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PCR–Enzyme-Linked Immunosorbent Assay and Partial rRNA Gene Sequencing: a Rational Approach to Identifying Mycobacteria

SUSHIL PATEL,[1] MALCOLM YATES,[2] AND NICHOLAS A. SAUNDERS[1]*

Molecular Biology Unit, Virus Reference Division, Central Public Health Laboratory, London NW9 5HT,[1] *and PHLS Mycobacterium Reference Unit, Public Health Laboratory Service, Dulwich Hospital, London SE22 8QF,*[2] *United Kingdom*

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A PCR-enzyme-linked immunosorbent assay (ELISA) for amplification and rapid identification of mycobacterial DNA coding for 16S rRNA was developed. The PCR selectively targeted and amplified part of the 16S rRNA gene from all mycobacteria while simultaneously labelling one strand of the amplified product with a 5′ fluorescein-labelled primer. The identity of the labelled strand was subsequently determined by hybridization to a panel of mycobacterial species-specific capture probes, which were immobilized via their 5′ biotin ends to a streptavidin-coated microtiter plate. Specific hybridization of a 5′ fluorescein-labelled strand to a species probe was detected colorimetrically with an anti-fluorescein enzyme conjugate. The assay was able to identify 10 *Mycobacterium* spp. A probe able to hybridize to all *Mycobacterium* species (All1) was also included. By a heminested PCR, the assay was sensitive enough to detect as little as 10 fg of DNA, which is equivalent to approximately three bacilli. The assay was able to detect and identify mycobacteria directly from sputa. The specificities of the capture probes were assessed by analysis of 60 mycobacterial strains corresponding to 18 species. Probes Avi1, Int1, Kan1, Xen1, Che1, For1, Mal1, Ter1, and Gor1 were specific. The probe Tbc1 cross-hybridized with the *Mycobacterium terrae* amplicon. Analysis of 35 strains tested blind resulted in 34 strains being correctly identified. This method could be used for rapid identification of early cultures and may be suitable for the detection and concurrent identification of mycobacteria within clinical specimens.

In the last few years, there has been a change in the spectrum of mycobacterial disease, with the incidence of infections due to mycobacteria other than tuberculosis increasing significantly (5, 7, 33, 36). This trend can be attributed to a number of factors, primarily the increasing numbers of immunocompromised patients susceptible to opportunistic mycobacteria. This increase is largely due to patients with AIDS, in whom the *Mycobacterium avium* complex of mycobacteria is particularly common (7). The correct identification of mycobacterial species is important for effective patient management, since different mycobacterial species can cause similar illnesses (33) and these species may differ in their susceptibilities to antimicrobial agents (12).

Currently, the most-rapid and cost-effective method for diagnosis of mycobacterial infection is microscopic examination of stained specimens (13). However, this method lacks sensitivity and cannot always identify the species present. Culture of the specimen and biochemical analysis of any isolate are slow, often taking many weeks. This delay has caused rapid genotypic methods to be investigated, resulting in the development of commercial nucleic acid probes applicable to isolates of the most-common mycobacterial species (GenProbe, Inc., San Diego, Calif.).

To improve the sensitivity of detection directly from specimens and thus circumvent the need for culture, PCR-based assays have been developed. These can amplify a single specific target from a *Mycobacterium* species or species complex (6, 27). However, this approach is of limited value for species identification, since different PCR primers are required for each species. Alternatively, numbers of targets can be simultaneously amplified by multiplex PCR, allowing identification of several species in a single reaction (4, 35). A different and commonly used strategy is to amplify a target found in all mycobacteria that contains within it species-specific polymorphisms. These species-specific regions can be analyzed in several ways, the simplest being restriction fragment length polymorphism analysis of the genus-specific product (23, 30, 31). Alternatively, the amplification product can be sequenced (16, 17, 24, 29) or hybridized to species-specific probes (1, 2, 8, 10, 18).

