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 IS6110 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, 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 pellet 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′ACACCGCATGACGGCGG3′) and spacer region 34 specific (5′CGACGGTGTGGGCAGGCGG3′); IS6110, MTC-specific primers (20), TB284 (sequence 5′GGACACGCGCAGAATGGGG3′) and TB850 (sequence 5′TAGCGGTGCCGTGACAAAGGCCAC3′); and Mycobacterium genus-specific (65-kDa antigen gene) primers (15), TB11 (sequence 5′ACCAACGATGTTGTGTCATCAT3′) and TB12 (sequence 5′CTTGCGAACGCAGCATACC3′).

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

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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 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 IS6110, 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), *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 kansasi* (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 IS6110-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

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains tested</th>
<th>Presence of band generated following multiplex PCR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>99 bp</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td><em>M. africanum</em> I</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>M. africanum</em> II</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>NTM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54</td>
<td>-</td>
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<sup>a</sup> NTM, nontuberculous mycobacteria.

FIG. 1. Products of multiplex PCR assay using the IS6110, 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. africanaum 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. africanaum II.

The fact that in this study M. africanaum I was similar to M. bovis whereas M. africanaum 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. africanaum isolates was intermediate between that of M. bovis and M. tuberculosis (18). Our assay could not differentiate between M. africanaum I and M. bovis or M. africanaum II and M. tuberculosis, but M. africanaum 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.

REFERENCES