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Rapid Identification of Mycobacteria to Species Level by PCR-Restriction Fragment Length Polymorphism Analysis of the *hsp65* Gene and Proposition of an Algorithm To Differentiate 34 Mycobacterial Species

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PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene (A. Telenti, F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer, J. Clin. Microbiol. 31:175–178, 1993) was applied to 108 mycobacterial isolates representing 34 species to evaluate its potential as a rapid reference method. A total of 49 distinct patterns were obtained; 25 species were characterized by a single PRA pattern, while 9 species gave more than one specific pattern. An algorithm describing these 34 species (which includes five additional species and new subgroups of *Mycobacterium kansasii*, *M. abscessus*, and *M. peregrinum*) is proposed. A relatively simple and inexpensive method, PRA may be particularly helpful in routine clinical microbiology laboratories.

Classical identification results for mycobacteria based on cultural and biochemical tests may take several weeks after reception of specimens, and the tests sometimes fail to produce a precise identification. Additional techniques such as thin-layer chromatography (4, 16), gas-liquid chromatography (14), high-performance liquid chromatography (5), and gene sequencing (3, 11, 12, 22, 23) are powerful tools but unfortunately remain limited to reference laboratories. Recent molecular techniques based on probe hybridization (7, 15) or on amplification of a specific genomic region, such as the Amplicor MTB test (6), provide results limited to a single species per experiment. In contrast to the above-mentioned techniques needing specialized equipment, PCR-restriction fragment length polymorphism analysis (PRA) (26) of the *hsp65* gene present in all mycobacteria (9, 20, 21) offers an easy, rapid, and inexpensive procedure to identify several mycobacterial species in a single experiment. *hsp65* PRA was also used to identify some slowly growing mycobacteria (18), as well as clinical isolates cultured in Bactec 460-TB vials (25). Other PCR-restriction fragment length polymorphism methods based on the analysis of digestion products of specific genes, such as 16S rRNA (2, 10, 29) or *dnaJ* genes (24), using three to five different restriction enzymes have also been reported but remain cumbersome compared to *hsp65* PRA, which uses only two restriction enzymes.

Our primary aim was to evaluate the PRA method and to extend it to other mycobacterial species that were not studied previously, with particular emphasis on the differentiation of *Mycobacterium chelonae*, *M. abscessus*, *M. fortuitum*, and *M. peregrinum*. These rapidly growing nonchromogenic mycobacteria are often found in drinking water and are of significant clinical importance because of their multiple drug resistance (8). These four species were previously referred as the *M. fortuitum* complex (composed of *M. fortuitum* var. *fortuitum*, *M. fortuitum* var. *peregrinum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus*) and could be differentiated from other rapidly growing nonchromogenic mycobacteria by an arylsulfatase-positive test result (4). Differentiation between *M. chelonae* and *M. abscessus* and between *M. fortuitum* and *M. peregrinum* has been achieved by cultural and biochemical tests, thin-layer chromatography of mycolic acids, and mycobactin analysis (4, 14). In the present investigation, we have applied PRA followed by computer analysis to both reference and clinical isolates and propose a more complete algorithm by adding five more species, as well as new subgroups of *M. kansasii*, *M. abscessus*, and *M. peregrinum.*

