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Application of a Simple Multiplex PCR To Aid in Routine Work of the Mycobacterium Reference Laboratory

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A PCR specific for spacer regions 33 and 34 of the direct repeat region of the *Mycobacterium tuberculosis* complex was developed to complement the biochemical differentiation of *M. tuberculosis*, *Mycobacterium bovis*, *M. bovis* BCG, and *Mycobacterium africanum* subtypes I and II. In addition, this approach was incorporated into a multiplex PCR that included primers specific for IS*6110* and the 65-kDa antigen gene in order to differentiate members of the *M. tuberculosis* complex from atypical mycobacteria.

There is value in the rapid differentiation of cultured *Mycobacterium tuberculosis* complex (MTC) from atypical mycobacteria. This confirms the initial diagnosis and treatment regimen being used. In addition, there is some concern that members of the MTC may not always be accurately distinguished from one another, which confounds accurate epidemiology and could prevent important outbreaks of infection from being observed or sources of infection from being identified. Traditionally, identification relies on a battery of biochemical tests (4), which are slow and time-consuming to set up. Commercial molecular tests are available for testing of cultured isolates, but they have some disadvantages. The tests are usually specific for a given species such that a negative result could be the result of inadequate biomass, as can occur with liquid cultures, or the inhibition of the test by that sample. In addition, these tests do not differentiate between members of the MTC (1, 2, 10). There is, therefore, a need to improve the existing methods.

A number of PCR assays based on observed genetic differences between mycobacterial species have been developed. One PCR assay for distinguishing between *M. tuberculosis* and *Mycobacterium bovis* was dependent on the fact that the former contains more IS*6110* copies than do *M. bovis* strains (14). However, further studies revealed that some strains of *M. bovis* have a high IS*6110* copy number, and the reverse is true for some *M. tuberculosis* strains (17). The gene *mtp40* was reported previously to be present only in *M. tuberculosis* and not in *M. bovis* (12), which seemed to offer an alternative approach for distinguishing these species, but further studies revealed that this gene is found in most, though not all, *M. tuberculosis* strains and is also found in some *M. bovis* strains (19).

In 1997, Kamerbeek et al. (9) reported a PCR-based method, spoligotyping, for the fingerprinting of MTC strains. This assay was based on earlier work that revealed polymorphism in the direct repeat (DR) region of the MTCs that depends on the presence or absence of specific spacer region sequences between two DR sequences (7). It has the added advantage of differentiating between *M. bovis* and *M. tuberculosis* (9), as *M. bovis* contains spacer regions 33 and 34, which are absent in *M. tuberculosis*. In addition, we have observed from published data (3, 16) that *M. bovis* BCG has two copies of spacer region 33 but only one of spacer region 34. We have used the observation to design a PCR that could differentiate among *M. bovis*, *M. bovis* BCG, and *M. tuberculosis*. We also incorporated this PCR into a multiplex PCR for use on cultured isolates, which could identify the genus *Mycobacterium*; identify members of the MTC; and distinguish *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

All the mycobacterial strains used in this study were obtained from the Mycobacterium Reference Unit of the Public Health Laboratory Service at Dulwich Hospital. The numbers of strains of each species tested are shown in Table 1. Cultures had already been identified to species level by conventional, culturing, and biochemical procedures.

The mycobacteria were inactivated by being heated at 80°C for 20 min prior to DNA extraction. DNA was extracted by modification of a simple, rapid method (20) using chloroform to assist in disrupting cells and to precipitate proteins. With a clean loop (1 μl), a small quantity of mycobacteria grown on solid agar (Lowenstein-Jensen) was harvested and placed into a microcentrifuge tube containing 100 μl of sterile distilled water. One hundred microliters of chloroform was then added and vortexed for 10 s. The mixture was then heated at 80°C for 20 min, followed by brief freezing at −20°C. The tubes were then removed from the freezer and allowed to thaw. Centrifugation at 13,000 × *g* for 3 min pelleted the cell debris to the chloroform aqueous interface. The clear lysate above the chloroform was used directly in PCR.

