. avium* I (128), *M. avium* II (31), *M. avium* III (11)
M. intracellulare	22	*M. intracellulare* I (11), new pattern 235/115/100-145/125[b] (11)
M. scrofulaceum	5	*M. scrofulaceum* I (1), new pattern 235/115/85-125/95 (4)
M. kansasii	20	*M. kansasii* I (19), *M. kansasii* III (1)
M. chelonae/abscessus	12	*M. abscessus* I (4), *M. abscessus* II (8)
M. fortuitum/perigrinum	11	*M. fortuitum* I (3), *M. fortuitum* III (3), new pattern 235/130/85-145/125/100/60 (1), *M. peregrinum* I (1), *M. peregrinum* II (1), new pattern 235/205-145/140/100/60 (2)
M. gordonae	19	*M. gordonae* I (4), *M. gordonae* II (1), *M. gordonae* III (5), *M. gordonae* VII (1), new pattern 1 (235/115/100-125/110/60) (1), new pattern 2 (235/115/100-140/120/95) (5), new pattern 3 (320/115-125/110) (2)
M. flavescens	2	*M. flavescens* I, New pattern 235/205-135/60
M. lentiflavum	2	*M. lentiflavum* I
M. triviale	1	*M. triviale* I
M. szulgai	3	*M. szulgai* I
M. leprae[c]	3	*M. leprae* I
M. marinum	1	*M. marinum* I
M. simiae	4	*M. simiae* I
M. terrae	3	New pattern 1 (320/115-145/115/70), new pattern 2 (235/115/100-145/70/60), new pattern 3 (320/115-140/85/60)
MAC-like	4	New pattern 441-125/100
Unidentified	2	New pattern 1 (441-160/115/100), new pattern 2 (235/115/100-140/110/100/90)

[a] As reported by Telenti et al. (24), Devallois et al. (5), Taylor et al. (23), Leão et al. (11), da Silva Rocha et al. (4), and Brunello et al. (2).
[b] The patterns are based on the molecular sizes of the bands after digestion with *Bst*EII and *Hae*III, which are specified for the new patterns.
[c] Amplified directly from clinical samples.

with a band of 55 bp upon digestion with *Hae*III, as described by Brunello et al. (2) and as present on the PRA website; one had pattern *M. peregrinum* II, as described by Devallois et al. (6) (pattern 235/205-140/120/100/60, also present on the website); and two had a new pattern identical to that of pattern *M. peregrinum* I except for the presence of a band of 60 bp instead of one of 55 bp. Among two strains of *M. flavescens*, one had a new pattern. The three strains characterized as *M. terrae* each had a different new pattern; the third pattern was almost identical to that obtained with our reference strain of *M. chitae*, except for a band in the 50- to 40-bp range. The pattern for *M. chitae* was identical to that present on the PRA website and recently published by Brunello et al. (2), but neither of those sources reported bands smaller than 50 bp. Finally, two strains that had been conventionally identified as MAC could not be characterized to the species level and had new patterns; reidentification by conventional procedures indicated that the phenotypic characteristics of the strains did not match those of any known species (data not shown).

Identification patterns of reference strains. The genetic patterns for 44 reference isolates belonging to 35 different species were determined, as demonstrated in Table 1. The identification patterns for species that had been published before (6, 23, 24) were mostly confirmed except for those for *M. chelonae*, *M. flavescens*, and *M. xenopi* type strains, which had an additional *Hae*III band of 60 bp, and for *M. fortuitum*, which had two unreported bands of 60 and 55 bp. Brunello et al. (2) very recently published identification patterns for 54 mycobacterial species and calculated restriction fragment profiles after polyacrylamide gel electrophoresis and sequencing analysis. In the present study, the molecular sizes interpolated after electrophoresis in agarose gels were very similar to those determined by Brunello et al. (2) after sequence analysis; and we confirmed their patterns for *M. agri*, *M. aichiense*, *M. chitae*, *M. diernhoferi*, *M. obuense*, *M. rhodesiae*, and *M. thermoresistibile*; *M. smegmatis*, however, had an *Hae*III band of 145 bp both in our study and on the PRA website instead of the band of 154 bp reported by Brunello et al. (2). The PRA patterns that we obtained for *M. aurum* and *M. tokaiense* were different from those described by Brunello et al. (2) (Table 2), but the pattern for one of our *M. aurum* strains was identical to pattern *M. aurum* 2, available on the PRA website. We have no explanation for the fact that the second *M. aurum* isolate, apparently with the same American Type Culture Collection strain number, had a different pattern. We also determined restriction profiles for *M. chelonae* subsp. *niacinogenes*, *M. chubuense*, and *M. komossense*, species for which no identification patterns have been reported earlier (Table 2).

