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Rapid Identification of Mycobacteria to the Species Level by Polymerase Chain Reaction and Restriction Enzyme Analysis

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A method for the rapid identification of mycobacteria to the species level was developed on the basis of evaluation by the polymerase chain reaction (PCR) of the gene encoding for the 65-kDa protein. The method involves restriction enzyme analysis of PCR products obtained with primers common to all mycobacteria. Using two restriction enzymes, *Bst*EII and *Hae*III, medically relevant and other frequent laboratory isolates were differentiated to the species or subspecies level by PCR-restriction enzyme pattern analysis. PCR-restriction enzyme pattern analysis was performed on isolates (n = 330) from solid and fluid culture media, including BACTEC, or from frozen and lyophilized stocks. The procedure does not involve hybridization steps or the use of radioactivity and can be completed within 1 working day.

Differentiation of mycobacteria to the species level is currently done by time-consuming evaluation of phenotypic and biochemical characteristics. Additional methods such as high-performance liquid chromatography (HPLC) or thin-layer chromatography are limited by the need for standardized growth conditions (4, 13, 18). Gen-Probe (Gen-Probe, Inc., San Diego, Calif.) is a rapid method, but it requires several probes and covers only a limited range of mycobacterial species (14). Sequencing of the 16S rRNA gene is a powerful technique of differentiating species; however, it is labor-intensive and difficult to implement for routine use in many clinical laboratories (16).

We developed a rapid method based on the evaluation of the gene encoding for the 65-kDa heat shock protein by the polymerase chain reaction (PCR). A similar approach to the differentiation of mycobacteria has recently been reported by Plikaytis et al. (15). The 65-kDa protein contains epitopes that are unique as well as epitopes that are common to various species of mycobacteria (17). The conserved nature of this gene allowed differentiation of mycobacteria within 1 day by restriction enzyme digestion of PCR products obtained by using primers common to all mycobacteria.

MATERIALS AND METHODS

Samples. Forty reference strains (Table 1) were investigated to establish the diagnostic algorithm. Thereafter, the assay was used to determine the species of 290 clinical isolates.

Sample preparation. A loop of mycobacteria grown on solid medium (Lowenstein-Jensen or Middlebrook 7H10 agar) was suspended in 1 ml of TE (10 mM Tris, 1 mM EDTA [pH 8]) and was heat inactivated for 10 min at 80°C. When working with liquid medium (Middlebrook 7H9, BACTEC 12B, or BACTEC 13A) or frozen or rehydrated lyophilized stocks, 250 μl was centrifuged and the pellet was resuspended in TE as described above. After inactivation, the bacteria were centrifuged for 15 min, the pellet was resuspended in 100 μl of TE, and 100 μl of glass beads (Sigma) was added; the suspended cells were mechanically disintegrated for 2 min in a Mickle apparatus (Mill Works, Gomshall, United Kingdom). After a 10-min centrifugation step, the supernatant was transferred to a new tube. All centrifugation steps were done at 13,000 rpm in an Eppendorf microcentrifuge.

Amplification. Five microliters of lysate was added to each reaction tube. The composition of the PCR mixture (50 μl) was 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 10% glycerol, 200 μM (each) deoxynucleoside triphosphate, 0.5 μM (each) primer, and 1.25 U of *Taq* polymerase (Cetus or Boehringer Mannheim). The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); this was followed by 10 min of extension at 72°C. Primers Tb11 (5′-ACCAACGATGGTGTGTCCAT) and Tb12 (5′-CTTGTCGAACCGCATACCCT) amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence (17).

Contamination precautions. Amplification tubes for PCR-restriction enzyme pattern analysis (PRA) were prepared in a "PCR-clean" room, while the mycobacterial isolates were inactivated and lysed by staff from the mycobacteriology laboratory. Thereafter, samples were added to the PCR mixture in a hood from an intermediate sample-loading area, and PCR and restriction analysis was performed in the "PCR-dirty" area. Other recommended precautions were respected (12).

