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Use of a Cutoff Range in Identifying Mycobacteria by the Gen-Probe Rapid Diagnostic System

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Commercial DNA probes (Gen-Probe Corp., San Diego, Calif.) for *Mycobacterium tuberculosis* complex *Mycobacterium avium*, and *Mycobacterium intracellulare* were compared with conventional methods for accuracy, applicability, and speed for the identification of putative isolates of the *M. tuberculosis* and *M. avium* complexes. Results are expressed as percent hybridization. Values of >15% were considered positive, and values of <5% were negative. Cultures having hybridization values within an indeterminate range of 5 to 15% were repeated. Mycobacterial isolates resembling *M. tuberculosis* and *M. avium* complex from cultures of 589 specimens, representing 432 patients, were entered into this study; 294 cultures were tested with the *M. tuberculosis* complex probe, and 326 cultures were tested with the *M. avium* probe. In all cases, probe results agreed with our biochemical identification of the isolates. The *M. intracellulare* probe was used with 117 isolates morphologically resembling *M. avium* complex, and one false-negative result was observed. Seventy-two cultures gave initial hybridization results that fell within the indeterminate range and were repeated. If the manufacturer's recommended 10% cutoff value had been used, the original hybridization values would have resulted in 27 misidentified cultures, 16 false-negatives and 11 false-positives.

In this report we describe the diagnostic evaluation of the Gen-Probe Rapid Diagnostic Systems (Gen-Probe Corp., San Diego, Calif.) assay for *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex in culture. The *M. tuberculosis* complex system detects *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*; the *M. avium* complex system distinguishes between *M. avium* and *M. intracellulare*. These assays are based on the ability of single complementary nucleic acid strands to hybridize and form stable double strands under appropriate conditions. The assays employ DNA probes complementary to the corresponding rRNA.

The objectives were (i) to compare the results of culture confirmation by nucleic acid hybridization probe with the results of our classical biochemical methods of identification (specificity), (ii) to evaluate the sensitivity of probes compared with biochemical methods, and (iii) to determine how much sooner the final results would be available with DNA technology.

MATERIALS AND METHODS

DNA probe test. All materials, lysing reagent, probe solutions, hydroxyapatite separation suspension, and wash solution, were obtained in kit form. The Gen-Probe Rapid Diagnostic Systems kit for *M. tuberculosis* complex contained only the *M. tuberculosis* complex probe solution; the *M. avium* complex kit contained probe solutions for both *M. avium* and *M. intracellulare*.

Control organisms. Positive controls included with the appropriate probes were *M. tuberculosis* H37Rv and ATCC 25618, *M. avium* serotype 4 (laboratory isolate), and *M. intracellulare* (laboratory isolate). These same organisms also were run as negative controls, i.e., *M. avium* was used with the *M. tuberculosis* complex probe, *M. intracellulare* and *M. tuberculosis* were run with the *M. avium* probe, and *M. avium* and *M. tuberculosis* were run with the *M. intracellulare* probe. All controls were included at the beginning of each run.

Preparation of organisms. Test organisms from solid media were suspended in 1 ml of sterile distilled water, two or three glass beads were added, and the suspension mixed on a Vortex mixer; the concentration was adjusted to a turbidity equivalent of a no. 1 McFarland standard. Each sample was transferred to a lysing reagent tube containing lysing reagent and glass beads and then sonicated for 15 min to release rRNA from the bacterial cells. After sonication, the [^{125}I]DNA probe solution was added to the sample. The reaction mixture was incubated at 72 ± 1°C for 1 h to allow hybridization between probe and target RNAs. A separation suspension, containing the solid adsorbent hydroxyapatite, was added to adsorb the probe-rRNA hybrid; unreacted probe that was not adsorbed was separated from the bound hybrid by low-speed centrifugation. After a single wash step, the hydroxyapatite-adsorbed hybrid pellet was quantitated in a gamma counter (model 1185-Z; Nuclear-Chicago Corp., Des Plaines, Ill.). The percent hybridization was calculated as follows: (sample counts per minute minus background)/(total counts per minute minus background) × 100.

Test interpretation. The manufacturer recommends interpretation of results as positive when the percent hybridization is ≥10%.

Sources of clinical specimens for hybridization assay. All cultures received in our laboratory over a 5-month period, which exhibited growth morphologically resembling *M. tuberculosis* or *M. avium* complex on slants of Lowenstein-Jensen medium (BBL Microbiology Systems, Cockeysville, Md.) or Mitchison 7H11 selective agar (Remel, Lenexa, Kans.) were entered into this study.

