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Routine Use of PCR-Restriction Fragment Length Polymorphism Analysis for Identification of Mycobacteria Growing in Liquid Media

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A PCR-restriction fragment length polymorphism (PCR-RFLP) procedure capable of rapidly identifying 28 species of clinically encountered mycobacteria was evaluated for use in the routine identification of acid-fast isolates growing in BACTEC 12B and 13A liquid media. PCR-RFLP identified 100 of 103 acid-fast isolates recovered from 610 patient specimens submitted for culture during the study. The three isolates unidentifiable by PCR-RFLP produced restriction patterns not included in the PCR-RFLP algorithm and could therefore not be assigned to a species. These isolates were characterized by their morphologic and biochemical characteristics. Two of the isolates were identified as *M. terrae* complex and *M. gordonae*. The third isolate could not be definitively identified and could only be characterized as a *Mycobacterium* sp. most closely resembling *M. chelonae*. PCR-RFLP identifications agreed with the conventional identifications for 96 of the 100 isolates identified by PCR-RFLP. Subsequent identification of the four discordant isolates by gas chromatography analysis supported the PCR-RFLP identification of each isolate. Amplification products were also obtained from isolates of *Streptococcus albus* and *Rhodococcus equi* recovered from patient specimens; however, the restriction patterns of these nonmycobacterial species did not resemble the patterns of any mycobacterial species included in the PCR-RFLP algorithm. PCR-RFLP seems to be a reliable procedure for the routine identification of mycobacteria and has the potential for providing identifications of mycobacterial isolates which are more accurate than conventional identification techniques based on morphologic and biochemical characteristics.

The resurgence of tuberculosis and the appearance of multidrug-resistant strains of *Mycobacterium tuberculosis* have intensified the need for the increased use of rapid methods for isolating and identifying clinically encountered mycobacteria. An important development in the rapid isolation of mycobacteria from clinical specimens has been an increase in the routine use of liquid media for primary cultures. It has been recognized for some time that the use of a liquid medium significantly increases isolation rates and decreases recovery times for mycobacteria (8). Because of these established benefits, the inclusion of a liquid medium as a primary culture is recommended for laboratories wanting to improve the sensitivity and speed of their acid-fast cultures (20).

While the use of liquid cultures improves the clinical laboratory's ability to isolate mycobacteria more rapidly, an inconvenience associated with any liquid medium can be the difficulty of quickly identifying an acid-fast isolate once it has been detected, due primarily to the limitations of the most commonly available identification techniques. Conventional identification techniques based on the cultural and biochemical characteristics of acid-fast isolates are the most commonly used methods for the determination of mycobacterial species (16), but these procedures are inappropriate for the identification of isolates from liquid cultures since they usually require cells grown on solid media. An acid-fast isolate growing in liquid medium can be presumptively identified as a species of the *M. tuberculosis* complex within 3 to 5 days by testing for susceptibility to *para*-nitro-alpha-acetylamino-beta-hydroxypropriophenone (NAP), but the NAP test cannot identify other species of mycobacteria. Molecular techniques such as thin-layer chromatography (5, 9), gas-liquid chromatography (21), high-performance liquid chromatography (4), and analysis with DNA probes (10) can provide rapid identifications of acid-fast isolates growing on solid media; however, these procedures are frequently too insensitive for the identification of isolates in liquid media due to the relatively low cell mass often produced or they are only capable of identifying a limited number of species.

In order to provide our laboratory with the ability to identify more rapidly a wide variety of mycobacteria from liquid media, we investigated the routine application of the PCR-restriction fragment length polymorphism analysis (PCR-RFLP) procedure of Telenti et al. (19) for the identification of acid-fast isolates growing in BACTEC 12B and BACTEC 13A liquid media. PCR-RFLP identifies an acid-fast isolate by first using PCR to amplify a 439-bp segment of the mycobacterial 65-kDa heat shock protein gene (13). The amplification product is then subjected to an enzyme restriction analysis capable of differentiating 28 species of clinically encountered mycobacteria.