A total of 108 mycobacterial strains analyzed by PRA comprised 43 reference strains (Table 1) and 65 clinical isolates, which were identified by classical biochemical identification tests and the Accuprobe tests and DT1-DT6 PCR (7) for the *M. avium* complex isolates. *M. chelonae*, *M. abscessus*, *M. fortuitum*, and *M. peregrinum* strains from a previous study (4) were identified on the basis of cultural and biochemical tests, mycobactin patterns, and mycolid acid content. Clinical isolates of *M. kansasii* were from a previous study (28) and were obtained from E. Tortoli, Florence, Italy. Bacterial DNA was prepared as follows: one loopful of bacteria was suspended in 300 μl of TE (10 mM Tris, 1 mM EDTA) and 100 μl of acid-washed glass beads (diameter, <106 μm; Sigma, St. Louis, Mo.), heated at 94°C for 15 min, and sonicated at 35 kHz for 15 min (water bath sonicator from Gen-Probe Inc., San Diego, Calif.). Five microliters of the supernatant containing the crude DNA extract was used for PCR. PRA based on the amplification of a 439-bp fragment of the *hsp65* gene was performed with primers Tb11 (5′-ACCAACGATGGTGTGT CCAT) and Tb12 (5′-CTTGTCGAACCGCATACCCT) by the method of Telenti et al. (26). Twenty-two microliters of the PCR product was digested by *Bst*EII (Promega, Madison, Wis.) or *Hae*III (BioLabs, Inc., Beverly, Mass.), and 12 μl of the restriction digest was loaded on a 4% (wt/vol) NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, Maine). A 100-bp ladder (Pharmacia Biotech, Uppsala, Sweden) served as an external molecular size marker and was added after every six lanes of migration to reduce migration-related errors. Restriction patterns were videocopied by using the Gel-Analyst software (Bioprobe Systems, Montreuil, France). The Taxotron package software (P. A. D. Grimont, Institut Pasteur, Paris,

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TABLE 1. Reference strains used in the present study

Species or isolate(s)	Strain[a]
M. abscessus	ATCC 19977
M. africanum	ATCC 25420
M. asiaticum	ATCC 25276
M. avium	ATCC 25291
M. bovis	ATCC 19210
M. bovis BCG	CIPT 140040001
Crohn's disease isolates	2569 (CDI), Lyon (EVL)
M. chelonae	NCTC 946
"*M. engbackii*"	ATCC 27353
M. flavescens	ATCC 14474
M. fortuitum	ATCC 6841
M. gastri	ATCC 15754
M. gordonae	ATCC 14470
M. haemophilum	ATCC 29548, CIPT 141420006
M. intracellulare	ATCC 13950
M. kansasii	ATCC 12478
M. malmoense	ATCC 29571
M. marinum	ATCC 927
M. paratuberculosis	ATCC 19698, 1077(CVM), 7912 (LCRV)
M. peregrinum	ATCC 19420
M. phlei	ATCC 11758
M. porcinum	ATCC 33775
M. scrofulaceum	ATCC 19981
M. senegalense	NCTC 10956
M. shimoidei	ATCC 27962
M. simiae	ATCC 25275
M. smegmatis	ATCC 19420
M. szulgai	NCTC 10831
M. terrae	ATCC 15755
M. tuberculosis	ATCC 27294, ATCC 25177, CIPT 140010059
M. triviale	ATCC 23292
M. ulcerans	ATCC 33728
Wood pigeon	6861 (LCRV), 6409 (LCRV)
M. xenopi	ATCC 19276, ATCC 19970

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CIPT, Collection Instituut Pasteur de Paris-Tuberculose; CDI, Centraal Diergeneeskundig Institut, Lelystad, The Netherlands; EVL, Ecole Vétérinaire de Lyon, Lyon, France; LCRV, Laboratoire Central de Recherches Vétérinaires, Maisons-Alfort, France; CVM, College of Veterinary Medicine, Ames, Iowa.

France) was used to convert the migration file into a molecular weight data file by the Schaffer and Sederoff method.

The results obtained in this investigation are summarized in Tables 2 and 3 and Fig. 1. As noted previously (26), restriction fragments shorter than 60 bp were not taken in account as they are suspected of being primer or primer dimer bands. Restriction fragments were visible for all the tested strains under normal conditions except for some *M. kansasii* strains which needed larger amounts of the PCR products for restriction analysis. All the 108 isolates were easily differentiated by PRA, and there was a perfect correlation between the PRA identification and the conventional identification. As summarized in Fig. 1, the restriction profiles obtained were in agreement with the algorithm of Taylor et al. (25), who have recently modified the initial algorithm of Telenti et al. (26). In addition, the algorithm proposed in Fig. 1 now incorporates newly found profiles that have not been described before (three *M. kansasii* subgroups, two *M. peregrinum* subgroups, and one *M. abscessus* subgroup) and also previously unanalyzed species such as *M. ulcerans*, "*M. engbackii*," *M. porcinum*, *M. phlei*, and *M. senegalense.*

