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Multiplex PCR Provides a Low-Cost Alternative to DNA Probe Methods for Rapid Identification of *Mycobacterium avium* and *Mycobacterium intracellulare*

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A multiplex PCR designed to differentiate *Mycobacterium tuberculosis* complex organisms from *M. avium* and *M. intracellulare* was used to test 105 isolates identified by DNA probe methods as *M. avium*, *M. intracellulare*, or *M. avium* complex type X. The multiplex PCR correctly identified 33 of 34 isolates identified by commercial probe methods as *M. avium* and all 51 isolates identified as *M. intracellulare*. The 20 isolates identified as *M. avium* complex type X by probe were identified as *Mycobacterium* spp. by the multiplex method. These results confirm that the multiplex PCR, which is simple to perform and cheaper than commercial probe methods, is suitable for routine identification of *M. avium* and *M. intracellulare*.

Although the term *Mycobacterium avium* complex (MAC) has no clear taxonomic status, it is widely used in the literature to cover a group of slowly growing mycobacteria that includes the well-defined species *M. avium* and *M. intracellulare*. Many laboratories identify these organisms with the broadly specific MAC probe of the AccuProbe series (Gen-Probe Inc., San Diego, Calif.), but it is now known that this probe detects organisms (the so-called MAC X group) that are neither *M. avium* or *M. intracellulare* and whose taxonomic status remains unresolved (3, 10, 14). Although information for the X group is sketchy, there are distinct differences in the clinical and epidemiological features of disease due to *M. avium* on the one hand and *M. intracellulare* on the other (7). Even so, workers in many laboratories choose not to subdivide the MAC. We believe that more precise identification will lead to a better understanding of clinical and epidemiological aspects of the disease caused by individual members of the MAC.

The three recognized clusters within the MAC are difficult to differentiate by using the standard panel of identification tests. High-performance liquid chromatography (HPLC) of mycolic acid profiles (2, 5) has been reported to be a rapid, efficient, and inexpensive method that can identify *Mycobacterium* isolates to the species level in a single day (12). However, this technique uses costly equipment (HPLC) and requires substantial amounts of the test organism. In recent years, methods employing molecular techniques have been developed as routine identification procedures. Restriction digestion of PCR products generated from the 16S rRNA (6) and the gene coding for the 65-kDa heat shock protein (11) can identify various species of mycobacteria. In addition, 16S rRNA sequencing has been proposed for routine identification of *Mycobacterium* spp. (1, 6, 8) but laboratories need access to expensive, specialist equipment for sequencing. The species-specific AccuProbes provide rapid and accurate results with the subgroups within the MAC and can be used with colonies from solid medium or the liquid culture medium of the BACTEC system (3, 4, 13). However, the cost of routine use of the full panel of commercial probes with every isolate would be difficult to justify. One of us (D.C.) was involved in the development of a multiplex PCR that allowed recognition of *M. tuberculosis*, *M. avium*, *M. intracellulare*, and the genus *Mycobacterium* in a single reaction (15). The test is both rapid (24 h) and inexpensive ($2 [United States] for reagents). Treatment of the sample is simple, requiring only a simple heating step to expose sufficient DNA for a successful PCR. In this study, we applied the multiplex PCR to 105 clinical isolates which had previously been identified as belonging to one of the three subgroups of the MAC by commercial probes.

The isolates tested were from patients living in Queensland (n = 78), Western Australia (n = 19), and Victoria (n = 8). Twenty-four of the isolates, i.e., 20 of *M. avium*, 1 of *M. intracellulare*, and 3 of the MAC X group, originated from patients known to be human immunodeficiency virus positive. The strains were members of the MAC (Table 1). Eight of the 20 strains in the X group were identified by the SNAP X-probe (Syngene Inc). The remainder were identified by reactions obtained with the AccuProbe panel. In addition to the strains listed, 11 isolates of *M. scrofulaceum* from our culture collection were also tested by the multiplex procedure.