We report here that PCR-RFLP appears to be a reliable method for the routine identification of mycobacterial isolates growing in liquid cultures. We found PCR-RFLP identifications to be potentially more accurate than conventional identifications of the same isolates, and PCR-RFLP identifications

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TABLE 1. Reference cultures

Species	Strain or source
M. tuberculosis	CAP E5-93, E8-93, E11-93, sputum
M. gordonae I	CAP E7-92, E3-93, sputum[a]
M. szulgai	CAP E9-92, E2-93
M. kansasii	CAP E10-92, E1-93, sputum
M. chelonae	CAP E4-93
M. avium-M. intracellulare complex	CAP E7-93
M. scrofulaceum	CAP E9-93, ATCC 35787
M. xenopi	CAP E10-93, ATCC 19250
M. trivale	ATCC 23292
M. vaccae	ATCC 15483
M. haemophilum	ATCC 29548
M. terrae	ATCC 15755
M. aurum	ATCC 23070
M. simiae	ATCC 25273
M. smegmatis	ATCC 607, right thigh
M. flavescens II	ATCC 14474
M. asiaticum	ATCC 25274
M. shimodei	ATCC 27962
M. gastri	ATCC 15754
M. malmoense	ATCC 29571
M. nonchromogenicum	ATCC 19530
M. fortuitum	Urine
M. intracellulare	Sputum
M. avium	Sputum
M. abscessus	Sputum
M. marinum	Sputum
M. peregrinum	Wound
M. gordonae IV	Sputum

[a] The isolate of *M. gordonae* I recovered from sputum was originally identified by conventional techniques as *M. scrofulaceum.*

were significantly more rapid since they were available within 24 h of detecting acid-fast cells in the liquid cultures.

MATERIALS AND METHODS

Reference cultures. The reference cultures that were used to become familiar with the PCR-RFLP procedure are listed in Table 1.

Patient specimens. All patient specimens other than blood submitted for acid-fast culture were processed by the *N*-acetyl-L-cysteine–NaOH decontamination procedure recommended by the BACTEC 460-TB system manufacturer (Bectin-Dickinson). A total of 0.5 ml of processed specimen was used to inoculate a BACTEC 12B medium vial and a Lowenstein-Jensen medium or Middlebrook 7H10 agar slant. Blood specimens were cultured by injecting approximately 5 ml of whole blood into a 13A medium vial. The liquid media and slants were incubated under conditions appropriate for the recovery of mycobacteria (14, 15) and were examined twice weekly for growth. Initially, liquid media were tested by PCR-RFLP when they produced a growth index (GI) greater than or equal to 100 and were Kinyoun smear positive for acid-fast bacilli. As the study progressed, vials with GI readings of less than 100 were tested. All positive liquid media were subcultured to chocolate agar plates and sheep blood agar plates, which were incubated at 35°C in 5% CO_2 for 72 h to detect possible contamination by non-acid-fast bacteria. Acid-fast isolates on solid media were tested by PCR-RFLP as soon as they were detected.

Positive 13A media were subcultured prior to nucleic acid extraction to remove inhibitors of the PCR present in the blood inoculum. Subcultures were performed by transferring approximately 5 ml of medium from a vial to a sterile 50-ml conical tube. After distilled water was added to a final volume of 35 ml, the tube was vortexed and then centrifuged at 2,000 × *g* for 30 min. After discarding the supernatant, the pellet was resuspended in 0.5 ml of distilled water and the mixture was then injected into a vial of 12B medium. This 12B medium subculture was tested by PCR-RFLP after incubation for 24 h at 35°C.

DNA extraction. The PCR-RFLP procedure described previously (19) uses mechanical disruption to release DNA from mycobacterial cells, but we find the following boiling technique to be an effective alternative. A sample extracted from a positive 12B medium consisted of 0.5 ml of medium transferred to a 1.5-ml screw-cap microcentrifuge tube. For isolates on solid media, a 1-μl loopful of cells or a single acid-fast colony was suspended in 0.5 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) in a 1.5-ml screw-cap microcentrifuge tube. Prior to extraction, the acid-fast bacteria in the samples of liquid media and suspensions were inactivated by heating the microcentrifuge tubes for 10 min at 80°C. The heat-treated samples were then centrifuged at 15,000 × *g* for 5 min. All but approximately 50 μl of the supernatant was removed, and 200 μl of well-mixed DNA Extraction Reagent (Perkin-Elmer) was added to the pellets, which were resuspended by vortexing. The samples were incubated at 56°C for 20 to 30 min, vortexed, and then placed in a boiling water bath for 10 min. After removal from the bath, the suspensions were vortexed and then centrifuged at 15,000 × *g* for 3 min. The supernatant containing the extracted DNA either was used for amplification immediately or was transferred to a clean microcentrifuge tube and stored at −70°C until needed.

