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Identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* Directly from Primary BACTEC Cultures by Using Acridinium-Ester-Labeled DNA Probes

KAYE D. EVANS, AUDREY S. NAKASONE, PAMELA A. SUTHERLAND, LUIS M. DE LA MAZA, AND ELLENA M. PETERSON*

Division of Medical Microbiology, Department of Pathology, Building 14, Route 84, 101 City Drive, University of California Irvine Medical Center, Orange, California 92668

Received 6 April 1992/Accepted 22 June 1992

Identification of members of the *Mycobacterium tuberculosis* complex and the *M. avium-M. intracellulare* complex (MAC) directly from primary BACTEC cultures was evaluated by using acridinium-ester-labeled DNA probes (AccuProbe; GenProbe, Inc., San Diego, Calif.). In preliminary experiments, blood present in samples was found to interfere with the assay because of nonspecific chemiluminescence, which was measured in relative light units (RLUs). There was a direct relationship between the age of the culture and the number of nonspecific RLUs. A protocol using 1% sodium dodecyl sulfate–5 mM EDTA to treat BACTEC broth cultures which, with specimens containing blood, gave on the average a ninefold reduction in nonspecific chemiluminescence was developed. By using this treatment protocol, 120 specimens were tested directly from BACTEC broth cultures with an AccuProbe for the *M. tuberculosis* complex and/or the MAC. In order to establish the background of the specimen, the patient sample was assayed without probe. The criteria for the inclusion of BACTEC cultures in the evaluation were a growth index of ≥100 and a positive smear for acid-fast bacilli directly from the BACTEC broth. For the 120 cultures tested, if a hybridization result of ≥30,000 RLUs was considered positive, the sensitivities for detecting the *M. tuberculosis* complex and the MAC were 47 and 90%, respectively, with a specificity of 100% for both. However, if a ratio of the RLUs obtained with the MAC or the *M. tuberculosis* complex probe to those obtained with the specimen background of ≥20 was considered positive, this gave 77% sensitivity and 100% specificity for BACTEC cultures containing *M. tuberculosis* complex isolates and 96% sensitivity and 100% specificity for those growing MAC isolates.

DNA probes have aided greatly in the rapid identification and detection of *Mycobacterium* spp. from cultures of clinical specimens. The first generation of probes to be used on colonies isolated on solid media utilized an isotopic label (2). In a previous report, we used isotopically labeled probes to directly identify *Mycobacterium* spp. from BACTEC cultures (12). A second generation of nonisotopic probes, the SNAP system (Syngene, Inc., San Diego, Calif.) which utilizes DNA probes labeled with horseradish peroxidase (10), and the AccuProbe system, which employs acridinium ester as the probe label (5), have been introduced. With this latter system, the hybridization reaction is detected by chemiluminescence upon hydrolysis of the label by H_2O_2 and NaOH. In addition to the advantages that come with the substitution of the isotopic label, the second generation of assays provides both probes for *Mycobacterium avium* and *M. intracellulare* as well as a combined *M. avium-M. intracellulare* complex (MAC) probe, whereas the first-generation system had only separate probes for *M. avium* and *M. intracellulare*. In many settings in which several cultures are obtained from a patient and/or serial cultures are used to monitor antimicrobic therapy, it is not necessary to distinguish between *M. avium* and *M. intracellulare* with every specimen, and it is in these circumstances that a combined MAC probe offers time and cost benefits. However, specific *M. avium* and *M. intracellulare* probes are also useful in initially establishing the species isolated from a patient so that information on treatment protocols can be gathered and, in some cases, antimicrobial therapy can be tailored to the species (7). The AccuProbes have been reported to be both specific and sensitive for identifying *Mycobacterium* spp. (5). In comparing the AccuProbe system for identifying colonies from solid media with the earlier, isotopically labeled version of these probes, Goto et al. (5) reported 100% sensitivity for both the *M. tuberculosis* complex probes and the MAC probe.

In this study, we evaluated the AccuProbe system for its ability to identify the *M. tuberculosis* complex and the MAC directly from BACTEC broth cultures. We have found that blood contributes to false-positive results with the AccuProbe system, and we have developed a method to reduce this problem.