Construction of a database. Although the band position on the gel was particular to each gel matrix, no influence of gel composition on band position was observed after normalization against external molecular size markers (data not shown) and the type of molecular size markers had no influence on the final band position; the 25- and 50-bp ladders were, however, more practical for visual recognition of band position. Staining with ethidium bromide before, during, or after electrophoresis also had no influence on the final band position. For automated recognition of PRA patterns with GelCompar software, optimal tolerance positions of 1.5% for *Hae*III fragments and 2% for *Bst*EII fragments were adopted; the use of more stringent or more relaxed tolerance position settings resulted in a failure to notice identical patterns or the consideration of similar patterns as identical, respectively (data not shown). By use of these stringency conditions, the *Hae*III and *Bst*EII patterns for all *M. tuberculosis* ($n = 15$) and *M. avium* ($n = 16$) strains that had been applied on seven different gels were 100% identical to their respective patterns in the database (data not shown). Also, the restriction patterns for species

TABLE 2. Restriction patterns of 43 reference isolates from 34 *Mycobacterium* species

Species	Strain[a]	Pattern[b]
M. africanum	UG MB3	*M. tuberculosis* complex I
M. agri	ATCC 27406	235/130/85-160/145/60
M. aichiense	ATCC 27280	320/115-195/70/60
M. asiaticum	ATCC 25276; RIVM Z26114	*M. asiaticum* I
M. aurum	ATCC 23366	441-125/115/70/60
	ATCC 23366	320/115-140/95/60/55
M. avium	ATCC 25291	*M. avium* I
M. bovis	ATCC 19210	*M. tuberculosis* complex I
M. bovis BCG-Moreaux	ATCC 35736	*M. tuberculosis* complex I
M. chelonae	NCTC 946; UG MB41	*M. chelonae* I + 60
M. chelonae subsp. *niacinogenes*	ATCC 19237	320/115-175/145
M. chitae	ATCC 19627	320/115-140/85/60
M. chubuense	ATCC 27278	235/205-140/85/60
M. diernhoferi	ATCC 19340	320/115-145/140/60
M. flavenscens	ATCC 14474	*M. flavescens* I + 60
M. fortuitum	ATCC 6841	*M. fortuitum* I + 60/55
M. gordonae	ATCC 14470	*M. gordonae* I
M. gordonae ureolyticum	RIVM MIS222	*M. gordonae* III
M. intracellulare	ATCC 13950	*M. intracellulare* I
M. kansasii	ATCC 12478	*M. kansasii* I
M. komossense	ATCC 33013	320/115-145/140/60
M. mageritense	UAM	*M. mageritense* I
M. marinum	ATCC 927; RIVM MIS 14	*M. marinum* I
M. neoaurum	ATCC 25790	*M. neoaurum* I -55
M. obuense	ATCC 27023	235/205-140/85/60
M. phlei	ATCC 11758	*M. phlei* I
M. porcinum	ATCC 33776	235/205-140/125/95/55
M. rhodesiae	ATCC 27024	320/115-160/125/60
M. simiae	ATCC 25275	*M. simiae* I
M. smegmatis	ATCC 19420	*M. smegmatis* I
M. smegmatis mc^2155	Pasteur Institute	*M. smegmatis* I
M. szulgai	NCTC 10831	*M. szulgai* I
M. terrae	ATCC 15755	*M. terrae* I
M. thermoresistibile	ATCC 19527	235/205-175/135/70
M. tokaiense	RIVM T 47502	235/130/85-140/95/80/60
M. triviale	ATCC 23292	*M. triviale* I
M. tuberculosis H37Rv	ATCC 27294	*M. tuberculosis* complex
M. tuberculosis H37Ra	ATCC 25177	*M. tuberculosis* complex
M. vaccae	ATCC 15483	*M. vaccae* I
M. xenopi	RIVM MYC527	*M. xenopi* I

[a] UG, University of Ghent, Ghent, Belgium; ATCC, American Type Culture Collection, Manassas, Va.; RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; NCTC, National Collection of Type Cultures; CRPHF, Reference Center for Tuberculosis Professor Hélio Fraga, Rio de Janeiro, Brazil; UAM, Autonomous University of Madrid.