Amplification. The following modification of the original PCR-RFLP amplification procedure (19) was used. A total of 20 μl of the DNA-containing supernatant was added to a reaction tube containing 50 μl of the PCR mixture (50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.001 [wt/vol] gelatin, 200 μM [each] deoxynucleoside triphosphate, 5 μM [each] primers Tb11 and Tb12, and 1.25 U of *Taq* polymerase [Perkin-Elmer Cetus]). The reaction tube was first heated for 5 min at 94°C and then for 1 min at 60°C. It was then subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C), followed by 10 min of extension at 72°C. The presence of amplified product was confirmed by agarose gel electrophoresis.

Restriction digestion and analysis. *Bst*EII and *Hae*II enzyme digestions of the amplification product were performed essentially as described previously (19) with restriction enzymes and buffers purchased from U.S. Biochemicals. Briefly, 10 μl of the amplified reaction solution was added to a mixture containing 1.0 μl (14.0 U) of enzyme, 3.0 μl of the appropriate restriction buffer, and 16.0 μl of autoclaved distilled water. The mixtures were incubated for 60 min at 60°C for the *Bst*EII digestions and at 37°C for the *Hae*III digestions. Restriction fragments were separated by agarose gel electrophoresis. Samples were prepared for electrophoresis by adding 4 μl of Gel Loading Solution (Sigma Chemical Co.) to a restriction digest. A total of 10 μl of this mixture was loaded onto an 8- by 10-cm 3% Metaphor agarose (FMC Bioproducts) gel, and the gel was electrophoresed with a 1:10 dilution of 10× Tris-borate–EDTA electrophoresis buffer (pH 8.3) (Promega Corp.) until the dye front migrated to approximately 1 cm from the end of the gel. Photographs of the restriction patterns were taken; the photographs were then analyzed visually to determine the number of fragments present, and the sizes of the fragments were compared with the molecular size standard (*Hae*III-digested ϕX174 DNA; Stratagene). Isolates were identified by using the PCR-RFLP algorithm (Fig. 1).

Comparison of PCR-RFLP with conventional identification methods. PCR-RFLP was compared with conventional identification techniques by subculturing liquid cultures containing acid-fast cells to Middlebrook 7H10 agar slants. Acid-fast isolates on the subcultures were identified by standard methods for the determination of the species of the mycobacterial isolates (16), and the results were compared with the PCR-RFLP result. Isolates having discordant identifications were also identified by gas chromatography (21).

RESULTS

Studies were initially conducted with reference cultures to validate the performance of PCR-RFLP and to familiarize ourselves with the procedure. First, all cultures were identified by PCR-RFLP with cells grown on solid media. These tests demonstrated that the restriction patterns of the cultures tested were easily interpreted, and the patterns obtained essentially agreed with those reported previously (19). Exceptions to the previously reported patterns were noted, however (Fig. 1). These involved minor but consistent variations in the apparent sizes of several restriction fragments, the observation of fragments not previously reported, and the inability to observe the 115-bp fragment of *M. haemophilum* ATCC 29548.

After adjustments were made to the PCR-RFLP algorithm for the observed restriction pattern discrepancies, PCR-RFLP was conducted on blinded subcultures grown in liquid media or on solid media. These identifications were performed to test our ability to interpret visually the restriction patterns of unknowns and to confirm that PCR-RFLP was capable of identifying isolates growing in a liquid medium. The results of this study showed that all of the blinded isolates were correctly identified and that an amplification product was obtained from all subcultures grown in liquid media. During these tests, an in-house reference culture originally isolated from sputum and identified by conventional techniques as *M. scrofulaceum* was identified by PCR-RFLP as *M. gordonae* pattern I (Table 1). Subsequent identification by gas chromatography supported the PCR-RFLP identification of this isolate (Table 2).