We report a rapid method based on species specific PCR and the use of an enzyme-linked immunosorbent assay (ELISA) capture probe method for identification to the species level. The combination of a simple DNA extraction procedure, PCR, and an ELISA that generates a colorimetric endpoint means that identification from visible growth in culture can be completed within a day and gives results that are simple to interpret. The system allows a variety of mycobacterial species to be screened, and it is particularly suitable as a rapid means of identifying isolates. The sensitivity of the method may also allow mycobacteria to be identified directly from clinical material.

MATERIALS AND METHODS

Bacterial strains and clinical specimens. The mycobacterial strains used in this study are listed in Table 1. Reference strains were obtained from the National Culture Type Collection (NCTC), Central Public Health Laboratory, London, United Kingdom. Clinical isolates (provided on Löwenstein-Jensen medium) were obtained from the Mycobacterium Reference Laboratory, London, United Kingdom, and the Regional Centre for Mycobacteriology, Cardiff, United Kingdom. The isolates were identified by standard biochemical methods in the source laboratories. Clinical specimens, i.e., sputa that were positive for acid-fast bacilli (AFB), were also provided by the Regional Centre for Mycobacteriology at Cardiff. DNA samples from other pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Klebsiella pneumoniae*,

* Corresponding author. Phone: 181 200 4400, ext. 3072. Fax: 181 200 1569. E-mail: nsaunder@phls.co.uk.

TABLE 1. Mycobacteria used in this study

Method and species (no.)	Source[a]
Reference strains	
M. aurum (1)	NCTC 10437
M. avium (1)	NCTC 08559
M. fortuitum (1)	NCTC 10394
M. gordonae (1)	NCTC 10267
M. kansasii (1)	NCTC 10268
M. malmoense (1)	NCTC 11298
M. tuberculosis H37Rv (1)	NCTC 07416
M. xenopi (1)	NCTC 10042
M. marinum (1)	NCTC 02275
M. chelonae subsp. *chelonae* (1)	NCTC 00946
M. microti (1)	NCTC 08710
M. phlei (1)	NCTC 08151
M. ulcerans (1)	NCTC 10417
M. intracellulare (1)	NCTC 10425
M. terrae (1)	NCTC 10856
Clinical strains[b]	
M. tuberculosis (1)	MRL clinical isolate
M. bovis (1)	MRL clinical isolate
M. avium complex (13)	MRL clinical isolates
M. avium complex (2)	RCM clinical isolates
M. intracellulare (1)	MRL clinical isolate
M. xenopi (5)	MRL clinical isolates
M. xenopi (2)	RCM clinical isolates
M. kansasii (4)	MRL clinical isolates
M. kansasii (2)	RCM clinical isolates
M. fortuitum (1)	MRL clinical isolate
M. fortuitum (2)	RCM clinical isolates
M. chelonae (3)	MRL clinical isolates
M. malmoense (2)	MRL clinical isolate
M. malmoense (2)	RCM clinical isolates
M. gordonae (2)	RCM clinical isolates
M. terrae group (2)	RCM clinical isolates

[a] MRL, Mycobacterium Reference Laboratory, London, United Kingdom. RCM, Regional Centre for Mycobacteriology, Cardiff, United Kingdom.
[b] Species determined biochemically by the source laboratories.

Staphylococcus aureus, *Escherichia coli*, *Coxiella burnetti*, *Corynebacterium diphtheriae*, *Corynebacterium xerosis*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*) were from the same sources and were prepared as described previously (34).

Extraction of DNA from cultures. DNAs from cultured isolates were extracted as previously described (21), and 1 μl of resuspended DNA extract was used for PCR. Alternatively, a rapid chelex extraction method was used. This involved removing a 1-μl loop of growth and adding it to a 0.5-ml Eppendorf tube containing 50 μl of 5% chelex–0.1% Nonidet P-40. The mixture was overlaid with mineral oil and heated to 99°C for 10 min. The mixture was cooled to −20°C, thawed, and centrifuged in a microcentrifuge for 5 min at 10,000 × *g*, and 2 μl of the supernatant was used for PCR.