[b] The numbers represent the molecular sizes (in base pairs) of the bands.

present on the PRA website, when identical, were 100% identical to those present in the database (data not shown). The molecular sizes interpolated with GelCompar software were very similar to those deduced from sequencing analysis by Brunello et al. (2).

Upon comparison of the patterns obtained in this study, those published by Brunello et al. (2), and those available and downloaded from the PRA website, we found that 13 patterns either are shared (9 patterns) or have differences of only 5 to 10 bp (4 patterns) and could be considered identical by visual or automated evaluation (Table 3).

DISCUSSION

Brazil has a vast territory of 8,547,404 km^2, and a considerable part of the country has a warm and humid climate, conditions favorable for the growth of mycobacteria. A systematic study of mycobacteria from the environment and sputum samples performed in India, in which soil, dust, and water samples were analyzed, suggested a relation between the *Mycobacterium* species present in water samples and in the sputa of humans with symptoms of lung infection (8). Favorable environmental conditions permitting the existence of a large variety of environmental strains could be one of the reasons why 10% of the clinical isolates in this study had PRA patterns that have so far not been published. The distribution of genetic subtypes by biogeography has been described for species such as *M. intracellulare* (5), *M. kansasii* (1), *M. simiae* (11), and *M. ulcerans* (7). In the present study and in accordance with the work of Telenti et al. (24), we almost exclusively encountered the *M. kansasii* I genotype; this is in contrast to the findings from other studies (6, 15), in which a considerable part of the *M. kansasii* strains had other genotypes, probably because isolates came from different continents. Significant genetic variability has also been described for *M. gordonae*, and although *M. gordonae* I is the most frequent allele in most studies (23, 24), other genotypes such as *M. gordonae* III and a previously undescribed pattern occur more frequently in Brazil. Also, in contrast to studies performed in other countries (6, 22, 23, 25), no PRA patterns were observed for *M. chelonae*, showing that *M. abscessus* is mostly responsible for infections caused by organisms of the *M. chelonae-M. abscessus* complex in Brazil. Although several reports have indicated that *M. chelonae* is pathogenic, the present observation suggests the need for a comparison of the clinical significance of the two species. Recently published identification algorithms describe a single allelic form for *M. scrofulaceum*, but significant phenotypic and genetic variability has been described for this species (10). In our study, four of the five *M. scrofulaceum* strains tested had a new unique pattern, and although this pattern could be one of the patterns described by Khosravi et al. (10), the molecular sizes presented in their paper are different from the ones that we determined and a comparison is not possible; this demonstrates the necessity for standardization of the PRA identification procedure (4).

Among organisms belonging to MAC, the largest group analyzed in the present study, 75% of the *M. avium* strains had pattern *M. avium* I, while the rest had patterns that have so far been described only in Brazil: patterns *M. avium* II and *M. avium* III (11). This suggests that particular genetic subtypes of this species are circulating in the country, but because *M. avium* II differs from *M. avium* I only by the presence of a 60-bp band in the *Hae*III digest, lack of consideration of this band could also be responsible for this genotype not having been observed in other studies. When the identification algorithm originally published by Telenti et al. (24) was compared with the most recent version of the identification algorithm (2) by using our present data, it was found that bands in the 55- to 70-bp range have been added, demonstrating observer-associ-

TABLE 3. Species with identical or similar restriction patterns

Species	PRA pattern		
	This study[a]	Literature[b]	PRA website[c]
M. lentiflavum I[d]	441-145/125	Springer et al. (21)	440-145/130
M. simiae 5[e]	ND[g]	ND	440-145/130
M. neoaurum	320/115-175/145	313/117-172/139	325/120-170/140
M. aurum	ND	Taylor et al. (23)	ND
M. kansasii I	235/205-130/105/80	234/211-127/103/78	240/210-130/105/80
M. branderi II	ND	234/211-140/106/76	240/210-130/105/80
M. marinum I	235/205-145/105/80	234/211-145/106/78	240/210-145/105/80
M. malmoense II	ND	Telenti et al. (1993)	240/210-145/105/80
M. gordonae V	ND	Telenti et al. (24)	240/210-125/110
M. interjectum 2	ND	ND	240/210-125/110
M. interjectum I	235/205-145/125	234/211-145/127	ND
M. avium III	235/205-145/125	Leão et al. (11)	240/210-145/130
M. simiae II-3	ND	Taylor et al. (23)	240/210-145/130
M. intracellulare 3	ND	ND	240/210-145/130
M. intermedium I	ND	ND	240/210-145/130
M. intracellulare new pattern	235/115/100 145/125	ND	ND
M. lentiflavum III-3	ND	Springer et al. (21)	240/120/100-145/130
M. obuense	235/205-140/85/60	234/211-139/87/58	ND
M. chubuense	235/205-140/85/60	ND	ND
M. diernhoferi	320/115-145/140/60	313/117-147/141/58	ND
M. komossense	320/115-145/140/60	ND	ND
M. chitae I[f]	320/115-140/85/60/40	313/117-139/87/58	325/120-140/85/55
M. terrae new pattern 3	320/115-140/85/60/45	ND	ND
M. gordonae I[f]	235/115/85-160/110/60	234/117/84-161/112/57	240/120/85-160/115/60
M. xenopi I	235/115/85-160/105/60	234/11784-161/104/59	240/120/85-160/105/60
M. kansasii II-2[f]	ND	Picardeau et al. (15)	340/130/85-130/105
M. lentiflavum 4	ND	ND	340/130/85-130/95
M. gastri I[f]	ND	Telenti et al. (24)	340/130/85-130/105/70
M. kansasii VI	ND	Telenti et al. (24)	340/130/85-130/100/75