Patient isolates were identified by PCR-RFLP and conven-

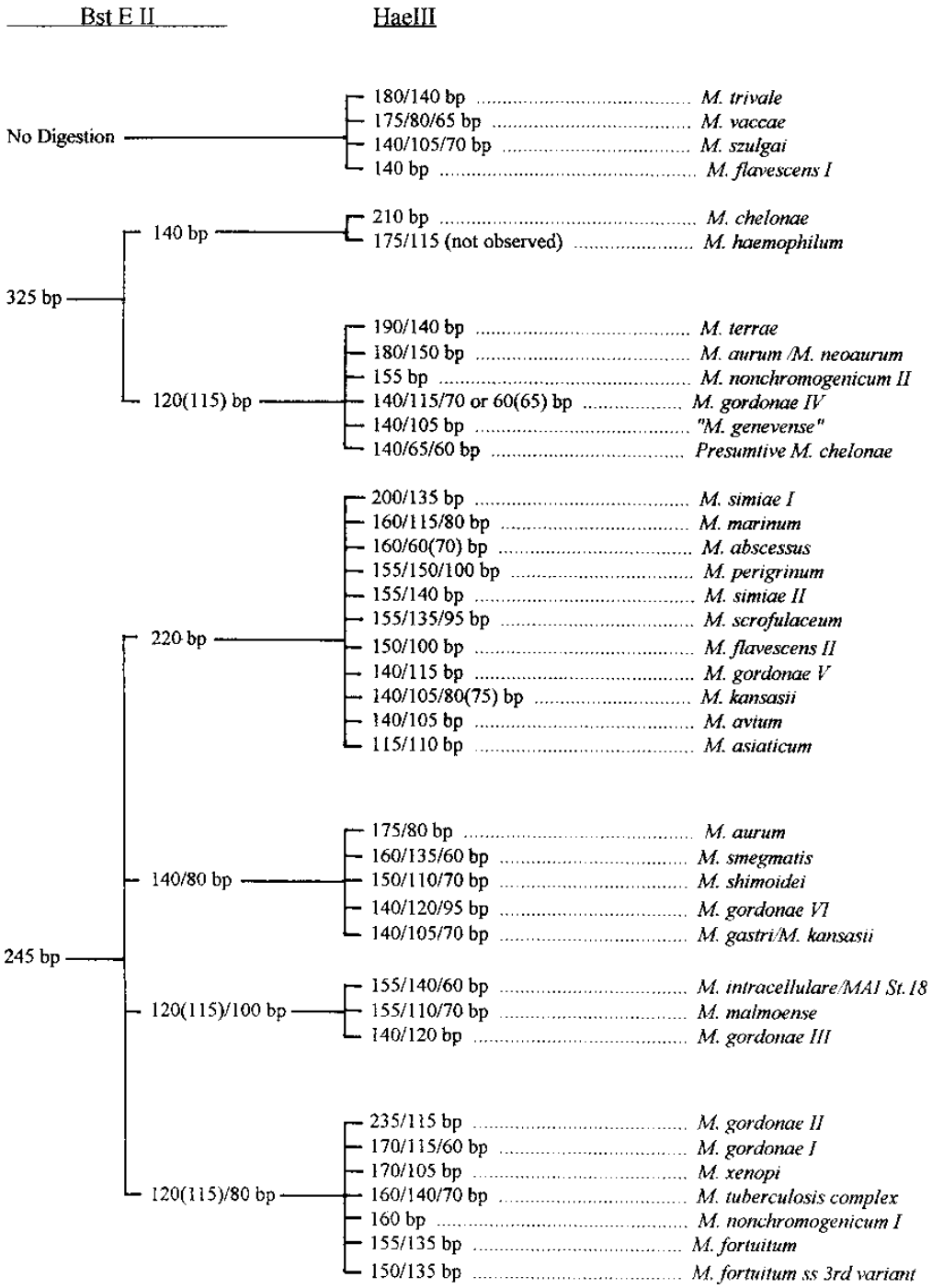

FIG. 1. Modified TB RFLP-PCR algorithm. The following modifications to the original algorithm (19), made with the permission and assistance of the authors, should be noted. All 120-bp *Bst*EII fragments and the 115-bp *Hae*III fragment of *M. haemophilum* were originally reported to be 125 bp. The 80-bp fragment of *M. kansasii*, the 135-bp fragment of *M. smegmatis*, and the 60-bp fragment of *M. gordonae* I were previously reported to be 70, 130, and 55 bp, respectively. The 60-bp fragment of *M. smegmatis* was not previously reported. The smallest restriction fragment of *M. gordonae* IV was originally reported to be 60 bp, but some isolates have been found to produce a 70-bp fragment. Fragment sizes within parentheses are the sizes observed for these fragments in the present study. The first restriction patterns described by Telenti et al. (19) for *M. simiae* and *M. nonchromogenicum* have been designated I. The fragment patterns of *M. nonchromogenicum* II, *M. simiae* II, and the presumptive *M. chelonae* isolate and the 65- and 70-bp fragments of *M. vaccae* and *M. szulgai*, respectively, were observed in the present study. The restriction pattern for *M. simiae* II was obtained from *M. simiae* ATCC 25273, which was not used to develop the original algorithm. The restriction pattern for *M. aurum* and *M. neoaurum* was obtained from both *M. aurum* ATCC 23070 and *M. neoarum* NCTC 1018. The 115-bp fragment of *M. haemophilum* ATCC 29548 was not observed in the present study. The previously listed names of *M. chelonae* subsp. *abscessus*, *M. chelonae* subsp. *chelonae*, *M. fortuitum* subsp. *fortuitum*, and *M. fortuitum* subsp. *peregrinum* have been changed to *M. abscessus*, *M. chelonae*, *M. fortuitum*, and *M. peregrinum*, respectively, to reflect current taxonomy.

TABLE 2. Comparison of PCR-RFLP, conventional, and gas chromatography identification methods for discordant isolates

Isolate no.	Result by:		
	PCR-RFLP	Conventional identification method	Gas chromatography
1	*M. avium*	*M. malmoense*	*M. avium*
2	*M. fortuitum*	*M. avium*	*M. fortuitum*
3[a]	*M. gordonae* I	*M. scrofulaceum*	*M. gordonae*
4	*M. gordonae* I	*M. flavescens*	*M. gordonae*
5	*M. kansasii*	*M. szulgai*	*M. kansasii*

[a] This isolate was recovered from sputum prior to this study and was used as a reference culture (Table 1). The remaining four isolates with discordant results were recovered from specimens cultured during the present study.

tional techniques after the completion of the familiarization studies. A total of 103 acid-fast isolates were recovered from 610 specimens submitted for acid-fast culture during the study (Table 3). All isolates from non-blood specimens submitted for acid-fast culture were recovered from both liquid and solid media and were first detected from the liquid medium. In addition, the four isolates of *M. smegmatis* and two isolates of *M. fortuitum* were initially detected in routine bacteriological cultures.