MATERIALS AND METHODS

Specimens. Specimens included in the study were those sent to the Medical Microbiology Laboratory at the University of California Irvine Medical Center with requests for mycobacterial cultures. Upon their receipt in the laboratory, the specimens were processed for culture within 24 h. Blood specimens were received in Isolator tubes (Wampole Laboratories, Cranbury, N.J.) which were centrifuged at 3,000 × *g* for 30 min, and the pellets were resuspended according to the instructions of the manufacturer. Specimens, including blood, that did not require further decontamination or digestion were inoculated directly into BACTEC 12B bottles (Becton Dickinson) as well as onto conventional media,

* Corresponding author.

including a biplate of Middlebrook 7H11 agar consisting of media with and without carbenicillin (50 μg/ml) and trimethoprim lactate (20 μg/ml) (Remel Laboratories, Lenexa, Kans.). Each BACTEC 12B bottle received 0.5 ml of specimen, and each conventional plate was inoculated with 0.1 ml of specimen. Specimens requiring decontamination, digestion, or both were treated with *N*-acetyl-L-cysteine and 2% sodium hydroxide, according to standard procedures (13). Treated specimens were suspended in phosphate-buffered saline (0.01 M, pH 7.2) containing penicillin (50 U/ml). BACTEC 12B bottles supplemented with antimicrobial agents (polymyxin B, 50 U/ml; amphotericin B, 5 μg/ml; nalidixic acid, 20 μg/ml; trimethoprim, 5 μg/ml; azlocillin, 10 μg/ml) were inoculated with 0.5 ml and conventional media were inoculated with 0.1 ml of treated specimen.

Culture identification. Conventional media incubated at 37°C in 5 to 10% CO_2 were read weekly for 8 weeks. Organisms were identified by using standard biochemical methods (13) or by using DNA-RNA hybridization (AccuProbe). BACTEC bottles were read twice a week by using the BACTEC 460 (Becton Dickinson). When a growth index (GI) of ≥100 was detected, an aliquot of the broth was stained, and when acid-fast bacilli were present, medium was withdrawn from the vial. A 1.8-ml volume was centrifuged at 15,000 × *g* for 10 min, the resulting pellet was used in hybridization assays (see below), 0.1 ml was inoculated onto chocolate agar to check for contamination, and 0.1 ml was inoculated onto a 7H11 agar plate to confirm hybridization results.

DNA probe assay. By using AccuProbe reagents, the pellet resulting from the centrifugation of 2 ml of BACTEC broth was directly suspended in 0.1 ml of specimen diluent (reagent 1) and 0.1 ml of probe diluent (reagent 2). A lysing pellet consisting of glass beads and lysing reagents was added to this, and cell suspensions were sonicated for 15 min. The suspension was then heat inactivated at 95°C for 10 min. This was followed by the addition of 0.1 ml of both the specimen diluent and the probe diluent. In this way, two hybridizations and a blank could be run with the lysed specimen, one hybridization with the *M. tuberculosis* complex probe and one with the MAC probe. To the hybridization tubes containing the specific probe, 0.1 ml of lysed organism was added. Hybridization tubes were incubated at 60°C for 15 min, 0.3 ml of selection reagent was added, and the tubes were incubated for 5 min at 60°C. Hybridization was read with a luminometer (Leader 50, GenProbe), and the results were reported in relative light units (RLUs).

Upon analyzing the initial study results, it was apparent that specimens containing blood gave high nonspecific values, i.e., ≥30,000 RLUs, that by the criteria of the manufacturer for broth specimens should be considered positive. In order to reduce these nonspecific values, specimens were subjected to one to three prewashes. To 1.8 ml of BACTEC broth, 0.2 ml of a solution of 10% sodium dodecyl sulfate (SDS)–EDTA (50 mM), pH 8.0, was added, and the specimen was vortexed and centrifuged for 10 min at 15,000 × *g*. To the resulting pellet, 0.2 ml of the SDS-EDTA solution was added, and the tubes were vortexed vigorously for approximately 20 s until the pellet was in solution. Sterile distilled water was then added to bring the volume to 2 ml, and the tube was vortexed again and centrifuged at 15,000 × *g*. At the end of the last SDS-EDTA treatment, the resulting pellets were washed with 2 ml of water and treated as described above for the hybridization assay. As a specimen background control, lysed specimens were added to tubes not containing probe and assayed in parallel with the other samples.