Restriction analysis. For *Bst*EII digestion, 10 μl of PCR product was added directly to a mixture containing 0.5 μl (≈5 U) of enzyme, 2.5 μl of restriction buffer (5× buffer B), and 11.5 μl of water, and the mixture was incubated for 60 min at 60°C. Similarly, 10 μl of product was digested at 37°C in a solution containing *Hae*III enzyme, the corresponding buffer (5× buffer M), and water. Enzymes and buffers were purchased from Boehringer Mannheim.

Evaluation of restriction patterns. After digestion, 4 μl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and 10 μl of the mixture was loaded onto a NuSieve 3:1 agarose gel (FMC Bioproducts). Fragments were visualized by ethidium bromide staining and UV light. For the interpretation of the PRA patterns we designed a computerized work sheet (Excel; Microsoft) to convert the running distance on electrophoresis (in millimeters) to ap-

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TABLE 1. Reference strains used for the development of the diagnostic algorithm

Isolate	Strain
M. tuberculosis H37Rv	14001.0001[a]
M. bovis	14002.0001[a]
M. avium-M. intracellulare serotype 1	Borstel[b]
M. avium-M. intracellulare serotype 4	Borstel[b]
M. avium-M. intracellulare serotype 15	Borstel[b]
M. avium-M. intracellulare serotype 18	ATCC 35770[c]
M. kansasii	NCTC 10268,[d] 14011.0001,[a] DSM 43224
M. gastri	14034.0001,[a] Borstel[b]
M. gordonae	14021.0001,[a] ATCC 14470[c]
M. shimoidei	14107.0001[a]
M. malmoense	NCTC 11298,[d] Borstel[b]
M. haemophilum	ATCC 29548[c]
M. terrae	DSM 10111.68[e]
M. nonchromogenicum	ATCC 19530[c]
M. scrofulaceum	Borstel[b]
M. triviale	ATCC 23292,[c] 14033.0001[a]
M. marinum	14012.0001[a]
M. flavescens	14023.0001,[a] ATCC 14474[c]
M. simiae	Borstel[b]
M. szulgai	Borstel,[b] 14024.0001[a]
M. xenopi	NCTC 10042[d]
M. smegmatis	14133.0001[a]
M. asiaticum	14108.0001[a]
M. aurum	14121.0005[a]
M. vaccae	14120.0006[a]
"*M. genavense*"	Hannover[f]
M. fortuitum subsp. *fortuitum*	ATCC 6841[c]
M. fortuitum subsp. *peregrinum*	ATCC 14467[c]
M. fortuitum third variant	ATCC 49403,[c] ATCC 49404[c]
M. chelonae subsp. *chelonae*	ATCC 35752[c]
M. chelonae subsp. *abscessus*	ATCC 19977[c]

[a] Institut Pasteur, Paris, France.
[b] National Research Institute for Mycobacteria, Borstel, Germany.
[c] ATCC, American Type Culture Collection, Rockville, Md.
[d] National Collection of Type Culture, London, England.
[e] Deutsche Stammsammlung für Mikroorganismen, Braunschweig, Germany.
[f] Institut für Medizinische Mikrobiologie, Hannover, Germany.

parent molecular size (in base pairs). The software builds a standard curve from the migration distance of a molecular size standard (*Hae*III-digested ϕX174 DNA). Thereafter, the distance values for each sample were automatically referred to the standard curve and expressed as the calculated molecular size. Results from sequential reading of *Bst*EII and *Hae*III digestion patterns were evaluated with the help of an algorithm (Fig. 1). To avoid confusion with primer and primer-dimer bands, restriction fragments shorter than 60 bp were disregarded.