Standard culture technique. Mycobacteria were isolated from clinical specimens by the *N*-acetyl-L-cysteine–2% NaOH digestion-decontamination method (4, 5); specimens were inoculated onto one slant each of Lowenstein-Jensen medium and Mitchison 7H11 agar. Cultures were incubated in a slanted position at 35 to 37°C (unless otherwise indi-

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cated) under 8% CO_2 for 2 weeks and then placed upright and incubated up to 60 days without CO_2.

Identification. Cultures of original specimens were observed for mycobacterial colonies at 14, 21, 40, and 60 days after inoculation. Cultures resembling mycobacteria were separated into three groups, those resembling *M. tuberculosis*, *M. avium* complex, and others. The Kinyoun stain was used to confirm the presence of acid-fast organisms in subcultures referred from other laboratories and when mycobacterial growth other than that of typical *M. tuberculosis* was observed in primary cultures. The colonies were removed from growth-positive slants for the gene probe assay and suspended in 5 ml of Middlebrook 7H9 broth. Inoculated broths were incubated for 5 days and used to perform drug susceptibility tests (2), determine purity of culture, and perform standard biochemical identification tests (2).

Drug susceptibility testing. Broth cultures were diluted to give a cell concentration approximating a no. 1 McFarland standard and used for drug susceptibility testing. Drug susceptibility of isolates to isoniazid, rifampin, streptomycin, ethambutol, pyrazinamide, kanamycin, cycloserine, and thiophen-2-carboxylic acid hydrazide was determined. Thiophen-2-carboxylic acid hydrazide is used to distinguish between *M. tuberculosis* and *M. bovis* (2, 3).

Observations for purity. Samples of four dilutions of broth (10^0, 10^{-3}, 10^{-4}, and 10^{-5}) were inoculated onto Middlebrook 7H10 quadrant plates and incubated for 21 days. Quadrant plate cultures were also used for repeated probe assay when initial probe results were in the borderline region and hence indeterminate (see Results).

Conventional biochemical identification. For conventional biochemical identification (2), undiluted 7-day-old broth cultures and growth from control plates were used to inoculate appropriate biochemicals and a Lowenstein-Jensen slant. Organisms were identified as *M. tuberculosis* if they yielded positive tests for both niacin production and nitrate reduction after incubation for 3 weeks. These organisms are generally susceptible to all antituberculosis drugs tested. Organisms were identified as belonging to the *M. avium* complex if they yielded a positive test for tellurite reduction but negative tests for niacin production, nitrate reduction, polysorbate-80 (Tween 80) hydrolysis, and urease production. Additional tests used in the identification of *M. avium* complex were growth at 22, 37, and 45°C and growth in the presence of 5% NaCl and on MacConkey agar (without crystal violet). Distinction between *M. avium* and *M. intracellulare* biochemically is not possible. Cultures biochemically identified as *M. avium* complex but not reacting with either *M. avium* or *M. intracellulare* probes were sent to the Centers for Disease Control (Atlanta, Ga.) for serotyping and confirmation of identification.

RESULTS

Early in this study, a number of the probe tests had to be repeated because the cultures selected were too young. Cell suspensions from these cultures equivalent to a no. 1 McFarland standard frequently did not contain sufficient nucleic acid to permit reliable probe analysis. Traces of medium, included when picking pinpoint colonies, may also have prevented accurate interpretation of cell suspension turbidity. Cultures were ready for probe testing when the colonies were large enough to be morphologically characterized macroscopically; in general, this corresponded to 2 to 3 weeks after inoculation.

A total of 589 clinical specimens from 432 different patients were identified by culture and Gen-Probe methods over a 5-month period. The inherent imprecision of this radioactive ligand assay (Table 1) did not permit use of the discrete 10% cutoff value recommended by the manufacturer. Rather, a borderline or indeterminate region of 5 to 15% was established, consistent with the assay's imprecision at the 10% binding range, and all assays having results within this range were repeated.