Amplification product was obtained from all patient isolates. While no formal attempt was made to correlate the GI of positive liquid cultures with the ability to obtain an identification by PCR-RFLP, we did note that approximately two-thirds of these cultures had an initial GI greater than or equal to 100. The majority of the remaining positive liquid cultures had initial GI values ranging between 50 and 100, and successful amplifications were obtained with vials having GI values as low as 6. As noted previously (19), isolates of *M. kansasii* were difficult to identify because of the weak amplification of the target sequence; this was true even for liquid cultures having a high concentration of cells. In our hands, the addition of 10% glycerol to the PCR mixture did not improve our ability to amplify the target sequence of *M. kansasii* more efficiently.

PCR-RFLP was able to provide identifications for 100 of the 103 acid-fast isolates recovered. The three isolates that could not be identified by PCR-RFLP were found to have novel restriction patterns when they were compared with the previously published patterns and the pattern variations cited above. Conventional identification techniques and gas chromatography analyses identified one of the unidentified isolates as *M. terrae* complex and another as *M. gordonae*. The restriction pattern produced by the *M. terrae* complex isolate was later determined by sequencing data to be produced by isolates of *M. nonchromogenicum* (18). The novel pattern for the isolate of *M. gordonae* was designated VI to differentiate it from the five previously described patterns for the *M. gordonae* complex (19). The last unidentified isolate could only be characterized as a rapidly growing *Mycobacterium* species most closely resembling *M. chelonae*. A comparison of the 100 PCR-RFLP identifications with the conventional identifications of the same isolates showed that both techniques agreed on the identifications for all but four isolates (Table 2); the subsequent gas chromatography analyses supported the PCR-RFLP results for the four isolates with discordant identifications.

A 439-bp amplification product was also obtained from two isolates each of *Streptomyces albus* and *Rhodococcus equi* recovered from vials of 12B medium with positive GIs that were tested by PCR-RFLP prior to the completion of the acid-fast staining of the vial contents. The restriction analyses of the amplicons of these isolates demonstrated no digestion with *Bst*EII, and the isolates produced *Hae*III fragment patterns which did not correspond to any mycobacterial species having an amplicon that was undigestible by *Bst*EII (Fig. 1). The PCR-RFLP results for both organisms were later confirmed by using subcultures grown on solid media. These findings were further tested by examining stock cultures of *S. albus* ATCC 3004 and *R. equi* ATCC 6939 by PCR-RFLP. The results of these analyses (Fig. 2) were identical to those obtained for the clinical isolates.