All the members of the *M. tuberculosis* complex (three reference strains of *M. tuberculosis* and one each of *M. africanum*, *M. bovis*, and *M. bovis* BCG [5, 4, 2, and 2 clinical isolates, respectively]), gave identical PRA profiles (Fig. 1). As illustrated in Fig. 1, within the *M. avium* complex *M. avium* and *M. intracellulare* species were easily differentiated; the three strains of *M. paratuberculosis* and the two isolates of wood pigeon and Crohn's disease mycobacteria all gave the *M. avium* profile, confirming their genetic relatedness to this species (27, 30).

Only one pattern each for *M. fortuitum* subsp. *fortuitum*, *M. fortuitum* subsp. *peregrinum*, *M. fortuitum* 3rd variant, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus* was initially described by Telenti et al. (26); this was followed by the description of an additional pattern for a single presumptive *M. chelonae* isolate by Taylor et al. (25). In this investigation a total of 39 isolates of this group (35 clinical isolates and 4 type strains) were studied, and in addition to the previously described patterns, one new pattern for *M. abscessus* and two for *M. peregrinum* were reported (Fig. 1). As all the 35 clinical isolates included in the present investigation have been extensively characterized (4, 14), we were able to add the new profiles to the algorithm proposed in Fig. 1. It should be further underlined that some miscellaneous strains which were not differentiated into either *M. chelonae-M. abscessus* or *M. fortuitum-M. peregrinum* in previous studies (4, 14) were easily discriminated by PRA (Table 2). In our opinion, it is important to properly classify the organisms before attributing a species-specific PRA pattern to them; e.g., an additional pattern reported for a "presumptive" *M. chelonae* isolate by Taylor et al. (25) was not reproduced for any of the 39 isolates studied here.

TABLE 2. Summary of PRA results for *M. chelonae*, *M. abscessus*, *M. fortuitum*, and *M. peregrinum*

PRA pattern	Bands with *Bst*EII and *Hae*III (bp)	Reference strain	No. of isolates per pattern (this study)	References for pattern
M. chelonae I	325/140 and 210	NCTC 946	10 *M. chelonae*, 3 miscellaneous[a]	25, 26, and this study
M. chelonae II[b]	325/120 and 140/65/60		None	25
M. abscessus I	245/220 and 160/60	ATCC 19977	2 *M. abscessus*, 1 miscellaneous[a]	25, 26, and this study
M. abscessus II	245/220 and 210/60		2 *M. abscessus*	This study
M. fortuitum I	245/120/80 and 155/135	ATCC 6841	4 *M. fortuitum*	25, 26, and this study
M. fortuitum II[c]	245/120/80 and 150/135		1 miscellaneous[d]	25, 26, and this study
M. peregrinum I	245/220 and 155/150/100	ATCC 19420	4 *M. peregrinum*, 1 miscellaneous[d]	25, 26, and this study
M. peregrinum II	245/220 and 150/135/100		2 *M. peregrinum*, 4 miscellaneous[d]	This study
M. peregrinum III	245/140/80 and 155/150/100		1 *M. peregrinum*	This study

[a] These isolates were previously classified as miscellaneous as differentiation between *M. chelonae* and *M. abscessus* was not achieved (4, 14).
[b] Pattern reported for a single presumptive *M. chelonae* isolate (25).
[c] Pattern previously reported for third-variant isolates ATCC 49403 and ATCC 49404 (26).
[d] These isolates were previously classified as miscellaneous as differentiation between *M. fortuitum* and *M. peregrinum* was not achieved (4, 14).

TABLE 3. Summary of PRA results for the five subgroups of *M. kansasii*

PRA pattern	Bands with *Bst*EII and *Hae*III	Reference strain	No. of isolates per pattern (this study)	References for pattern
M. kansasii I	245/220 and 140/105/80	ATCC12478	7	17, 25, 26, and this study
M. kansasii II	245/140/80 and 140/105[a]		8	17 and this study
M. kansasii III	245/140/80 and 140/105/70[a]		None	17, 26
M. kansasii IV	245/120/80 and 140/115/70[a]		None	17
M. kansasii V	325/120 and 140/100/80		None	17

[a] A minor (5-bp) difference upon *Bst*EII digestion was found by Picardeau et al. (17).