TABLE 1. Inter-run precision data

Probe	Control	No. of runs[a]	% Binding		Coefficient of variation
			Mean	SD	
M. tuberculosis complex	*M. tuberculosis*	23[b]	52.1	8.9	17.1
	M. avium	20[b,c]	1.7	0.6	38.2
M. avium	*M. avium*	18[b,c]	45.8	7.6	16.6
	M. intracellulare	17[b,c]	8.6	5.7	66.3
	M. tuberculosis	22	1.8	1.0	55.6
M. intracellulare	*M. intracellulare*	13[c]	49.3	7.6	15.4
	M. avium	13[c]	3.0	1.9	62.3
	M. tuberculosis	16	1.5	0.8	53.3

[a] For each run with the probe indicated in the first column, one culture of the control organism indicated in the second column was used.

[b] Hybridization values greater than 3 standard deviations of the mean were dropped as outliers, and the remaining data were used to produce the above results.

[c] New ATCC cultures were used as controls in three runs; hence hybridization values of these runs were not included in the above calculations.

A total of 73 specimens fell into this indeterminate range, and a summary of their reconciliation is presented in Table 2. A single repeat result was accepted as accurate in those instances where the new result was consistent with the organism's biochemical reactions, susceptibility pattern, and colonial morphology. When two or more indeterminate values occurred, the assay was repeated until a determinate value consistent with the above classical characterization was obtained. In a number of instances with *M. tuberculosis* complex and *M. avium* complex probes, clear-cut classical characterization was not apparent; in these instances the probe assays were repeated until two consistent determinate values were obtained. Of these repeated specimens, 19 were *M. tuberculosis* complex; 16 had to be assayed twice, and 3 had to be assayed three times. Of the remaining 54 specimens (43 *M. avium* and 11 *M. intracellulare*), the *M. avium* isolates were the most troublesome; 19 were assayed twice, 16 were assayed three times, 6 were assayed four times, and 2 were assayed five times; all of the *M. intracellulare* specimens were assayed twice. Had the 10% cutoff value been used, 16 specimens would have been reported as false-negative (5 *M. tuberculosis* complex and 11 *M. avium*), and 10 would have been reported as false-positive (6 *M. avium* and 5 *M. intracellulare*).

TABLE 2. Resolution of results in the indeterminate range

Specimen	No./total in the following hybridization range:	
	5 to 9%[a]	10 to 15%[b]
M. tuberculosis complex	5/5	0/14
M. avium	11/25	6/18
M. intracellulare	0/5	5/6

[a] With the 10% hybridization value given in the manufacturer's insert, these results would have been false-negative.

[b] With the 10% hybridization value given in the manufacturer's insert, these results would have been false-positive.

Fourteen problem cultures reacted with both *M. avium* and *M. intracellulare* probes. Some were strongly reactive with one probe and borderline or slightly positive with the other probe. These probe inconsistencies were reconciled only after multiple assays and in some instances after subculture. Eight of the most troublesome of these cultures, including one that consistently reacted with both *M. avium* and *M. intracellulare* probes, were sent to the Centers for Disease Control and the probe manufacturer (Gen-Probe) for confirmation. Similar problems were observed by Drake et al. (1), who described isolates initially reacting with both *M. avium* and *M. intracellulare* probes but, upon retesting of subcultures, found them to react with only one probe.

The Centers for Disease Control identified two of the cultures as *M. avium* complex serotypes 18B and 20B, consistent with our probe identification as *M. intracellulare*, and found five of the cultures to be nontypable with *M. avium* complex antisera. Gen-Probe used a modified nuclear probe procedure to assay the eight cultures and confirmed the two *M. intracellulare* cultures; in addition they found four of the nontypeable cultures to be *M. intracellulare* and one to be *M. avium*. These results were in agreement with our probe identification. However, one culture identified by the Centers for Disease Control as serotype 19B, indicating it to be *M. intracellulare* was identified by Gen-Probe and by our laboratory as *M. avium*. We could not determine the reason the discrepancy; the culture was no longer viable, and the assay could not be repeated. One additional culture sent only to the Centers for Disease Control was nontypable.

We tested cultures from 294 specimens with the *M. tuberculosis* complex probe and applied the following modified criteria for unequivocal results: (i) only hybridization values of >15% were considered positive, (ii) values of <5% were considered negative, and (iii) all assays having hybridizative values of 5 to 15% were repeated or subcultured and repeated until unequivocal results were obtained. Under these conditions, we found both the sensitivity and specificity of the Gen-Probe system to be 100%. Cultures from 257 specimens were *M. tuberculosis* complex probe positive, and 29 were probe negative; 1 of the probe positive specimens was subsequently identified as *M. bovis*.