For DNA extraction from sputum specimens, an equal volume of 1 M NaOH was added to the sample and the solution was gently mixed and incubated at 37°C for 2 h. Distilled H_2O (1.5 volumes) was added, and the solution was gently mixed and then centrifuged at 3,000 × *g* for 20 min. The supernatant was discarded, and the pellet was resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.0). The cell suspension was incubated at 80°C for 25 min. To remove PCR inhibitors, the cell suspension was laid on a 0.5-ml 50% sucrose cushion within a microcentrifuge tube and centrifuged for 5 min at 10,000 × *g* (32). A 10-μl loop of the cell pellet was chelex extracted as described above.

Primer and probe selection. 16S rRNA gene sequences of 38 mycobacterial species were aligned with the CLUSTAL V program (11). This alignment, part of which is shown in Fig. 2, allowed the design of PCR primers and species-specific capture probes. The capture probes (Table 2) were designed on the basis of sequence information for the hypervariable region of the 16S rRNA gene (3, 15, 17, 24, 25, 28, 29). Probes with similar lengths and G+C:A+T ratios were selected so that they would all have similar optimum temperatures for hybridization. A probe able to hybridize to all mycobacterial DNAs was also designed and included in the probe panel. The oligonucleotide primers and probes were synthesized by Cruachem Ltd. (Glasgow, Scotland), and each species capture probe was produced with an incorporated 5′ biotin. Oligonucleotides P1-F and P1-F2 primed synthesis of the DNA strand which was complementary to the capture probes, and P1-F was 5′ fluorescein labelled. The reverse PCR primer (P2-R) was unlabelled.

PCR amplification. Amplification was performed in a volume of 50 μl overlaid with mineral oil (Sigma) by using a thermocycler (Omnigene; Hybaid, Teddington, Middlesex, United Kingdom). The PCR mix contained 1 μl (10 ng) of DNA (or 2 μl of the chelex-extracted DNA), 10 mM Tris-HCl (pH 8.8 [at 25°C]), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each deoxynucleoside triphosphate (Boehringer, Mannheim, Germany), 0.15 μM primers P1-F (5′-XGCGTGCTTAACACATGCAA-3′ [positions 49 to 67, where X = fluorescein]) and P2-R (5′-CGCTCACAGTTAAGCCGT-3′ [positions 608 to 625]), and 1.5 U of *Taq* DNA polymerase (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.). The amplification conditions used were 1 cycle at 95°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension cycle at 72°C for 5 min.

To increase the sensitivity of the PCR, a heminested primer was used in a second amplification (22). Amplification was carried out under the cycle conditions and with the PCR mix described above, except that 1 μl of extracted DNA and 0.15 μM primers P1-F2 (5′-CTGGCTCAGGACGAACGCT-3′ [positions 26 to 44]) and P2-R were added to give a product of 601 bp from *M. tuberculosis*. Second-round amplification was as described for the first round, except that 1 μl of the first round product and 0.15 μM primers P1-F and P2-R were used. The PCR products were visualized by ethidium bromide staining after electrophoresis in a 1% agarose gel (Gibco BRL, Life Technologies, Inc.).

Capture probe hybridization. The 5′ biotinylated probes were immobilized to the wells of a streptavidin-coated microtiter plate (Labsystems, Basingstoke, United Kingdom) as follows. A 50-μl volume of phosphate-buffered saline containing 0.01% Tween (PBST) and 0.5 μM species-specific probe were added to each well, and the wells were incubated at 37°C for 30 min. The plate was washed three times with PBST and cooled on ice. The PCR product was denatured by heating to 99°C for 10 min and then chilled on ice. A 900-μl volume of ice-cold hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 1% [wt/vol] blocking agent [Boehringer], 0.1% [wt/vol] *N*-lauroylsarcosine) was added to the denatured PCR product. A 100-μl volume of this mix was added to each well in a horizontal row, and the plate was incubated at 55°C for 30 min. The plate was then washed three times with PBST. Hybridization of labelled PCR product to probe was detected colorimetrically with an anti-fluorescein horseradish peroxidase conjugate (Amersham) and TMB substrate (Sigma, Poole, United Kingdom). The conjugate was diluted 1 in 500 in hybridization buffer, and 100 μl of diluted conjugate was added to each well. The plate was incubated at 37°C for 30 min and washed three times with PBST. A 100-μl volume of 3,3′,5,5′-tetramethylbenzidene (TMB) solution (100-μg/ml TMB substrate [Sigma] in TMB buffer [0.1 M NaAc, 0.01% H_2O_2; pH 7.0]) per well was added, and color was allowed to develop for 30 min. The reaction was stopped by adding 50 μl of 2 M H_2SO_4, and the optical density (450 nm) was measured with a plate reader (Multiskan MCC/340; Wellcome Diagnostics, Beckenham, United Kingdom).