We are consequently unable to reconfirm the inclusion of this pattern for *M. chelonae* for the time being. A summary of previous results and those found in this study is provided in Table 2.

All the 15 clinical isolates of *M. kansasii* studied here came from a previous study describing *M. kansasii* biotypes associated with AIDS in Italy (28). Indeed, on the basis of phenotypic traits based on Tween 80 hydrolysis and the α-fucosidase test and genotypic criteria such as positive or negative hybridization with the AccuProbe test (13), two significant biotypes within the *M. kansasii* species were shown to exist (28). It is interesting to note that in our study, all of the pattern I isolates belonged to biotype 1 (i.e., AccuProbe test-positive isolates) (28), whereas pattern II isolates belonged to biotype 2 (i.e., AccuProbe test-negative isolates) (28). A majority of *M. kansasii* isolates from France also presented PRA patterns I and II (17) and had genotypes corresponding to those reported by Ross et al. (19). Apart from these two major subgroups, three other minor subgroups have also been reported (17, 26), confirming the heterogeneous nature of *M. kansasii*. These results are summarized in Table 3.

All the results obtained in this investigation, along with an update from previously published observations, have been integrated in the algorithm illustrated in Fig. 1. However, some

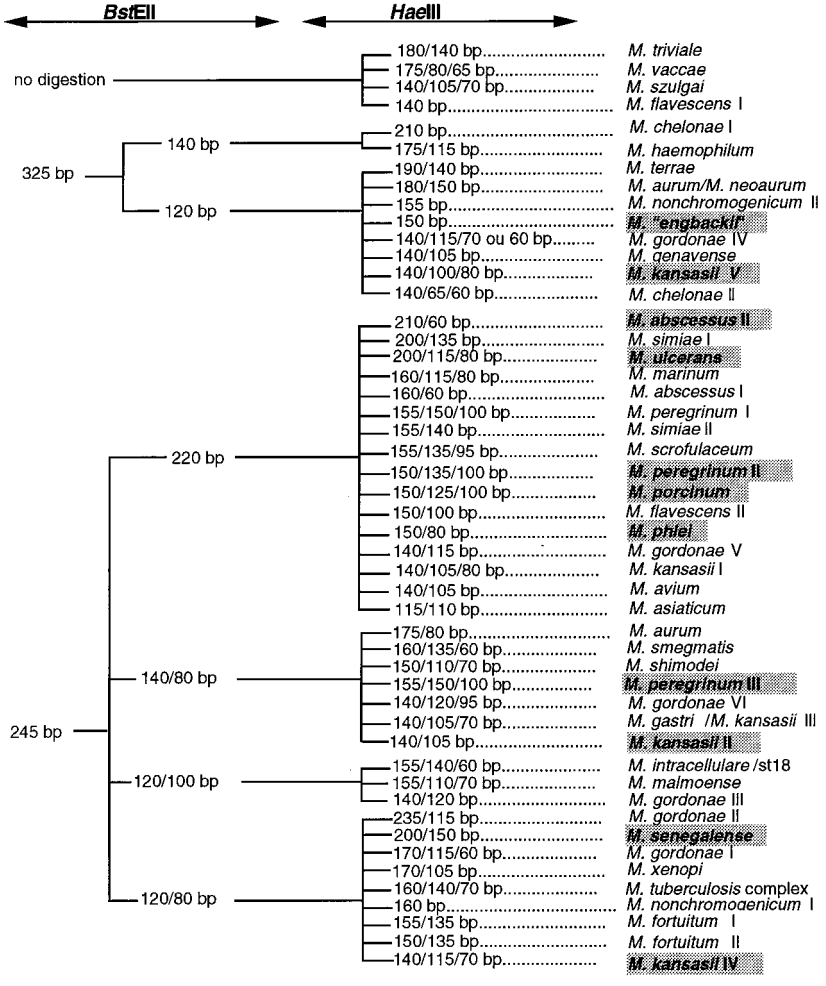

FIG. 1. Algorithm of PRA patterns for 34 mycobacterial species. Shaded species illustrate newer PRA patterns that were not reported in the previous algorithms of Telenti et al. (26) and Taylor et al. (25).