TABLE 1. Effects of washing the BACTEC pellet to reduce nonspecific chemiluminescence

No. of SDS-EDTA treatments	RLU values for specimen no.:				
	1	2	3	4	5
None	74,269	63,355	38,396	66,326	60,321
1	55,597	13,003	3,750	12,974	14,884
2	21,724	12,129	3,151	9,348	15,234
3	14,049	10,543	2,044	9,940	10,993

RESULTS

In the initial work to adapt the AccuProbe assay to amplified primary cultures, broth from BACTEC cultures with a GI of ≥100 was centrifuged and directly tested with both the MAC and *M. tuberculosis* complex AccuProbes. Of the eight cultures tested that contained visible blood, seven blood cultures and one knee aspirate, all seven blood cultures grew *M. avium* and the knee aspirate grew *M. tuberculosis* on 7H11 agar. With the probe that corresponded to the organism that grew on 7H11 agar (relevant probe), the RLUs ranged from 2 to 11 times the positive cutoff value of 30,000 RLU set by the manufacturer for broth cultures. However, the RLU values for the probe not corresponding to the organism (irrelevant probe) were also either positive (≥30,000) or borderline positive, with values ranging from 27,314 to 42,584 RLUs. Seven specimens not containing visible blood that were culture positive for *M. tuberculosis* (3) or *M. avium* (4) were also tested by using the same protocol. The RLU values for the relevant probe were 2.5 to 20 times the positive cutoff value. In contrast to those with the specimens containing blood, the RLU values for the irrelevant probe were low, ranging from 1,344 to 2,581 RLUs. These findings suggested that blood contributed to the elevated RLU values seen with the cultures containing visible blood.

In an attempt to reduce the nonspecific effect of blood, five specimens from patients that after eight weeks of incubation were culture negative for *Mycobacterium* spp. were processed in the following manner: 1 ml of BACTEC broth was centrifuged as described above, and the resulting pellet was subjected to the same hybridization protocol described earlier, except that the probe was omitted. Therefore, any RLU values would represent nonspecific chemiluminescence. Three other 1-ml aliquots were treated with 1% SDS–5 mM EDTA and centrifuged. One pellet received one wash with 1% SDS–5 mM EDTA followed by a final water wash, while the other two pellets received two or three SDS-EDTA washes followed by a final wash with water. The nonspecific RLU values for these specimens can be seen in Table 1. The BACTEC broth from specimens containing blood that were centrifuged without SDS-EDTA treatment and tested by using the AccuProbe system gave high hybridization signals in the absence of a DNA probe, therefore corroborating the findings that the blood in specimens accounted for the high nonspecific RLUs. From the RLUs obtained after SDS-EDTA treatment, it was clear that from two to three SDS-EDTA washes were necessary to minimize the nonspecific chemiluminescence. It was also noted that the background RLUs with four of these five specimens were even higher in the nontreated group than those nonspecific RLUs that were

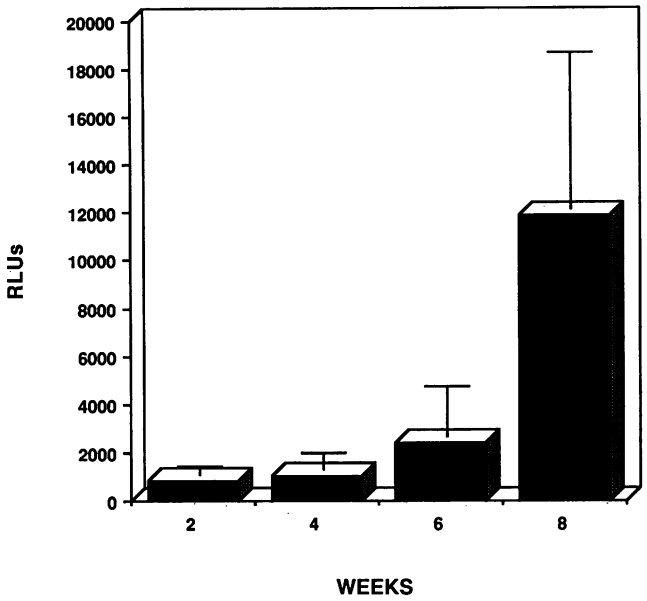

FIG. 1. Effect of age of BACTEC blood cultures on nonspecific RLUs. BACTEC 12B cultures inoculated with blood specimens were incubated from 2 to 8 weeks and then processed as described in Materials and Methods to measure the contribution of the sample to the background RLUs.

seen with the eight specimens containing blood which were discussed above. In examining the age of these cultures, it was found that they were ≥8 weeks old, in contrast to the 2- to 4-week age of the eight cultures initially tested. Therefore, in order to determine whether the age of the blood culture had an effect on the nonspecific RLUs, 23 BACTEC broth blood cultures were tested for nonspecific chemiluminescence at 2, 4, 6, and 8 weeks of incubation. In Fig. 1, which shows results from an experiment in which all BACTEC broths received three 1% SDS–5 mM EDTA washes, it can be seen that the nonspecific chemiluminescence as determined by RLUs increased with the age of the culture. The mean values of the 2-, 4-, 6-, and 8-week-old cultures were 801, 1,004, 2,426, and 11,915 RLUs, respectively.