RESULTS

Tested mycobacteria were differentiated by using two restriction enzymes (Fig. 1 to 3). The members of the *Mycobacterium tuberculosis* complex were not differentiated by PRA. The *M. avium-M. intracellulare* complex was discriminated to *M. avium* and *M. intracellulare* species (Fig. 2). *M. avium-M. intracellulare* ATCC 35770 serotype 18 displayed an *M. intracellulare* PRA pattern. The *M. fortuitum* complex was separated to the subspecies level

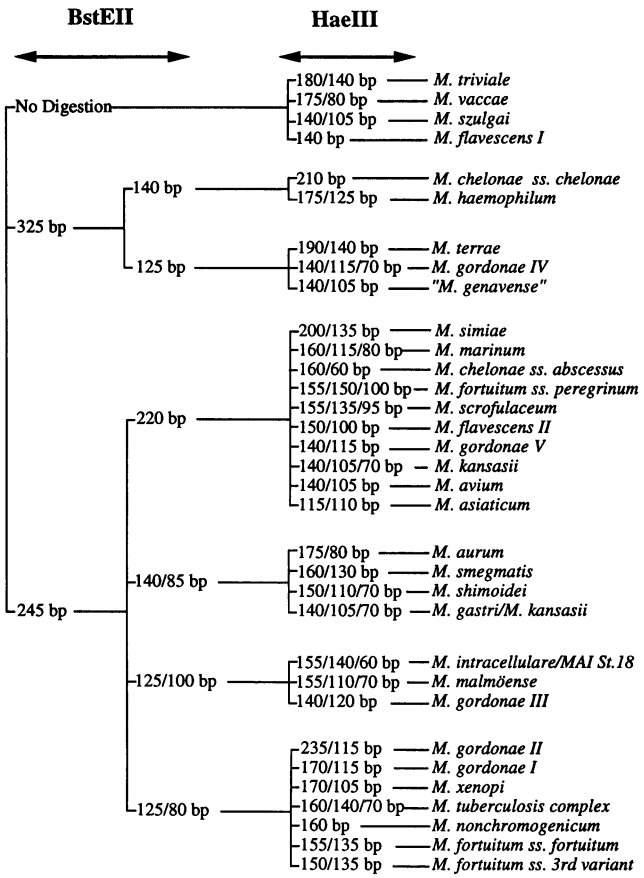

FIG. 1. Algorithm for the differentiation of significant medical and laboratory mycobacterial isolates to the species level. Apparent molecular sizes can vary within a ±5 bp range, but this rarely represents a problem for a correct identification.

(Fig. 3). Reference strains of *M. kansasii* and *M. gastri* exhibited distinct PRA patterns; however, two clinical isolates had biochemical characteristics of *M. kansasii*, but they were Gen-Probe negative and exhibited an *M. gastri* PRA pattern. Two *M. gordonae* reference strains evaluated and 38% of the clinical *M. gordonae* isolates exhibited the pattern described in Fig. 1 as *M. gordonae* I. However, other *M. gordonae* clinical isolates generated the following additional patterns: II (17%), III (12%), IV (21%), and V (12%) (Fig. 1). Two reference strains of *M. flavescens*, one from the Institut Pasteur and one from the American Type Culture Collection, presented different PRA patterns (*M. flavescens* I and II, respectively); two clinical strains exhibited pattern I.

Most PRA patterns could be recognized visually. Patterns with the greatest degree of similarity were those from *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. third variant (Fig. 3, lanes 3 and 4, respectively), *M. fortuitum* subsp. *peregrinum* and *M. flavescens* II (Fig. 1), and *M. xenopi* and *M. gordonae* I (Fig. 1). *M. tuberculosis* and *M. fortuitum* subsp. *fortuitum* displayed similar patterns on *Bst*EII digestion; however, they were discriminated at the *Hae*III level by the presence of a characteristic ≈70 bp *M. tuberculosis* restriction band (Fig. 2, lane 1), or a triplet pattern (≈3 low-molecular-mass bands) common to *M. fortuitum* subsp. *fortuitum* and *M. chelonae* (Fig. 3, lane 3).