The following primers were used: spacer region-specific primers, spacer region 33 specific (5′ACACCGACATGACG GCGG3′) and spacer region 34 specific (5′CGACGGTGTG GGCGAGG3′); IS*6110*, MTC-specific primers (20), TB284 (sequence 5′GGACAACGCCGAATTGCG3′) and TB850 (sequence 5′TAGGCGTCGGTGACAAAGGCCAC3′); and *Mycobacterium* genus-specific (65-kDa antigen gene) primers (15), TB11 (sequence 5′ACCAACGATGGTGTGTCCAT3′) and TB12 (sequence 5′CTTGTCGAACCGCATACCCT3′).

PCR mixtures contained 20 μl of 2× PCR mix (20), 10 μl of

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TABLE 1. Results of multiplex PCR

Species	No. of strains tested	Presence of band generated following multiplex PCR			
		99 bp	172 bp	439 bp	550 bp
M. tuberculosis	30	−	−	+	+
M. bovis	18	+	−	+	+
M. bovis BCG	18	+	+	+	+
M. africanum I	5	+	−	+	+
M. africanum II	5	−	−	+	+
NTM[a]	54	−	−	+	−

[a] NTM, nontuberculous mycobacteria.

primer mix with each primer at 0.66 pmol/μl, 0.2 μl of *Taq* polymerase enzyme (Roche Diagnostics Ltd.), and 10 μl of extracted DNA. The PCR conditions were 95°C for 3 min; 30 cycles of 95°C for 20 s, 65°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. After PCR, the products were analyzed by electrophoresis on a high-resolution 2% (wt/vol) Metaphor agarose matrix (Flowgen).

As expected, all 18 strains of *M. bovis* BCG produced two bands of 172 and 99 bp corresponding to amplification products from both of the spacer regions 33 in conjunction with spacer region 34 (Fig. 1 and Table 1). All 18 strains of *M. bovis*, which has only one spacer region 33, produced only the 99-bp band, whereas these bands were absent in all 30 strains of *M. tuberculosis*, as *M. tuberculosis* does not contain either spacer region. Interestingly, when *Mycobacterium africanum* strains were tested, all five strains of *M. africanum* I produced the 99-bp *M. bovis*-specific band, but all five strains of *M. africanum* II, similarly to *M. tuberculosis*, did not produce any band in this region. All the MTC strains produced a band of 550 bp corresponding to amplification of IS*6110*, and the mycobacterium-specific 65-kDa antigen gene resulted in a band of 439 bp (Fig. 1 and Table 1).

A number of strains of six different atypical mycobacterial species including *Mycobacterium chelonae* (a total of 10 strains tested), *Mycobacterium malmoense* (a total of 10 strains tested), the *Mycobacterium avium* complex (a total of 12 strains tested), *Mycobacterium fortuitum* (a total of 10 strains tested), *Mycobacterium marinum* (a total of 2 strains tested), and *Mycobacterium kansasii* (a total of 10 strains tested) were also analyzed. None of these species produced either of the *M. bovis*-specific bands of 99 and 172 bp or the IS*6110*-specific band (Fig. 1 and Table 1). However, all the atypical mycobacterial species produced the mycobacterium-specific 65-kDa antigen gene band.

Many reference laboratories perform a molecular test on cultured isolates so that identification of MTC is not delayed by subculturing for biochemical testing. These tests do not have a mycobacterium-specific internal control and do not differentiate between the members of the MTC (2, 10). Our multiplex PCR approach complements the biochemical testing in rapidly differentiating MTC from other mycobacteria and in confirming subsequent species-level identification of MTC. The procedure is simple, using a simple chemical extraction for preparation of the sample. In addition, the method is also rapid, taking a total of 5 h when using a conventional thermal cycler, which could be reduced to 3 h if a rapid capillary cycler were used. The sample throughput is limited only by the capacity of the cycler used, which could range from 24 to 96 samples per run. Unlike other PCR-based protocols, this method does not require hybridization or restriction enzyme analysis (PCR-restriction fragment length polymorphism) (5, 8, 13, 15).