Partial 16S rRNA gene sequencing. The region of the 16S rRNA gene to be sequenced was first amplified. PCR was carried out by the standard amplification protocol as described above, except that primer P1-F2 instead of P1-F was used. The amplified product was electrophoresed in 1% agarose (Gibco-BRL, Life Technologies, Inc.), and the band was excised. DNA was removed from the gel slice with a DNA recovery kit (Gene Clean II; Bio 101, La Jolla, Calif.). The purified DNA was prepared for sequencing with a PCR sequencing kit (Abi Prism; Applied Biosystems, Warrington, United Kingdom) and primer P2-F (5′-GCGTGCTTAACACATGCAA-3′), a non-fluorescein-labelled version of primer P1-F. Sequencing was carried out on a 373A Sequencer (Applied Biosystems, Warrington, United Kingdom).

TABLE 2. Nucleotide sequences of capture probes used for 16S rDNA sequence hybridization

Oligonucleotide probe	Position[a]	Sequence (5′ to 3′)[b]
All1	175–155	GGTATTAGACCCAGTTTCCCA
Tbc1	203–183	ACAAGACATGCATCCCGTGGT
Avi1	203–183	AGAAGACATGCGTCTTGAGGT
Int1	204–184	CTAAAGACATGCGCCTAAAGG
Xen1	203–184	ACCCCACATGCGCAGAATGG
Kan1	203–183	ACAAGGCATGCGCCAAGTGGT
Mal1	189–169	TCGGGGTCCTATTCGGTATTA
Mal2	215–195	AAGCTTTCCACCCCAAGGCAT
Che1	210–190	TTGCACCACTCACCATGAAGT
For1	197–177	CATGAAGCGCGTGGTCATATT
Ter1	203–183	ACAGAACATGCATCCCATGGT
Gor1	197–177	CATGTGTCCTGTGGTCCTATT

[a] Numbering corresponds to *E. coli* 16S rRNA positions.
[b] Each sequence shown represents the noncoding strand.

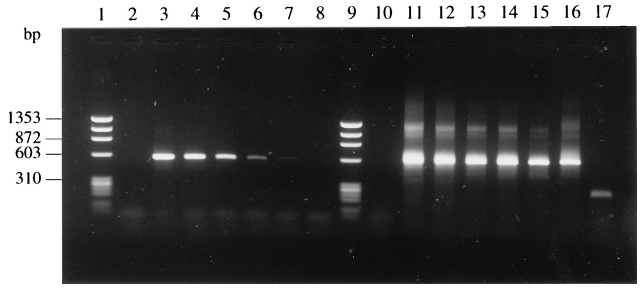

FIG. 1. Comparison of the sensitivities of standard and heminested PCR. Dilutions of purified *M. tuberculosis* DNA were subjected to PCR. Lanes: 1 and 9, molecular size markers (ΦX174 replicative-form DNA-*Hae*III fragments); 2 and 10, negative controls containing no template DNA; 3 to 8, standard PCR-generated products from serial (10-fold) dilutions of 1 ng to 10 fg of DNA; 11 to 17, heminested PCR products from serial (10-fold) dilutions of 1 ng to 1 fg of DNA.