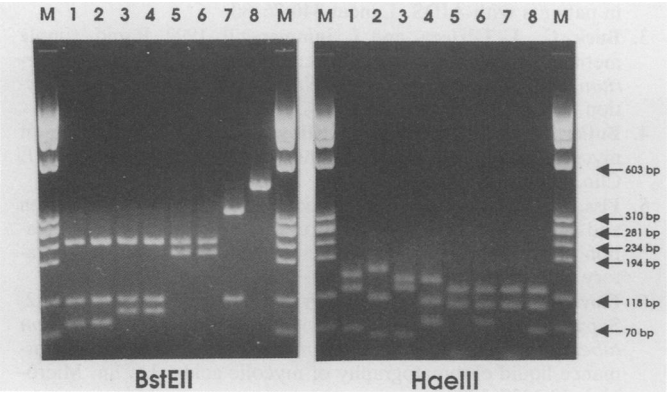

FIG. 2. PRA of the following selected slow-growing mycobacteria: *M. tuberculosis* complex (lane 1), *M. gordonae* (lane 2), *M. intracellulare* (lane 3), *M. malmoense* (lane 4), *M. avium* (lane 5), *M. kansasii* (lane 6), and *M. szulgai* (lane 8; not digested with *Bst*EII). Lane M, molecular mass markers. Fastidiously growing mycobacteria such as the recently described "*M. genavense*" (lane 7) can also be identified by PRA.

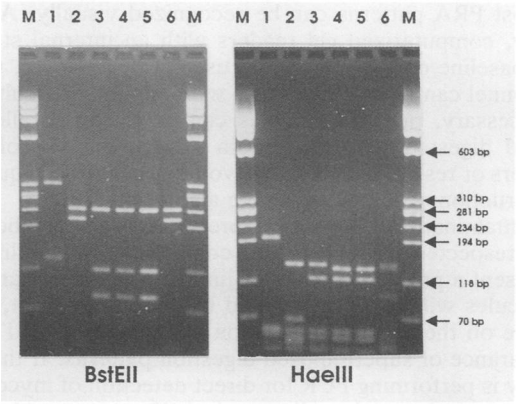

FIG. 3. PRA allows differentiation of the following *M. fortuitum* complex strains to the subspecies level: *M. chelonae* subsp. *chelonae* (lane 1), *M. chelonae* subsp. *abscessus* (lane 2), *M. fortuitum* subsp. *fortuitum* (lane 3), *M. fortuitum* subsp. third variant ATCC 49403 and ATCC 49404 (lanes 4 and 5, respectively; identical patterns), and *M. fortuitum* subsp. *peregrinum* (lane 6). Lane M, molecular mass markers.

Agarose gel artifacts (i.e., distorted or unequal migration patterns) and gel-to-gel differences may result in variations in the apparent molecular size of ±5 bp.

The following clinical isolates were evaluated by PRA: *M. tuberculosis* complex (n = 109, including 5 *M. bovis* and 1 *M. africanum*), *M. avium* (n = 50), *M. intracellulare* (n = 12), *M. gordonae* (n = 24), *M. xenopi* (n = 9), *M. kansasii* or *M. gastri* (n = 8), *M. malmoense* (n = 5), different subspecies of the *M. fortuitum* complex (n = 30), 2 isolates each of *M. marinum*, *M. szulgai*, *M. smegmatis*, *M. flavescens*, and *M. shimoidei*, and 1 isolate each of *M. terrae* and *M. simiae*. DNA prepared from six clinical isolates containing the newly described fastidiously growing "*M. genavense*" (2) was successfully amplified and investigated by PRA (Fig. 2, lane 7). PRA results were confirmed by standard biochemical patterns, Gen-Probe, or 16S rRNA gene sequencing. There were 25 isolates with patterns not represented in the reference algorithm.

DISCUSSION

The two main problems in the differentiation of mycobacteria to the species level are the diversity of techniques and tests that are necessary and the time required for a full identification. The method described here contributes to the solution of these two problems: it can be completed in 1 day, it represents a universal system of identifying mycobacteria to the species level, and it does not require hybridization to a panel of species-specific probes, which is a limitation of other PCR-based and hybridization methods for differentiating mycobacterial species (1, 5, 7, 9, 11, 14).

Preparation of samples was accomplished by simple mechanical disintegration of the bacteria. Alternative protocols, such as the enzymatic digestion with lysozyme and proteinase K, have not been as effective in producing a consistent yield of amplified product and are more time-consuming. Other techniques for the mechanical disruption of the mycobacterial cell wall, such as sonication, are likely to be equally effective (3, 15). The isolates were obtained from solid or liquid medium, including BACTEC, and from frozen or lyophilized mycobacterial stocks without the need for standardization of growth conditions that limit other rapid methods of differentiating mycobacterial species (4, 6). The addition of 10% glycerol to the amplification mixture was essential for consistent PCR results. In the absence of glycerol, PCR yields were reduced, in particular for species such as *M. avium*, *M. kansasii*, and *M. flavescens*.