Results of eight cultures tested with the MTBC probe were not used in sensitivity and specificity calculations because they could not be identified biochemically. Two of the probe-positive cultures and three of the probe-negative cultures were grossly contaminated; the remaining three probe-positive cultures would not growth on subculture.

The *M. avium* probe was used with cultures from 326 specimens resembling the *M. avium* complex. When comparing the *M. avium* probe results (using the 5 to 15% cutoff range as described above) to the culture results for *M. avium* complex, we found the sensitivity and specificity to be 100%. The *M. avium* probe was positive with cultures from 220 specimens and negative with cultures from 106 specimens. Three probe-positive and two probe-negative cultures were grossly contaminated and were not used in sensitivity and specificity determinations. Upon reexamination of cultures from the 106 *M. avium* probe-negative specimens, 30 required no further probe analysis because they did not resemble *M. avium* complex or *M. tuberculosis* complex and hence were identified biochemically. These results are summarized in Table 3.

The *M. intracellulare* probe was used with cultures from 117 (76 *M. avium* probe-negative and 41 *M. avium* probe-positive) specimens morphologically resembling the *M. avium* complex; 39 were *M. intracellulare* probe positive and 78 were *M. intracellulare* probe negative. When the *M. intracellulare* probe results were compared with the culture results for the *M. avium* complex, we found the sensitivity to be 100% and specificity to be 96.9%. A single *M. avium* complex positive culture that was *M. avium* probe negative gave an *M. intracellulare* probe result in the indeterminate range (10% hybridization). Growth from subculture gave a negative *M. intracellulare* probe hybridization value of 2% and thus met the criteria for identification as *M. intracellulare* probe negative (false-negative result). However, since the Centers for Disease control had confirmed our biochemical identification as *M. avium* complex, the subculture was retested; a positive hybridization value of 22% was obtained with the *M. intracellulare* probe.

TABLE 3. Sensitivity and specificity of *M. avium* and *M. intracellulare* probes[a]

Probe and result	No. with the following *M. avium* complex identification	
	Positive	Negative
M. avium positive	220	0
M. intracellulare positive	39	0
M. avium negative	0	64
M. intracellulare negative	1	31

[a] The sensitivity was as follows: overall, 259 of 260 (99.6%); for *M. avium*, 220 of 220 (100%); for *M. intracellulare* 39 of 40 (97.5%). The specificity (overall) was 95 of 95 (100%). The positive predictive value was 259 of 259 (100%). The negative predictive value was 95 of 96 (99.0%) overall (64 of 64 [100%] for *M. avium* and 31 of 32 [96.9%] for *M. intracellulare*).

Of the *M. intracellulare* and *M. avium* probe-negative cultures identified conventionally as *M. avium* complex, five were found to be errors in conventional biochemical speciation due in part to problems with Tween 80 degradation and urease testing. The use of the new assays permitted us to identify members of the *M. avium* and *M. tuberculosis* complexes 3 to 6 weeks earlier than was possible with our conventional biochemical systems.

DISCUSSION

Traditional mycobacterial identification involves growing the organism and using biochemical, serological, or chromatographic methods for identification; this process may take 3 to 12 weeks, occasionally longer. In addition, traditional methods require that only pure cultures be used and cannot distinguish between *M. avium* and *M. intracellulare*. Recombinant DNA technology is providing an alternative identification method that offers rapid 1-day test results, does not require pure cultures, is characterized by its high sensitivity and specificity, and permits the distinction between *M. avium* and *M. intracellulare*.

In 1987, we received 30,229 primary clinical specimens and 547 subcultures, of which 1,619 were positive for mycobacteria; 779 (48.1%) were identified as *M. tuberculosis*, and 637 (39.3%) were biochemically identified as *M. avium* complex. Many subcultures arrived weeks to months after the initial isolation and were often in poor condition and contaminated with non-acid-fast organisms. Thus, 87.4% of our positive cultures could be identified by DNA probes within 1 day of the appearance of sufficient positive growth, in general 3 to 12 weeks sooner than was possible by conventional methods.