RESULTS

Following optimization, the sensitivity of the standard PCR was assessed by analysis of a serial dilution of template *M. tuberculosis* DNA (Fig. 1, lanes 1 to 8). The PCR was able to generate a product of the desired size, detectable by visual analysis of an ethidium bromide-stained gel, from 1 pg of chromosomal DNA. Heminested amplification was able to generate a product from 100 times less DNA, i.e., 10 fg (Fig. 1, lanes 9 to 17).

The sensitivity of PCR-ELISA was determined by colorimetric analysis of the product generated from serially diluted *M. tuberculosis* DNA. The use of the standard PCR protocol required a DNA concentration of 10 pg to produce an optical density (OD) reading greater than 0.1 OD U (at 450 nm), which was the cutoff value set for a positive reading. However, by a heminested PCR, 10 fg of template DNA was sufficient to produce a positive reading.

A search of the GenBank database with the BLAST program indicated the level of specificity of each primer used in the PCR. Specificity was conferred on the PCR primarily by the reverse unlabelled oligonucleotide primer P2-R, which appears to be specific to mycobacteria. The labelled primer P1-F was not completely specific for mycobacteria species and was found within the 16S rRNA gene sequences of species belonging to closely related genera. Amplification with this primer pair would be expected to be specific for the mycobacteria, and PCR with DNA samples from respiratory pathogens not belonging to the genus *Mycobacteria* did not produce an amplicon, whereas amplification of DNA from all mycobacterial isolates tested (Table 1) gave a product of approximately 565 to 590 bp, the precise size depending on the species (e.g., 578 bp for *M. tuberculosis*) (results not shown).

The specificities of the capture probes were assessed by analysis of 60 cultured strains with known identities (Table 1). Fifteen of these were reference strains. The remainder of the isolates had been identified biochemically by the source laboratories. All reference strains for which probes were available were specifically identified by their corresponding species probes under the hybridization conditions selected, except for *M. terrae*, which also reacted with the *M. tuberculosis* probe (Tbc1). The cross-reacting signal generated by the *M. terrae*-derived amplicon with the *M. tuberculosis* probe was approximately half that typically produced by hybridization of the probe to *M. tuberculosis*-amplified product but clearly a positive result when the cutoff of 0.1 OD U was applied. Increasing the hybridization temperature did not abolish the cross-reaction without also attenuating the *M. tuberculosis* signal to an unacceptable level. Several alternative *M. tuberculosis* probes were designed in an attempt to prevent this cross-reaction, but none performed as well overall as probe Tbc1. Probe Tbc1 also hybridized to amplicons derived from members (*M. bovis* and *M. microti*) of the *M. tuberculosis* complex. The All1 probe, which was designed to identify all members of the genus mycobacteria, hybridized to amplicons from all of the isolates.

The *M. intracellulare* probe (Int1) was designed to hybridize to strains of the group of serovars that includes the type strain, but it also cross-hybridized to a strain of *M. intracellulare* serovar 18, which differs from the type strain sequence at two base positions. The *M. fortuitum* probe (For1) hybridized to PCR product from all *M. fortuitum* strains tested. However, the probe also cross-hybridized weakly to some *M. chelonae* strains, although on no occasion was a positive result (signal greater than 0.1 OD U) obtained. Probes for *M. avium*, *M. malmonense*, *M. gordonae*, *M. chelonae*, *M. kansasii*, *M. xenopi*, *M. terrae*, and *M. gordonae* identified their corresponding species in all cases.

The specificity of probe binding was affected by the temperature at which hybridization of the PCR amplicon to the species capture probes was carried out. A hybridization temperature of 50°C produced a strong positive signal, but cross-reactions were stronger. Although a temperature of 60°C resulted in excellent specificity, the signal intensities were considerably lower. A temperature of 55°C provided an optimal combination of signal intensity and probe specificity (results not shown).