patterns may be close enough even upon *Hae*III digestion (isolates with a 5-bp difference for some of the bands or those varying in a single band) and it may be difficult to differentiate between such isolates without appropriate software. Examples may include differentiation between *M. scrofulaceum* and *M. peregrinum* II and between *M. fortuitum* I and *M. fortuitum* II.

Mycobacterial identification to the species level is critical step in patient management, as the results obtained influence both the choice of proper treatment and the eventual need for patient isolation. It is therefore important to develop methods that provide rapid results and that may be used in a wide variety of laboratories around the world. The latter criterion invariably requires that such methods be both simple and cost-effective.

The PRA method was selected in this investigation because of its ease and rapidity and because it may help speciate numerous species of mycobacteria within a single experiment. This broader spectrum of the PRA method, as compared to other molecular methods, is based on the amplification of a conserved *hsp65* gene present in all mycobacteria and in some other bacteria such as *Nocardia* spp. (9). The restriction analysis of a 439-bp fragment within this gene after *Bst*EII and *Hae*III digestions is highly effective for differentiating mycobacteria at the species level (26). The results obtained clearly showed distinct profiles for all the 34 species. For some species, such as *M. kansasii*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. peregrinum*, *M. gordonae*, *M. nonchromogenicum*, *M. simiae*, and *M. flavescens*, the discrimination was even obtained at the subgroup level. For *M. kansasii*, this subdivision was clearly linked to bacteriological and clinical specificities (28) and was also reflected by 16S rRNA sequencing (13) and other molecular techniques (1, 17, 19). It is therefore possible that the discrimination at a subgroup level for other species, although not investigated in detail for the time being, could be similarly linked to bacteriological and clinical specificities. On the other hand, the four members of the *M. tuberculosis* complex were characterized by similar PRA profiles, confirming that they do belong to a similar group genetically.

A substantial number of mycobacterial species and clinical isolates have now been run in parallel by using PRA and other reference methods (17, 25, 26; this study), and the available data have been integrated in the algorithm presented in Fig. 1. It is apparent that some species harbor unique patterns while others present several distinct profiles, which may complicate the interpretation of the results obtained. For example, a lot of PRA profiles were generated in this investigation, and had it not been for the computer-assisted analysis and interpretation of the gels, which significantly reduced the gel-to-gel variations, we may have faced problems in resolving all the patterns obtained in a short time. Furthermore, the computer-assisted analysis was particularly useful for analyzing fragments of between 100 and 200 bp.

Despite the fact that we recommend analysis of PRA profiles using appropriate software, it is important to underline that visual analysis of PRA profiles remains appropriate if related type strains are run in parallel. In our opinion, PRA is particularly useful for identifying clinical isolates giving mixed biochemical results, a situation which may require choosing between two or three closely related species. Last but not least, PRA is a rapid method which, like multiplex PCR or 16S rRNA sequencing, permits the identification of several species in a single experiment.

As far as smaller technical details are concerned, the NuSieve gel is well adapted to the migration of small DNA fragments and less cumbersome than polyacrylamide gels. As 5-bp differences in the sizes of restriction fragments may arise between different users, we recommend that each laboratory willing to use this method first validate PRA on reference strains. Last but not least, the usual recommendations to prevent PCR-linked contamination may be useful to avoid false identification. We personally use separate manipulation rooms for reagent preparation, specimen preparation, and amplification and detection. Even specimen centrifugations were performed in a separate aerosol-free area (distinct from the above three areas).

In conclusion, PRA is a rapid, cost-effective, and efficient method for the identification of mycobacteria in a clinical microbiology laboratory. Recently, PRA was used on Bactec cultures (25), and it should now be evaluated directly for clinical specimens. If successful, PRA will be the method of choice, as opposed to nucleic acid probes, which require large number of bacilli.

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