Rapid Identification of Mycobacterium avium Complex in Culture Using DNA Probes

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A commercial DNA probe procedure for identification of Mycobacterium avium complex isolates was evaluated for accuracy and applicability for use in the clinical laboratory. The test (Gen-Probe Rapid Diagnostic System for Mycobacterium Avium Complex; Gen-Probe Corp., San Diego, Calif.) uses hybridization in solution of two 125I-labeled cDNA probes. One probe is complementary to rRNA from M. avium, and the other is complementary to rRNA from M. intracellulare. Results are expressed as absolute percent hybridization, with values ≥10% considered positive. The procedure accurately identified all 134 M. avium complex isolates and concomitantly identified them to species level. There were no false-positives with 66 other mycobacteria, including 22 M. tuberculosis and 18 M. kansasii isolates, or with 8 Nocardia isolates. The mean percent hybridization (± the standard deviation) of M. avium probe-positive isolates (94 isolates) was 48.0 ± 9.9 (range, 11.5 to 72.7); for M. intracellulare (40 isolates), it was 45.7 ± 8.8 (range, 22.7 to 60.7). Among the 74 non-M. avium complex isolates, the percent hybridization range was 1.0 to 4.2, except for a single value of 9.7 which was <5 when the test was repeated. Four M. avium complex isolates reacted positively with both probes on initial testing, and three were confirmed. On repeat testing of subcultures, each reacted with only one probe, suggesting the presence of a mixed culture. The procedure can be completed in as little as 2 h and could easily be performed in most clinical laboratories.

Identification of mycobacteria other than Mycobacterium tuberculosis requires considerable time and expertise. The development of rapid and simplified identification techniques has long been desired and is now possible through nucleic acid probe technology. A commercial kit (Gen-Probe Rapid Diagnostic System for Mycobacterium Avium Complex; Gen-Probe Corp., San Diego, Calif.) is available for culture confirmation and identification to species level of organisms belonging to the Mycobacterium avium complex. This test uses two separate 125I-labeled cDNA probes, one homologous to rDNA derived from the type strain of M. avium and the other homologous to rDNA from Mycobacterium intracellulare. Hybridization is performed in solution with sonic extracts of mycobacterial suspensions, and the entire procedure can be completed in as little as 2 h.

M. avium complex organisms are of major clinical importance, causing tuberculosis-like pulmonary disease, as well as local and disseminated infections in immunosuppressed patients (3, 6, 7). The broadening acquired immune deficiency syndrome epidemic has significantly increased the frequency with which M. avium complex is isolated in the clinical laboratory. At the University of California at Los Angeles, M. avium complex accounted for approximately 55% of all mycobacteria isolated in 1986. Prompt and accurate identification of these organisms is important since therapy for M. avium complex infections differs significantly from that for tuberculosis and other mycobacterial diseases. On primary isolation, the colony morphology and pigmentation of M. avium complex varies such that biochemical tests are necessary to distinguish them from other mycobacterial species. The purpose of this study was to evaluate the accuracy and applicability of these DNA probe procedures for identification of M. avium complex isolated in the clinical laboratory.

MATERIALS AND METHODS

Preparation of organisms. A total of 134 M. avium complex isolates, 66 non-M. avium complex mycobacterial isolates, and 8 Nocardia isolates were tested (Table 1). These isolates were obtained from clinical specimens submitted to the University of California at Los Angeles Clinical Microbiology Laboratory, except for 31 stock strains of M. avium complex provided by L. Wayne, Long Beach Veterans Administration Hospital, Long Beach, Calif.; 4 Mycobacterium kansasii and 9 Mycobacterium fortuitum clinical isolates from N. Glover, Olive View Medical Center, Los Angeles, Calif.; and 1 isolate each of Mycobacterium xenopi, Mycobacterium terrae, and Mycobacterium haemophilum obtained from College of American Pathologists proficiency samples. Only one isolate per patient was included for study. M. avium complex isolates represented a mix from acquired immune deficiency syndrome (76 isolates) and other (58 isolates) sources. All isolates had been previously identified by conventional testing (5), which included growth rate, gross and microscopic colony morphology, pigmentaion, and tests for niacin, semiquantitative catalase, nitrate reduction, urease, and tellurite reduction. M. haemophilum was grown at 30°C on Middlebrook 7H10 medium supplemented with hemin, whereas M. avium complex, M. terrae, M. xenopi, and Nocardia isolates were grown on Wallenstein medium and other mycobacteria were grown on Lowenstein-Jensen medium, all at 35°C in 8% CO2. Culture media were obtained from Cal Scott, Inc., Carson, Calif.; and BBL Microbiology Systems, Cockeysville, Md. Cultures were tested within 4 weeks of subculturing except for M. fortuitum and Mycobacterium chelonae, which were tested within...
7 days. Colonies were removed from the agar surface, and the organisms were suspended in 2 ml of distilled water to obtain a turbidity equivalent to a no. 1 McFarland standard. A few glass beads were added to the suspension tubes to achieve homogeneity when M. tuberculosis isolates were tested. Suspensions not used immediately were stored at 4°C and tested within 24 h of preparation.