For multiplex PCR, a single colony was inoculated into 100 μl of purified water and mixed, heated for 10 min at 94°C, and cooled. After brief spinning to deposit cell debris, 5 μl of the supernatant was inoculated into a 20-μl PCR mixture overlaid with 50 μl of paraffin oil and tested with the multiplex PCR by the method of Wilton and Cousins (15), with the following modifications. The concentration of primers was altered so that 50 ng of MYCOGEN-F and MYCOGEN-R, 50 ng of MYCAV-R, 20 ng of TB-1 A and B, and 75 ng of MYCINT-F were used in each reaction. Genus-specific primers MYCOGEN-F and MYCOGEN-R were originally described by Böddinghaus et al. (1). After 35 cycles of amplification, 5 μl of the amplified product was loaded onto a 2% agarose gel and subjected to electrophoresis at 100 V for 0.6 h. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV light by using a transilluminator (UVP, San Gabriel, Calif.). Reference strains of *M. avium* and *M. intracellulare* were run as positive controls, and water was used as a negative control. The sizes of amplified products were compared with a

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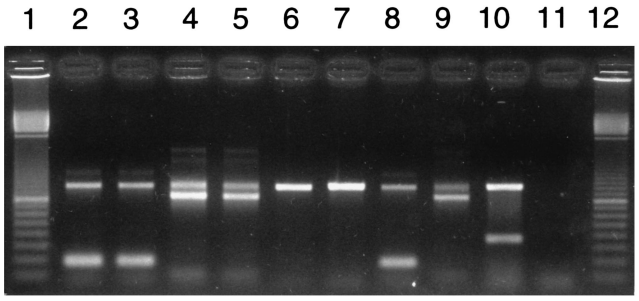

FIG. 1. Results of testing of *Mycobacterium* strains with the multiplex PCR. Lanes: 1 and 12, 100-bp ladder molecular size marker (the 800-bp band is the most intense); 2 and 3, *M. avium*; 4 and 5, *M. intracellulare*; 6, MAC group X; 7, *M. scrofulaceum*; 8, *M. avium* control; 9, *M. intracellulare* control; 10, *M. tuberculosis* complex control; 11, negative control.

molecular size marker (100 base pair ladder; AMRAD Pharmacia Biotech, North Ryde, New South Wales, Australia) run with the samples on the gel. Isolates were identified as *M. avium* in the multiplex PCR if they produced two amplified products of 1,030 and 180 bp, as *M. intracellulare* if the amplified products were 1,030 and 850 bp, and as *Mycobacterium* spp. if they produced a single amplified product of 1,030 bp (Fig. 1). No bands of any other size were observed with the multiplex PCR. The PCR tests were interpreted blindly without knowledge of the probe result, and results were collated only after the PCR testing was finalized.

Table 1 shows the results of the multiplex PCR for the 105 strains. Thirty-three (97.06%) of the 34 isolates identified as *M. avium* by AccuProbe were identified as *M. avium* by PCR. The remaining isolate (strain S81/222, from an infant's lymph node) was identified as *M. intracellulare* by multiplex PCR. In addition to testing performed locally, this isolate has also been tested at the laboratories of Gen-Probe Inc., where it was identified as *M. avium* by AccuProbe (7a). It is noteworthy that, unlike the large majority of *M. avium* isolates that we have tested, the isolate does not grow at 44°C. Although both multiplex PCR and AccuProbe target the rRNA gene, the degree of complementarity is not known; it seems likely that strain S81/22 is aberrant in the region targeted by the AccuProbe *M. avium* probe. All of the 51 isolates identified as *M. intracellulare* by AccuProbe were confirmed as *M. intracellulare* by multiplex PCR. The 20 isolates identified as members of the MAC X group were shown to be neither *M. avium* nor *M. intracellulare* by the multiplex PCR, producing only the single 1,030-bp band indicative of *Mycobacterium* spp. Similarly, the 11 isolates of *M. scrofulaceum* were identified as *Mycobacterium* spp. in the multiplex PCR.