TABLE 3. Acid-fast isolates recovered from patient specimens

Species	No. of isolates recovered
M. avium	29
M. abscessus	3
M. chelonae	1
Presumptive *M. chelonae*	1
M. fortuitum	9
M. peregrinum	1
M. gordonae I	11
M. gordonae IV	1
M. gordonae VI	1
M. intracellulare	15
M. kansasii	8
M. marinum	1
M. scrofulaceum	1
M. simiae	4
M. smegmatis	4
M. nonchromogenicum[a]	1
M. tuberculosis complex	12
Total	103

[a] Initially identified by conventional techniques and gas chromatography as *M. terrae* complex.

DISCUSSION

Our first priority after acquiring a PCR capability was to adopt a PCR-based procedure that would allow us to identify acid-fast isolates growing in BACTEC 12B and 13A media. We also wanted a procedure that was capable of identifying multiple species of mycobacteria since, like most laboratories, the majority of the acid-fast isolates recovered from our specimens are not *M. tuberculosis*. While other PCR-based procedures capable of identifying multiple species of mycobacteria have been described (1, 2, 11, 12) we chose to investigate the PCR-RFLP procedure of Telenti et al. (19) because of its ability to identify the greatest number of species without the need for probing or sequencing of the amplicons. Also, the authors stated that their PCR-RFLP could identify acid-fast isolates growing in BACTEC 12B medium, although they did not indicate how many of the isolates in their study were identified from liquid cultures. Our major concerns about implementing PCR-RFLP were our ability to analyze restriction fragment patterns successfully using visual inspection alone, the frequency of encountering isolates having unidentifiable restriction patterns, and the reliability of PCR-RFLP identifications compared with conventional identifications.

The PCR-RFLP procedures of both Telenti et al. (19) and of Plikaytis et al. (11) used computers for the analysis of the restriction patterns produced in their studies. Even though both groups stated that it was possible to analyze the patterns without the aid of computers, we thought visual interpretations would be difficult. This was because many of the restriction fragments differ by only 5 bp, and the apparent sizes of the

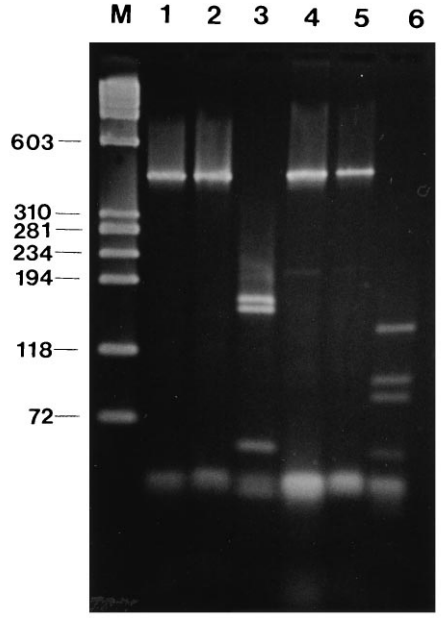

FIG. 2. Restriction analysis of *R. equi* ATCC 6939 and *S. albus* ATCC 3004 amplification products. Lanes: M, molecular size markers (numbers on the left are in base pairs); 1, undigested 439-bp *R. equi* amplification product; 2, *Bst*EII digest of *R. equi* amplification product; 3, *Hae*III digest of *R. equi* amplification product showing two fragments of 180 and 170 bp; 4, undigested *S. albus* amplification product; 5, *Bst*EII digest of *S. albus* amplification product; 6, *Hae*III digest of *S. albus* amplification product showing three fragments of 140, 100, and 90 bp.

fragments were reported to vary by as much as ±5 bp due to gel artifacts (19). Our experience in the familiarization studies and with the clinical isolates confirmed, however, that visual determination of the sizes of the restriction fragments relative to the position of the molecular size standards is reliable. Trained personnel could consistently distinguish fragments of similar size, even fragments in the range of 118 to 194 bp. These fragments are the most difficult to identify because many of them are similar in size and because the size standard contains no reference fragments in this region of the gel for comparison. Absolute correspondence between the restriction pattern of an isolate and an algorithm pattern was required for an identification. Occasionally, faint bands not corresponding to algorithm fragments were encountered in certain patterns, but these were discounted if they were less intense than the algorithm fragments. By lowering the primer concentrations to 2.5 μM, reducing the number of amplification cycles to 30, and increasing the primer annealing temperature in the cycling program to 62°C, these nonspecific amplification bands are eliminated (data not shown). The accuracy of visual analysis was such that parallel testing of reference culture DNA to confirm questionable fragment identifications was seldom needed.