**Assay procedure.** Reagents were provided by the manufacturer in kit form (Gen-Probe Rapid Diagnostic System for Mycobacterium Avium Complex). Each kit included the following: lysing reagent; two probe solutions, one each for *M. avium* and *M. intracellulare*, with 125I activities of 0.5 to 1.0 μCi/20-ml vial; hydroxyapatite separation suspension; and wash solution. Positive and negative control strains, prepared as described above for test organisms, were included in each run. The control strains used were *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950, and *M. tuberculosis* ATCC 25177. For each isolate, two separate tubes containing lysing reagent were inoculated with 100 μl of bacterial suspension and then immersed in a sonicating water bath for 15 min at 60 to 70°C. Probe solution (1 ml) for either *M. avium* or *M. intracellulare* was added to each tube. All tubes were vortexed and then incubated in a 72°C water bath for 1 h. Separation suspension (4 ml) was added, and the tubes were vortexed and incubated for an additional 5 min. After removal of the tubes from the water bath, they were inverted 20 times before centrifugation (2,000 × g for 2 min; model TJ-6; Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was decanted and discarded, and 4 ml of wash solution was added to the pellet. The pellets were resuspended by vortexing for 20 s and centrifuged as before. The supernatant was decanted and discarded, and the radioactivity of the adsorbent pellet was counted in a gamma counter (Picker Pace-1; Picker International, Inc., Norwalk, Calif.). Each tube was counted for 1 min. Total counts for *M. avium* and *M. intracellulare* probe solutions were determined by counting the radioactivity of 100 μl of solution. The test results are expressed as percent hybridization, determined as follows: percent hybridization = [(sample cpm − background cpm)/(total cpm − background cpm)] × 100, where cpm is counts per minute. Tests were considered positive when the percent hybridization was ≥10. Statistics were determined by standard methods (2).

**Organism viability and containment.** Reaction tubes had internally threaded caps with rubber O-ring seals and were kept tightly closed throughout the procedure except when contents were added or removed. Steps requiring opening of tubes were performed in a biosafety hood used for mycobacterial specimen handling. Samples of the pellets and residual moisture in reaction tubes for 27 *M. avium* complex isolates, 56 non-*M. avium* complex mycobacterial isolates (including 22 *M. tuberculosis* isolates), and 8 *Nocardia* isolates were subcultured to Wallenstein medium to determine organism viability after the completion of testing. Additionally, the moisture surrounding the pellets and on the walls of the reaction tubes for 50 representative isolates was subcultured after the first decanting step.

**Results**

All 134 *M. avium* complex isolates were identified as such by reaction with either the *M. avium* or *M. intracellulare* probe (Table 1). The mean percent hybridization for positive isolates was 10 to 20 times that for negative isolates. The recommended breakpoint of 10% hybridization for separating positive and negative reactions appeared to be valid. Only 3 of the 134 *M. avium* complex isolates showed less than 20% hybridization; these reacted positively with the *M. avium* probe with values of 16.7, 17.8, and 11.5%. With one exception, all non-*M. avium* complex isolates had percent hybridization values of <5 (Table 1). A single *M. kansasi* isolate had 9.7% hybridization on initial testing, but when testing was repeated on two occasions, the values were <5%. Among the 450 total tests performed on 208 isolates, percent hybridization values between 5 and 10 were observed on only 10 occasions: 7 times for *M. avium* or *M. intracellulare* probe-positive (≥30% hybridization) *M. avium* complex isolates which reacted with the converse probe with values between 5.7 and 8.1%, twice during repeat testing of low-positive *M. avium* complex (*M. avium*) isolates, and once for the *M. kansasi* isolate described above. Four *M. avium* complex isolates reacted positively with both probes when first tested, and these initial results were confirmed for three of the four isolates on retesting. However, when subcultures of these three isolates were evaluated, suspensions from individual colonies (which included several morphotypes) consistently reacted with only one of the two probes.

Precision data for control organisms are shown in Table 2. There was moderate run-to-run variability for the positive control organisms. However, in all 15 runs the values were well above the recommended acceptability level of 20% hybridization. Likewise, negative control values were below the recommended limit of 5% hybridization in all runs. The three isolates mentioned above with relatively low percent hybridization (<20%) were tested on two additional occasions, using freshly prepared bacterial suspensions. Two of the three isolates reacted below 10% on one occasion each (values of 9.1 and 7.4%).