TABLE 1. Results of testing of 105 MAC isolates with commercial DNA probe methods and multiplex PCR

Identity determined by commercial probe	No. of isolates identified by multiplex PCR as:			Total no. of isolates
	M. avium	*M. intracellulare*	*Mycobacterium* spp.	
M. avium	33	1	0	34
M. intracellulare	0	51	0	51
MAC X group	0	0	20	20
Total	33	52	20	105

The multiplex PCR that we employed in this study was devised as a rapid and inexpensive technique for identification of the *M. tuberculosis* complex, *M. avium*, and *M. intracellulare*—the pathogens isolated most commonly in clinical laboratories. Because the multiplex employs an amplification step and requires minimal growth, it could be expected that results will be obtainable earlier than with hybridization-based procedures such as the AccuProbe. We have already used the multiplex PCR to correctly identify more than 304 isolates of the *M. tuberculosis* complex (9). Here, we have shown that the multiplex PCR agreed with the AccuProbe results for 84 of 85 isolates identified as either *M. avium* or *M. intracellulare.*

It must be noted that the multiplex PCR does not identify members of the MAC X group. Members of this group are potential pathogens (13), and our experience in Australia suggests that the MAC X group accounts for around 5% of all clinical cases of atypical mycobacteriosis (3a). The rationale

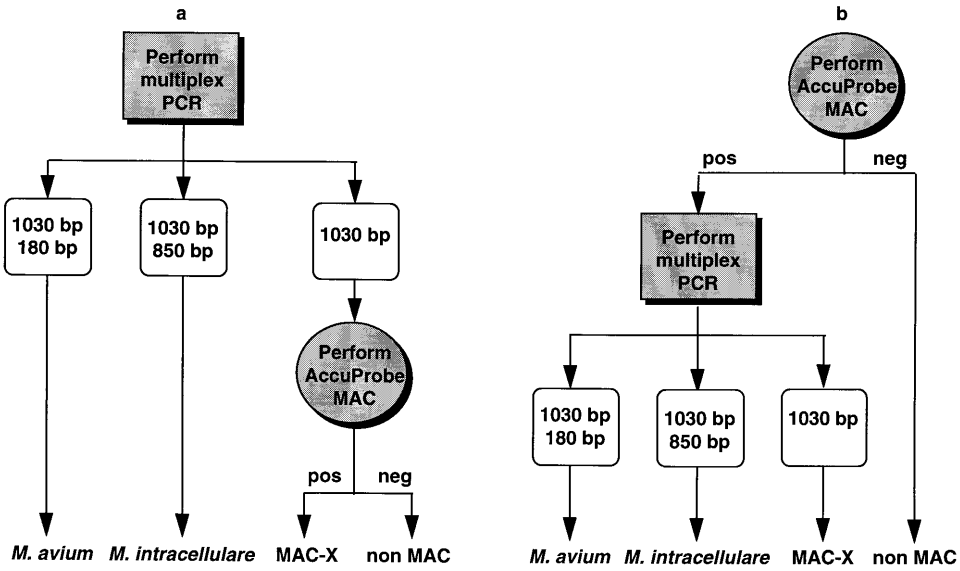

FIG. 2. Alternative algorithms for testing of presumptive MAC strains. Algorithm a is recommended to minimize the cost of identification of *M. avium*, *M. intracellulare*, and members of the MAC X group. pos, positive; neg, negative.

for inclusion of the X cluster in the MAC is problematic. HPLC study of mycolic acid esters suggests that the cluster probably contains more than one cluster (13). Soini et al. (10) have recently demonstrated genetic diversity in the 32-kDa sequences of the MAC X group.

Furthermore, conventional properties vary between strains (13). It seems that there is a need to resolve the taxonomic status of the X group to better define the specificity of the MAC probe. Perhaps the manufacturers should consider changing the composition of the MAC probe so that it reacts with only *M. avium* and *M. intracellulare* and renaming it the *M. avium-M. intracellulare* probe.