The species of most of the clinical isolates investigated were determined by PRA. These included the newly described fastidiously growing "*M. genavense*" (2) and the less common isolates *M. malmoense*, *M. marinum*, *M. shimoidei*, and *M. simiae*. The *M. tuberculosis* isolates consistently displayed the same PRA patterns. However, members of the *M. tuberculosis* complex, which are difficult to separate by means of other methods such as 16S rRNA gene sequencing or HPLC analysis of mycolic acids (4, 6, 16), could not be discriminated by PRA. In contrast, the *M. fortuitum* complex was separated to the subspecies level, and the *M. avium-M. intracellulare* complex was discriminated to *M. avium* and *M. intracellulare* species in a manner that correlated with the Gen-Probe results. Reference strains of *M. kansasii* and *M. gastri*, which cannot be differentiated by 16S rRNA gene sequencing, exhibited distinct PRA patterns; however, two clinical isolates had biochemical characteristics of *M. kansasii* but were Gen-Probe negative and had an *M. gastri* PRA pattern. These observations underscore the current problem in the taxonomy of these mycobacteria (19). In contrast to other mycobacteria, *M. gordonae* isolates generated multiple patterns. This finding is consistent with the *M. gordonae* pattern "clustering" described by Plikaytis et al. (15) and the results presented in a prior report on microheterogeneity within the rRNA-encoding region of *M. gordonae* (10).

Many isolates whose patterns were not present in the reference algorithm were environmental mycobacteria previously identified by conventional means as "*M. terrae* complex" or "*Mycobacterium* species." Investigation by 16S rRNA gene sequencing was not conclusive. Ten of them were identical isolates from a bronchoscopy contamination outbreak and are being evaluated by 16S rRNA gene sequencing and biochemical means. In fact, PRA would be an efficient method for establishing the nature of such pseudoepidemics (8).

Most PRA patterns can be recognized visually. Alternatively, computerized gel readers with an internal standard and baseline correction can be used (15); however, trained personnel can interpret the gels with minimal difficulty, and if necessary, problem samples can be run in parallel with stored digested reference strain DNA. The use of other primers or restriction enzymes would necessarily require the construction of a new reference algorithm (15).

Contamination (carryover) precautions should be carefully respected (12). However, contamination is unlikely to represent a problem because a limited number of carryover molecules will be overwhelmed by the main target, and if visible on the gel, contamination can be recognized by the appearance of superimposed digestion patterns. If the laboratory is performing PCR for direct detection of mycobacteria in clinical samples (diagnostic PCR), we would recommend the use of primers directed to a completely unrelated genomic region to the one targeted for PRA. This is important because the very high copy number of PCR products generated during PRA could represent a source of contamination for a sensitive diagnostic PCR.

We are evaluating the use of PRA for routine identification of mycobacteria from positive BACTEC (growth index, ≥100) or solid medium cultures. In the future we will apply PRA to the direct investigation of smear-positive clinical samples. However, many of these render insufficient quantities of amplified product to allow a satisfactory visualization of digestion fragments on the agarose gel, and an additional step, such as chemoluminescence detection of restriction fragments, may be necessary. In addition, our preliminary experience with direct determination of mycobacterial species from smear-positive sputa suggests the presence of inhibitors of the restriction enzymes, and a further step of PCR product purification could be necessary. PRA will also prove to be useful for the identification of fastidiously or nongrowing mycobacteria, such as "*M. genavense*," that are currently differentiated to the species level by 16S rRNA gene sequencing (2).

In summary, PRA appears to be a very rapid method of differentiating mycobacteria to the species level. Other advantages are the simplicity of sample preparation and its independence from hybridization steps, multiple probes, or radioactivity. In addition, it can be upgraded to allow for automation, such as the use of fluorescent primers and automated gel readers.

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