The ability of the assay to identify strains of mycobacteria was assessed in a study of 35 coded strains. While blinded to information about the sources of the strains and their other test results, these were identified by PCR-ELISA (Table 3). The PCR-ELISA results were concordant with the results of biochemical testing, which was carried out for all strains, and of sequencing, which was used for six strains. In only one case did the PCR-ELISA result disagree with the identity of the strain as determined by biochemical analysis and/or sequencing. The amplicon from this isolate hybridized to probe Mal1, indicating that it belonged to the species *M. malmoense*. However, biochemical analysis suggested that this isolate belonged to the *M. terrae* complex. Sequencing showed that this isolate belonged to the species *M. nonchromogenicum*, a member of the *M. terrae* complex (26).

To determine if PCR-ELISA was applicable to clinical material, six sputum samples containing AFB on direct microscopy were analyzed. A PCR amplicon was generated from all six and was found to hybridize to the *M. tuberculosis* complex probe but not to the *M. terrae* probe, indicating that the isolates belonged to the *M. tuberculosis* complex. Culture and biochemical analysis of these sputum samples by the source laboratory confirmed that they contained *M. tuberculosis.*

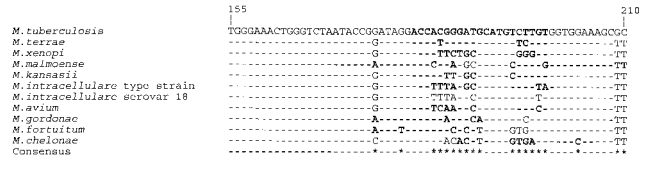

FIG. 2. 16S rDNA sequence alignment of mycobacterial species identified and comparison to the *M. tuberculosis* sequence. *, variable bases; −, sequence homologous to *M. tuberculosis*. The location of each species probe is indicated by boldface type. The probes Mal1 (bases 169 to 189) and Mal2 (bases 195 to 215) are both shown on the *M. malmonense* sequence. The position of the all-capture probe All1 is shown in boldface type on the consensus sequence.

TABLE 3. Comparison of results from PCR-ELISA with those from biochemical-16S rDNA sequence analysis of 35 isolates tested blind

Biochemical designation	PCR-ELISA result	16S rRNA sequencing result
M. tuberculosis complex	*M. tuberculosis* complex	—[a]
M. tuberculosis complex	*M. tuberculosis* complex	—
M. xenopi	*M. xenopi*	—
M. fortuitum	*M. fortuitum*	—
M. avium	*M. avium*	—
M. chelonae	*M. chelonae*	—
M. avium	*M. avium*	—
M. fortuitum	*M. fortuitum*	—
M. kansasii	*M. kansasii*	—
M. fortuitum	*M. fortuitum*	—
M. kansasii	*M. kansasii*	—
M. intracellulare	*M. intracellulare*	—
M. fortuitum	Unidentified	Unique sequence
M. kansasii	*M. kansasii*	—
M. chelonae	*M. chelonae*	—
M. chelonae	*M. chelonae*	—
M. kansasii	*M. kansasii*	—
M. chelonae	*M. chelonae*	—
M. kansasii	*M. kansasii*	—
M. kansasii	*M. kansasii*	—
M. malmoense	*M. malmoense*	—
M. xenopi	*M. xenopi*	—
M. gordonae	*M. gordonae*	—
M. xenopi	*M. xenopi*	—
M. fortuitum	Unidentified	Unique sequence
M. avium	*M. avium*	—
M. avium	*M. avium*	—
M. avium	*M. avium*	—
M. malmoense	*M. malmoense*	*M. malmoense*
M. fortuitum	*M. fortuitum*	—
M. tuberculosis complex	*M. tuberculosis* complex	—
M. tuberculosis complex	*M. tuberculosis* complex	—
*[b]	*M. kansasii*	*M. kansasii*
M. terrae group	*M. malmoense*	*M. nonchromogenicum*
*	*M. gordonae*	*M. gordonae*

[a] —, sequence not determined.
[b] *, not identified biochemically.