We currently have no definitive explanation for the restriction fragment size variations initially observed in our familiarization studies. These discrepancies are not likely due to gel artifacts or strain variation since they have consistently been observed under various conditions of electrophoresis and with different agarose gel preparations. They have also been encountered in all reference and clinical isolates of the species possessing these fragments. Although the cause of these fragment size variations is unknown, they have been consistent and have therefore caused no difficulties for isolate identifications once the PCR-RFLP algorithm was modified to account for them. Also, we are investigating the cause of our inability to observe the 115-bp fragment of *M. haemophilum* ATCC 29548. This finding is unusual since this was the same strain used to develop the original algorithm.

We expected to encounter isolates with unidentifiable patterns since several were detected in our reference cultures (Fig. 1) and because a number of isolates in the original PCR-RFLP study were not recognized by the algorithm (19). Presumably, these novel patterns are due to strain variations in the sequence of the 439-bp target. Novel patterns were produced by patient isolates, but not frequently enough to compromise the usefulness of PCR-RFLP. Our observation of a sixth pattern for *M. gordonae* was not unexpected given the recognized genetic heterogeneity of this species (6). Like the fragment size variations described above, the novel restriction patterns of reference cultures and patient isolates have been added to the algorithm. The performance of PCR-RFLP with the patient isolates with discordant results and the misidentified *M. gordonae* reference culture demonstrated that the PCR-RFLP identifications of the acid-fast isolates are potentially more reliable than the conventional identifications. Identification procedures based on cultural and biochemical characteristics are the most commonly used techniques for determining mycobacterial species, but obtaining correct results by these procedures can sometimes be difficult even for experienced personnel. This is because many of the phenotypic characteristics used to identify mycobacteria are significantly affected by variations in media and reagents and by the size, age, and physiological condition of the inoculum. Also, it is possible for the interpretation of cultural characteristics or test results to vary from individual to individual. Conventional procedures are therefore prone to erroneous results if they are conducted under suboptimal conditions or if their results are misinterpreted. In contrast, PCR-RFLP is readily standardized, and the analysis of PCR-RFLP restriction patterns is less subject to methodological variation or misinterpretation than conventional procedures. We believe that these two characteristics give PCR-RFLP the potential to provide more accurate identifications than conventional techniques, especially for isolates having atypical phenotypic profiles. This conclusion is similar to that reached by a recent study comparing another molecular identification technique, 16S rRNA sequencing, with traditional methods of identifying mycobacteria (17). PCR-RFLP identifications are also very rapid. Most were available within 24 h of detecting acid-fast isolates in liquid media or on solid media, although the procedure can be completed within 8 h if necessary.

One shortcoming of PCR-RFLP is its inability to differentiate *M. tuberculosis* from the other species of the *M. tuberculosis* complex (19). This is a common failing of most PCR procedures that detect the *M. tuberculosis* complex and reflects the high degree of genetic relatedness of this group (7). Another potential drawback of PCR-RFLP is the ability of the Tb11 and Tb12 primers to amplify the 65-kDa heat shock protein gene of nonmycobacterial species. This ability was recently demonstrated for *Nocardia braziliensis* (22) and in this study for *S. albus* and *R. equi*. Given the highly conserved nature of the 65-kDa protein, it is likely that amplification could be obtained with other genera of actinomycetes. We are investigating the extent of amplification by the Tb11 and Tb12 primers using reference cultures of *Streptomyces* spp., *Rhodococcus* spp., and other genera of actinomycetes. Preliminary experiments with a 266-bp probe internal to the *M. tuberculosis* amplicon (base pairs 465 to 731) (13) suggest that the sequences of the *S. albus* and *R. equi* amplicons detected in this

study are less similar to the sequence of the *M. tuberculosis* amplicon than are the amplicon sequences of other mycobacteria; this is because the probe hybridizes with the amplicons of other mycobacteria but fails to hybridize with the *Streptomyces* and *Rhodococcus* amplicons under identical levels of stringency (data not shown). In practice, the amplification of species other than mycobacteria has not caused problems for PCR-RFLP identifications, since positive cultures are usually confirmed to contain acid-fast bacteria prior to PCR-RFLP testing and because the PCR-RFLP restriction patterns of the nonmycobacterial species tested so far would not be confused with the restriction pattern of any mycobacteria listed in the algorithm (data not shown).