[a] The molecular sizes (in base pairs) of the bands after digestion with *Bst*EII and *Hae*III are given; although the molecular sizes present on the PRA website were sometimes corrected upon introduction into the database, we adopted the values presented on the website and by Brunello et al. (2).
[b] Literature data are either the reference or the molecular sizes (in base pairs) obtained by sequencing, as recently described by Brunello et al. (2).
[c] http://www.hospvd.ch:8005.
[d] Pattern number used in the algorithm.
[e] Number used on the PRA website.
[f] Patterns with differences of 5 to 10 bp.
[g] ND, not determined.

ated variability in pattern interpretation and, once more, the need for standardization of the methodology. Additionally, we have demonstrated here that patterns that differ only by the presence of a 60- and/or a 55-bp band coexist in species such as *M. avium*, *M. intracellulare*, *M. gordonae*, and *M. peregrinum*; and this could have phylogenetic importance.

An increase in the accuracy of identification to the species level by conventional identification by PRA was most pronounced for MAC strains, as 10% of MAC strains were neither *M. avium* nor *M. intracellulare* upon genotyping. This was partly due to misinterpretation of *M. scrofulaceum*, *M. simiae*, and *M. lentiflavum* as members of MAC, to the presence of MAC-like organisms, and to the presence of mixtures of MAC strains with other species. The presence of mixtures of MAC strains with other species was determined by the detection of mixed PRA patterns or during reidentification after contradictory results were obtained by both identification procedures (data not shown). Evaluation of PRA as an identification procedure in Brazil has been performed earlier by either commercial identification procedures (4) or conventional phenotypic identification (3) for comparison.

Because of the growing amount of reported PRA restriction patterns, a database containing DNA patterns was constructed by using GelCompar software. Plikaytis et al. (16) used computer-mediated identification of restriction fragments of clinically significant mycobacteria using a position tolerance of

0.6%, independent of the restriction enzyme; that tolerance was different from the position tolerances of 1.5 and 2% for *Hae*III and *Bst*EII, respectively, used in the present study. The former study, however, besides using restriction enzymes that generated DNA fragments in the 800- to 100-bp range that were separated on polyacrylamide gels, used internal size standards, and conversion and normalization were not fully automated. A more recent study on the semiautomated identification of aerobic *Actinomyces* isolates by PCR-restriction enzyme analysis gave no information on the stringency of analysis (25). Besides aiding in identification, we believe that the construction of databases permits, with the use of proper molecular size markers, the introduction of patterns that have been printed in the literature and that are available on the Internet, as verified by the use of patterns downloaded from the PRA website.

Although shared patterns between species have so far been reported only for *M. gastri-M. kansasii* and *M. avium-M. paratuberculosis*, with the introduction of foreign patterns (i.e., patterns not generated in our laboratory) into the database, more shared or almost identical patterns between species were observed, with the most striking example being the pattern shared by *M. avium*, *M. intracellulare*, *M. simiae*, *M. intermedium*, and *M. interjectum*. Unfortunately, no data on the correctness of the species determination before the introduction of patterns into the PRA website are available. If our observation is correct, the consequences of shared patterns on the use of PRA as a single identification procedure will depend on the frequency of occurrence of such genotypes in the region of interest, and the introduction of an additional identification procedure of either a conventional or a genetic nature will be necessary in these cases. Fortunately, several of the shared patterns have been observed in species that are not isolated frequently.

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