DISCUSSION

The single-round PCR followed by ELISA was able to detect approximately 10 pg of DNA with concurrent identification to the species level. This sensitivity was sufficient to allow amplification from culture, resulting in a simple rapid identification procedure. Identification of mycobacterial species directly in clinical materials, which contain far fewer target organisms, would require a second, heminested amplification (22). The heminested PCR-ELISA was able to amplify small quantities of mycobacterial DNA which allowed the identification of species from as little as 10 fg of DNA (equivalent to three genomes of strain H37Rv [2.5×10^9 Da]). Examination of six AFB smear-positive sputa demonstrated that identification directly from clinical material was feasible.

While the probes were designed to be species specific, they could not always distinguish between closely related species. This is because differently named species may have identical 16S rRNA sequences or similar sequences that do not differ within the hypervariable region employed in this study. For example, the members of the *M. tuberculosis* complex were indistinguishable with probe Tbc1. In the same way, it can be predicted (although no experimental evidence is yet available) that probe Avi1 would not distinguish *M. avium* and *M. paratuberculosis* strains. The *M. chelonae* probe Che1 would be expected to hybridize to amplification product from the closely related species *M. abscessus* (15, 19, 28), and the *M. kansasii* probe Kan1 might hybridize to *M. gastri*, *M. simiae*, and *M. scrofulaceum* amplicons. This is a limitation that is inherent in using 16S rRNA as an identification target. Although further discrimination might be possible in some cases by using more than one probe to identify a particular species, this would mean enlarging the identification panel. The additional probes would bring little advantage, since most of the mycobacteria that might otherwise be misidentified are rarely encountered clinically.

The Tbc1 probe not only identified all strains belonging to the *M. tuberculosis* complex but also reacted with *M. terrae*-amplified DNA, even though there were 3-bp differences between the relevant sequences. Cross-hybridization has been reported with other *M. tuberculosis* probes based on this region (9, 20), although this has been corrected for the current TB AccuProbe (Gen Probe, Inc., San Diego, Calif.). Kox et al., who also reported cross-reactivities between *M. terrae* DNA and a *M. tuberculosis* probe based in the same hypervariable region, proposed that the cross-reaction of their probe was due either to the spacing between mismatches or to the length of the run of matching nucleotides (18). With the probes described in this study, it was possible to distinguish between strains of the species *M. terrae* and *M. tuberculosis*, since the probe Ter1 did not cross-react with strains of the latter species. Thus, analysis of both Tbc1 and Ter1 probe results enabled *M. tuberculosis* complex isolates to be clearly differentiated from *M. terrae* isolates.

The probe Int1 hybridized to the type strain of *M. intracellulare* and to strains with related sequences. The serovar 18 amplicon, which hybridized weakly with the probe, differs from Int1 by two mismatches located 12 bp apart (3). Int1 would also be expected to hybridize weakly with amplicons derived from strains of *M. intracellulare* serovar 7, which are identical to serovar 18 strains in this region. The type strain *M. intracellulare* is indistinguishable at the 16S rRNA level from *M. intracellulare* serovars 12, 13, 14, 15, 16, 17, 19, and 20. Together with serovars 7 and 18, these are considered true members of the species *M. intracellulare* (3). The other *M. intracellulare* serovars (4 to 6 and 8 to 11) are indistinguishable from *M. paratuberculosis* at the 16S rRNA level and would not be expected to hybridize to probe Int1. At the molecular level, strains of these serovars are much more closely related to *M. avium* than to *M. intracellulare* (3) and would be indistinguishable from *M. avium* strains by using the *M. avium* probe Avi1. Therefore, the single probe (Int1) was able to give a straightforward identification of all strains of *M. intracellulare* that are closely related to the type strain. Strains of *M. intracellulare* serovars that have a closer genotypic affiliation with *M. avium-M. paratuberculosis* than with the type strain would not be expected to give a signal with Int1, but they should react with Avi1.