Our results confirm the usefulness of the multiplex PCR for rapid and accurate identification of the two most commonly encountered pathogens within the MAC. We believe it offers an inexpensive alternative to commercial probe tests for clinical and veterinary laboratories with expertise in PCR. The usefulness of the PCR would be optimized if suspect MAC organisms were tested in the multiplex PCR in the first instance as shown in Fig. 2a. Any strains identified as *Mycobacterium* spp. by multiplex PCR could subsequently be tested with the AccuProbe MAC probe. AccuProbe test-positive isolates could then be reported as MAC X. Alternatively, the AccuProbe MAC probe test could be used first and positive isolates could be tested in the multiplex PCR for identification as shown in Fig. 2b, although we believe the option shown in Fig. 2a to be far more economical. Identification of the cause of infections as *M. avium*, *M. intracellulare*, and members of the MAC X group will lead to a better understanding of the epidemiology and clinical importance of the clusters that make up the MAC. The fact that multiplex PCR, unlike probe-based procedures, can recognize instances of mixed cultures is a further attribute. This is an important consideration in cases in which *M. tuberculosis* complex organisms are among the component species.

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REFERENCES

1. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
2. **Butler, W. R., and J. O. Kilburn.** 1988. Identification of major slowly growing pathogenic mycobacteria and *Mycobacterium gordonae* by high-performance liquid chromatography of their mycolic acids. J. Clin. Microbiol. **26:**50–53.
3. **Cregan, P., D. M. Yajko, V. L. Ng, P. C. Gonzalez, P. S. Nassos, C. A. Sanders, and W. K. Hadley.** 1992. Use of DNA probes to detect *Mycobacterium intracellulare* and "X" mycobacteria among clinical isolates of *Mycobacterium avium* complex. J. Infect. Dis. **166:**191–194.

3a. **Dawson, D.** Unpublished data.

4. **Evans, K. D., A. S. Nakasone, P. A. Sutherland, L. M. de la Maza, and E. M. Peterson.** 1992. Identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* directly from primary BACTEC cultures by using acridinium-ester-labeled DNA probes. J. Clin. Microbiol. **30:**2427–2431.
5. **Floyd, M. M., V. A. Silcox, W. D. J. Jones, W. R. Butler, and J. O. Kilburn.** 1992. Separation of *Mycobacterium bovis* BCG and *Mycobacterium bovis* by using high-performance liquid chromatography of mycolic acids. J. Clin. Microbiol. **30:**1327–1330.
6. **Hughes, M. S., R. A. Skuse, L.-A. Beck, and S. D. Neill.** 1993. Identification of mycobacteria from animals by restriction enzyme analysis and direct cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences. J. Clin. Microbiol. **31:**3216–3222.
7. **Inderlied, C. B., C. A. Kemper, and L. E. M. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.

7a. **Jonas, V.** Personal communication.

8. **Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F.-C. Bange, and E. C. Böttger.** 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. **31:**2882–2889.
9. **Liébana, E., A. Aranaz, B. Francis, and D. Cousins.** 1996. Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **34:**933–938.
10. **Soini, H., E. Eerola, and M. K. Viljanen.** 1996. Genetic diversity among *Mycobacterium avium* complex AccuProbe-positive isolates. J. Clin. Microbiol. **34:**55–57.
11. **Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer.** 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. **31:**175–178.
12. **Thibert, L., and S. Lapierre.** 1993. Routine application of high-performance liquid chromatography for identification of mycobacteria. J. Clin. Microbiol. **31:**1759–1763.
13. **Viljanen, M. K., L. Olkkonen, and M.-L. Katila.** 1993. Conventional identification characteristics, mycolate and fatty acid composition, and clinical significance of MAIX AccuProbe-positive isolates of *Mycobacterium avium* complex. J. Clin. Microbiol. **31:**1376–1378.
14. **Wayne, L. G., and H. A. Sramek.** 1992. Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin. Microbiol. Rev. **5:**1–25.
15. **Wilton, S., and D. Cousins.** 1992. Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. PCR Methods Applications **1:**269–273.