The major problem we found with the test was the discrete manufacturer recommended cutoff value of 10%. Drake et

al. (1) recommended repeating all values between 5 and 10%; our results indicated that an indeterminate region of 5 to 15% should be employed and that all values falling within this range should be repeated. If we had used the 10% cutoff value (Table 2), we would have reported 27 of the 72 specimens falling within the indeterminate region incorrectly; there would have been 16 false-negative and 11 false-positive results. The false-negative cultures (5 *M. tuberculosis* complex and 11 *M. avium* complex) had values that originally fell within the 5 to 9% hybridization range. The false-positive cultures (six *M. avium* and five *M. intracellulare*) had values that originally fell within the 10 to 15% hybridization range.

Failure to have made this modification to the manufacturer's endpoint interpretation would have resulted in a decrease in the sensitivity of the *M. tuberculosis* complex probe from 100 to 98.0%; the specificity would have been unchanged at 100%. Correspondingly, the sensitivity for the *M. avium* probe would have decreased from 100 to 95%; the specificity would have decreased from 100.0 to 90.6%. For the *M. intracellulare* probe, the sensitivity would have been unchanged at 97.5%, but the specificity would have decreased from 100 to 83.9%. The overall sensitivity of the *M. avium* plus *M. intracellulare* probes for the *M. avium* complex culture would have decreased from 99.6 to 95.4%; the specificity would have decreased from 100 to 88.4%. Cultures from the remaining 46 specimens with hybridization results in the 5 to 15% indeterminate range gave consistent and clear negative or positive values upon reassay.

Unusual binding was observed occasionally when *M. avium* and *M. intracellulare* were used as negative controls. Binding as high as 7% was observed when *M. avium* was used as the negative control with the *M. intracellulare* probe and as high as 30% when *M. intracellulare* as used as a negative control with the *M. avium* probe. Manufacturer explanation for this high background cross-reactivity is hybridization reaction temperatures of less than 71°C. Although we had no reason to suspect temperature fluctuations in our water bath during the initial phases of our study, we took extra care to insure that the incubation temperature was maintained at 72 ± 1°C. These precautions included replacing our old water bath with a new thermostatically controlled water bath, use of a National Bureau of Standards calibrated thermometer, and frequent temperature checks by the analyst.

It should be emphasized that all of the manufacturer's assay recommendations were scrupulously followed during this study. In spite of the attention paid to the details of the assay, occasional unusual binding occurred throughout the study when *M. avium* and *M. intracellulare* were used as negative controls. We suspect that some of the difficulties observed were due to the organisms and not method, for we observed four instances in which *M. avium* was isolated from multiple cultures from the same patient; in each case the probe assay had to be repeated several times before unambiguous results were obtained. The manufacturer suggested that ambiguous results might be resolved by washing and counting the radioactive pellet again. Although this did prove helpful in one instance, in all other instances it had no effect and was discontinued. On rare occasions, organisms that do not react with the probes may be encountered. Recently, two isolates (not included in this study) that did not react with either the *M. avium* or *M. intracellulare* probe were identified by the Centers for Disease Control as *M. avium* complex by high-performance liquid chromatography.

It should be stressed that failure to include sufficient sample for analysis (uniform turbidity upon suspension equivalent to a no. 1 McFarland standard) may result in hybridization values in the indeterminate region. Our main problem with the *M. tuberculosis* complex probe was difficulty in determining the amount of growth needed to produce a uniform suspension of the organisms. Nineteen specimens were repeated; three had very scant growth, one was heavily contaminated, and in one case the tube containing the probe was spilled. Seven additional specimens that were initially negative but positive upon retesting were also noted by the technologists to have scant growth. We suspect that this may also have been the problem with most of the other 14 retested *M. tuberculosis* cultures as well as with some of the *M. avium* complex cultures.

It was necessary to eliminate 13 specimens from this study because either the cultures were too heavily contaminated to use or no growth was obtained on subculture. Interestingly, eight of these cultures (five *M. tuberculosis* complex and three *M. avium*) yielded positive probe results and were satisfactory for patient reporting. In addition, this study demonstrated that five errors in biochemical speciation were made and thus pointed out quality control problems with two routine biochemical tests.

The probe assays evaluated in this study were rapid and, under our modification of the test interpretation definition, accurate for the identification of *M. tuberculosis* complex, *M. avium*, and *M. intracellulare*. Only 1 false-negative result would have been reported from the 589 clinical specimens that we studied.

We recommend that the probes be used without biochemical confirmation in all cases (provided experienced personnel are reading the cultures) except when ambiguous results are obtained in spite of repeat testing. The probes would be more versatile, however, if nonradioactive probes were available, because currently their use is limited to laboratories having facilities for radioligand analysis.

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