Analysis of the 35 strains tested blind led to the discovery that probe Mal1 cross-reacted with amplified *M. nonchromogenicum* DNA, although there were 3-bp mismatches between the Mal1 probe and the corresponding *M. nonchromogenicum* 16S rRNA region. However, all three mismatches were located at one end (5′) of the probe. Previous work suggested that mismatches are more likely to effect probe specificities when they are close to the middle of oligonucleotide probes than when they are at the ends (1). A second *M. malmoense* probe

(Mal2) did not hybridize to *M. nonchromogenicum*. However, further evaluation of this probe showed slight cross-hybridization to *M. kansasii*. Accordingly, the use of a combination of Mal1 and Mal2 allowed genuine *M. malmonese* strains to be differentiated from *M. nonchromogenicum* or *M. kansasii* isolates.

The inclusion of an all-*Mycobacterium* species capture probe (All1) allowed the presence of any mycobacterial species to be detected, even when it did not hybridize to one of the species-specific probes. The identity of any such isolate could then be determined by either reprobing against a second panel of less commonly encountered species or, as we did, by partial 16S rRNA gene sequencing followed by comparison to a data base of known mycobacterial DNA coding for 16S rRNA (rDNA) sequences which are available in the EMBL, GenBank, and the University of Illinois small-subunit rRNA databases.

Sequencing proved useful on several occasions in resolving discrepancies between the results of PCR-ELISA and biochemical testing. Two isolates could not be identified by PCR-ELISA but hybridized to the All1 capture probe, suggesting that they were mycobacteria. Biochemical analysis had indicated that the isolates were *M. fortuitum*, but sequencing showed that both isolates had indistinguishable 16S rRNA sequences which were unique. The sequence was different from that of the reference strain *M. fortuitum*. However, the sequences had only a 1-bp difference from a previously reported *M. fortuitum* subtype sequence, which was represented by *M. fortuitum* ATCC 49404 (15), supporting the biochemical designation. Sequencing is particularly suitable for identifying subtypes that are rarely encountered. For example, although the *M. gordonae* type strain probe Gor1 specifically identified all *M. gordonae* strains in the test study, other subtypes with different 16S rRNA hypervariable regions that would not be expected to hybridize to probe Gor1 have been described elsewhere (14).

Recently, other probe-based assays capable of identifying a more-restricted range of mycobacteria have been described (1, 18). One of these methods, oligonucleotide-specific capture plate hybridization (OSCPH), also uses an ELISA format (1). However, the OSCPH hybridization protocol is more complicated and consequently takes longer than the PCR-ELISA described here. In addition, OSCPH requires the incorporation of digoxigenin-11-dUTP nucleotides into the PCR amplicon during amplification. This might be expected to adversely affect the reliability and sensitivity of the test, besides increasing its cost versus that of primer labelling due to the requirement to add the modified base to each reaction mixture.

While identification directly from clinical material is desirable, there are potentially problems in interpreting the results. For example, the sensitivity of these assays means that they might detect and identify mycobacteria that are present in samples but that do not cause disease. Environmental mycobacteria are ubiquitous and can colonize patients without causing invasive illness (36). More practical applications for this assay would be for the rapid identification to species level of smear AFB-positive samples, as shown here, and for rapid examination of specimens for which there is a strong suspicion of infection. Alternatively, the assay would be suitable for identification to species level of early growth in mycobacterial cultures or as a means of rapidly and objectively assessing established cultures. Combined with liquid culture systems, it might be expected that a PCR-ELISA identification for most samples could be made within 1 week of inoculation. Screening of all clinical specimens by PCR-ELISA would be expensive and perhaps unjustified, since the great majority of samples submitted to most laboratories are negative and are examined for precautionary reasons.

The identification of cultured isolates by sequencing of amplified 16S rRNA has already been advocated by several workers (16) but is relatively complex and too expensive to be used routinely in diagnostic laboratories. However, if the cultured isolates were screened by PCR-ELISA, a large proportion of isolates, representing the most common clinically encountered species, could be identified and very few isolates would require sequencing. Such a strategy would reduce the cost and the time required to make a genotypic identification.

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