This study confirms and complements the observations of

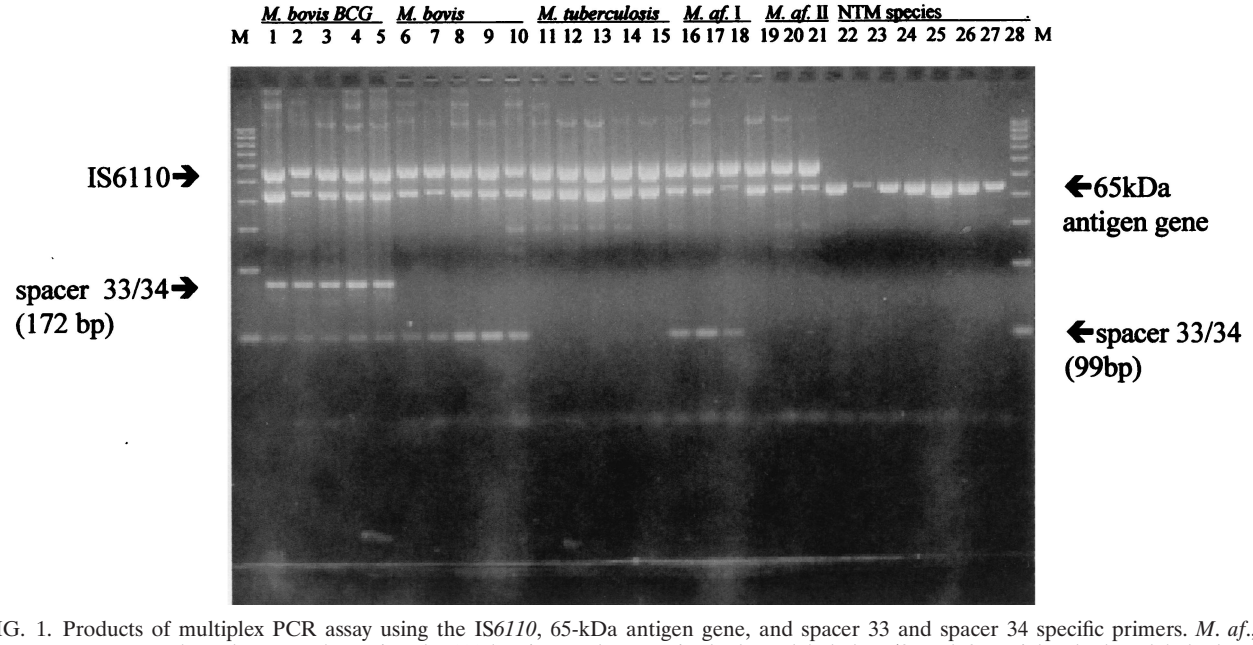

FIG. 1. Products of multiplex PCR assay using the IS*6110*, 65-kDa antigen gene, and spacer 33 and spacer 34 specific primers. *M. af.*, *M. africanum*; NTM, nontuberculous mycobacteria. The 100-bp size markers are in the lanes labeled M (from left to right, the lane labels shown at the top of the figure have a one-to-one correspondence with the lanes shown).

Niemann et al. (11), who used PCR-restriction fragment length polymorphism of the *gyrB* gene to investigate members of the MTC. Using this method, in contrast to our study, *M. africanum* I could be differentiated from other MTC members but *M. bovis* could not be differentiated from *M. bovis* BCG. As in our study, *M. tuberculosis* could not be differentiated from *M. africanum* II.

The fact that in this study *M. africanum* I was similar to *M. bovis* whereas *M. africanum* II, in both this and the study by Niemann et al. (11), was similar to *M. tuberculosis* is an interesting observation that perhaps strengthens the view that these species are intermediate between *M. bovis* and *M. tuberculosis*. A recent publication also concluded that the spoligotyping pattern produced by the analysis of the DR region of *M. africanum* isolates was intermediate between that of *M. bovis* and *M. tuberculosis* (18). Our assay could not differentiate between *M. africanum* I and *M. bovis* or *M. africanum* II and *M. tuberculosis*, but *M. africanum* is rare in western Europe (6), and our test was designed to complement rather than replace biochemical testing and to aid in workload management. It is envisaged that our test would be used after confirmation of mycobacterial cultures by Ziehl-Neelsen staining. The results of the multiplex PCR enable a rapid identification of MTC, indicate whether the result is likely to be *M. bovis* or *M. bovis* BCG, and facilitate the setting up of the appropriate biochemical test.

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