The results of preliminary studies performed by Telenti et al. (19) suggested that the use of the unmodified PCR-RFLP for the direct detection of mycobacteria from smear-positive sputa is difficult because of the presence of inhibitors of the restriction enzymes in the specimens. We also had limited success in using PCR-RFLP for the direct detection of mycobacteria from processed patient specimens. We found that PCR-RFLP would only detect and identify the mycobacteria in 9 of 16 smear-positive specimens from a series of 223 respiratory and nonrespiratory specimens submitted for acid-fast culture (data not shown). Specimen aliquots used for PCR-RFLP in this limited study were washed three times in TE buffer prior to DNA extraction to remove inhibitors of the restriction enzymes, but the washing apparently failed to remove inhibitors of the PCR, since many smear-positive specimens failed to produce amplification products.

The ability to identify more rapidly a variety of mycobacterial species growing in liquid media has provided several benefits. We are now able to alert physicians to the presence or absence of an *M. tuberculosis* complex isolate days sooner than would be possible with NAP susceptibility testing. Rapid identifications have also reduced the number of unnecessary respiratory isolations for patients who are infected with acid-fast bacilli other than *M. tuberculosis*. This has resulted in the more efficient use of our limited number of respiratory isolation rooms, a reduction in the costs and inconveniences associated with respiratory isolation and a reduction in the time expended on unnecessary contact investigations by infection control and occupational health personnel.

These benefits are the result of PCR-RFLP's ability to differentiate the *M. tuberculosis* complex more rapidly from other mycobacteria growing in liquid media. As recently demonstrated by Forbes and Hicks (3), this capability is not unique to PCR-RFLP and could probably be performed by many of the PCR-based procedures that have been described for the direct detection of *M. tuberculosis* from patient specimens. Isolates of the *M. tuberculosis* complex growing in liquid media can also be identified by using the Gen-Probe system used by many clinical laboratories for the rapid identification of a number of clinically significant microorganisms, including the most frequently encountered species of mycobacteria (10). Our experience with referred liquid cultures has shown, however, that many do not have adequate cell mass for probe identifications. This conclusion is based on the observation that PCR-RFLP was often able to provide an identification for an isolate growing in a liquid medium that was unidentifiable by probes. This would suggest that amplification-based identification procedures could be more cost-effective than probe-based systems, since identifying a non-*M. tuberculosis* complex isolate from a patient in respiratory isolation just 1 day earlier could mean substantial cost savings to the institution.

An advantage of PCR-RFLP compared with *M. tuberculosis* complex-only PCR tests and probe identifications is its ability to identify most clinically encountered species of what have been referred to as potentially pathogenic environmental mycobacteria (PPEM) (23). The rapid identification of PPEM isolates growing in liquid media avoids potentially misleading information conveyed by reports of "acid-fast positive organisms" for cultures growing usually nonpathogenic species. Also, PPEM are not communicable, but their rapid identification can be of importance to infected individuals because of the established pathogenicity of many species (23). An example of how PCR-RFLP's universal identification capability can influence the management of infections caused by PPEM was its identification of the *M. smegmatis* isolates (Table 3) as the cause of an extensive mycobacterial osteomyelitis in a severely traumatized leg. The rapid identification of this unusual etiology eliminated the need for an empiric antimicrobial therapy and permitted the use of a directed treatment within 48 h of recovering the isolate.

In summary, PCR-RFLP appears to be a reliable technique that has given us the ability to identify more rapidly mycobacteria growing in BACTEC 460-TB system media. PCR-RFLP seems to compare favorably with conventional identification techniques and is easily incorporated into the routine work flow of a microbiology laboratory. We recommend that laboratories having PCR capabilities consider investigating the use of PCR-RFLP for detecting and determining the species of a wide variety of clinically encountered mycobacteria from liquid or solid media.

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