In a limited evaluation, the type of medium on which *M. avium* complex isolates were cultured did not influence reactivity with the probes. Four *M. avium* complex isolates

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>% Hybridization of probe (X ± SD [range])</th>
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<tbody>
<tr>
<td><em>M. avium</em> complex</td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em> probe positivea (94)</td>
<td>48.0 ± 9.9 (11.5–72.7)</td>
</tr>
<tr>
<td><em>M. intracellulare</em> probe positivea (40)</td>
<td>3.9 ± 1.4 (2.1–8.1)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> (22)</td>
<td>1.9 ± 0.5 (1.2–3.6)</td>
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<tr>
<td><em>M. kansasii</em> (18)</td>
<td>2.4 ± 1.9 (1.2–9.7)</td>
</tr>
<tr>
<td><em>M. fortuitum</em> (19)</td>
<td>2.0 ± 0.5 (1.1–3.0)</td>
</tr>
<tr>
<td><em>M. chelonae</em> (2), <em>M. xenopi</em> (3), <em>M. terrae</em> (1), <em>M. haemophilum</em> (1)</td>
<td>2.1 ± 0.6 (1.5–3.1)</td>
</tr>
<tr>
<td><em>Nocardia asteroides</em> (4), <em>Nocardia brasiliensis</em> (4)</td>
<td>2.1 ± 0.7 (1.2–3.1)</td>
</tr>
</tbody>
</table>

a Data used for isolates which reacted with both probes on initial testing were from repeat tests in which only one probe reacted positively.
(three *M. avium* probe-positive isolates, including one with a relatively low percent hybridization, and one *M. intracellulare* probe-positive isolate) were grown on Wallenstein, Middlebrook 7H10, and Lowenstein-Jensen media before testing. The percent hybridization values for each isolate from the three media, respectively, were as follows: 53.4, 45.2, and 50.7; 48.2, 41.8, and 47.1; 52.2, 52.0, and 52.0; and 20.0, 19.1, and 17.0.

Portions of the assay tube contents for 27 *M. avium* complex and 64 non-*M. avium* complex isolates (including 22 *M. tuberculosis* isolates) were cultured after completion of the assay to determine whether any viable organisms remained. In all instances, no growth was detected. Samples from 50 assay tubes taken after the initial decanting step showed that this total loss of organism viability had occurred by the completion of the sonication and hybridization steps.

### DISCUSSION

These nucleic acid probe assays for culture identification and concomitant identification to species level of *M. avium* complex isolates constitute a significant advance for clinical mycobacteriology. As a method of culture confirmation of potential *M. avium* complex isolates, testing with both probes in parallel was accurate for all 134 *M. avium* complex isolates examined, with no false-positive reactions among 66 isolates from seven other mycobacterial species and 8 *Nocardia* isolates. Since a 10-fold or greater difference in percent hybridization separated mean positive and negative reaction values, the interpretation of results was straightforward and the results were rarely close to the cutoff value of 10% hybridization. We concur that this cutoff level is appropriate but recommend for isolates negative with both probes that the tests be repeated if either value is between 5 and 10% and that the organism be identified by traditional biochemical testing if values remain in this range.

A unique aspect of these tests is their apparent ability to distinguish between *M. avium* and *M. intracellulare*. Although this study was not designed to validate the use of these probes to accurately determine species, as established by serotyping or DNA-DNA hybridization (1, 4), other studies in progress (R. Enns, personal communication) indicate that they do. The finding of four *M. avium* complex isolates that reacted with both probes on initial testing was therefore unexpected. However, the three isolates for which the initial results were confirmed reacted with only one of the probes (two isolates with the *M. avium* probe and one isolate with the *M. intracellulare* probe) when individual colonies from subcultures were tested. This suggested that the original cultures contained previously unrecognized mixtures of strains of each *M. avium* complex species. These infrequent reactions (4 of 134 isolates) do not affect the accuracy of the test, however, since a reaction with either probe is considered positive and there is no recognized clinical significance in distinguishing between the two *M. avium* complex species.

These probe tests can be performed rapidly and could easily be adopted by most clinical microbiology laboratories. The procedure can be completed in as little as 2 h, depending on the number of samples tested at one time, compared with several days to weeks for conventional methods. For a laboratory already performing mycobacterial isolation, the additional equipment necessary for implementation of these tests includes a sonicating water bath, 72°C water bath, centrifuge, and gamma counter. Approval for use of 125I is also required. Middlebrook 7H10 and Wallenstein media, as well as Lowenstein-Jetnsen medium, may be used for the culture of organisms before testing. Biosafety guidelines for handling mycobacteria should be followed through sonication and incubation in the 72°C water bath. Exposure to these conditions was sufficient to render organisms nonviable in the representative samples we examined. However, a conservative approach would be to treat the specimens as potentially infectious throughout the procedure and to decontaminate assay tubes and liquid before discarding them as radioactive waste.

As with other molecular techniques for clinical laboratory use, the cost of reagents for the *M. avium* complex probe tests is considerably greater than that for the reagents used in conventional testing. Although it was beyond the scope of this study to perform accurate work time studies, the time savings realized through the use of the probe procedures would partially offset the difference in reagent costs. In addition, the availability to clinicians of a definitive organism identification days to weeks sooner than has been possible should reduce the overall cost of care and potential patient morbidity.

The successful application of nucleic acid probe technology for clinical microbiology diagnosis has been eagerly awaited. The probe test evaluated in this study was found to be a rapid and highly accurate procedure for identification of
M. avium complex in culture and one which most laboratories should be capable of performing.

ACKNOWLEDGMENTS

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LITERATURE CITED