A total of 120 clinical samples, 40 blood samples, 54 respiratory specimens, 14 stool samples, and 12 specimens from other sources were tested directly from BACTEC broth by the AccuProbe. The conventional culture results for the 120 specimens tested were 102 MAC complex isolates, 17 *M. tuberculosis* complex isolates, and 1 *M. xenopi* isolate. To be included in the evaluation, each BACTEC broth had to be from a culture ≤8 weeks old that had a GI of ≥100 and was positive for AFB when stained. Broth from BACTEC cultures meeting these criteria was centrifuged and washed three times as described above with SDS-EDTA and then washed finally with 2 ml of water. Depending on the specimen source and whether the patient had previous positive mycobacterial cultures, the sample was tested with the MAC and/or the *M. tuberculosis* probes. Also included with each specimen was a reaction mixture containing the patient sample but no probe, in order to determine the background chemiluminescence contributed by the sample.

The range of RLUs for the MAC probe when tested with the BACTEC broth for cultures growing MAC on conventional media was 1,033 to 741,173 RLUs, with an average of 212,861 RLUs. Cultures growing an *M. tuberculosis* complex isolate on conventional media had BACTEC broth hybridization values of 3,333 to 259,193 RLUs and an average of 51,491 RLUs when assayed with the *M. tuberculosis* complex probe.

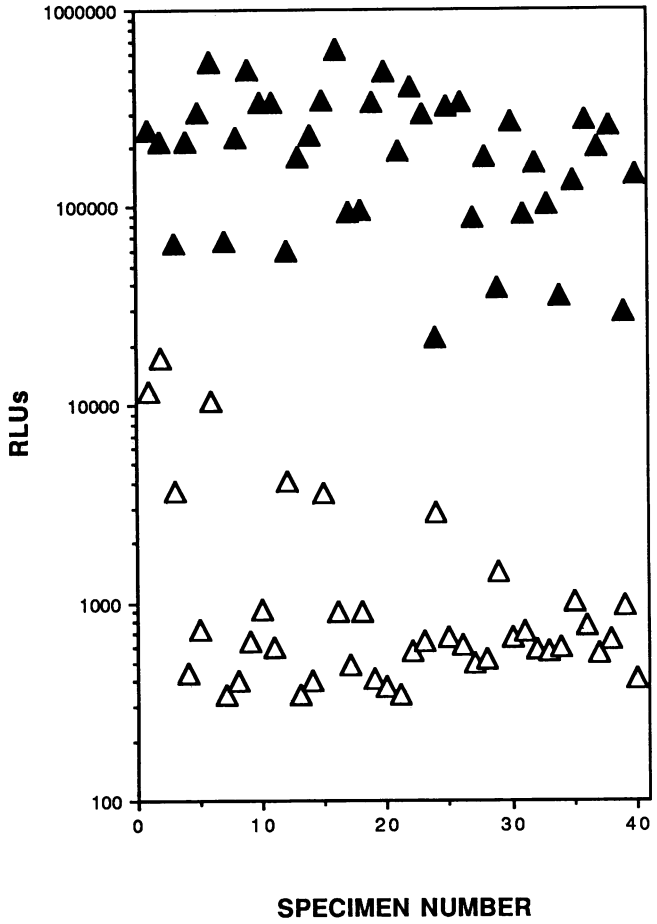

FIG. 2. Comparison of RLUs obtained with the MAC probe (▲) with the RLUs contributed by the specimen alone (△); 40 MAC culture-positive blood specimens were tested.

The average specimen background chemiluminescence of the 40 blood specimens tested was 1,862 RLUs (standard deviation [SD], 3,501 RLUs), and the mean specimen background chemiluminescence of the 80 other specimens that did not contain visible blood was 428 RLUs (SD, 228 RLUs). Therefore, while treatment of the pellets with SDS-EDTA reduced the background chemiluminescence of the specimen, specimens containing blood still had higher background RLU values than specimens which did not contain visible blood. However, the average ratio of the RLUs obtained with the MAC probe to the specimen background RLUs for the blood cultures that grew the MAC was 327 (SD, 287) with a range from 8 to 1,323. In Fig. 2, this is illustrated by the background RLUs and the RLUs obtained with the MAC probe being displayed for the MAC-positive blood cultures. The mean of this ratio for cultures from sources other than blood growing the MAC was 494 (SD, 422), with a range from 2 to 1,728. The mean ratio of the *M. tuberculosis* complex probe hybridization RLU values to specimen background RLUs for cultures growing the *M. tuberculosis* complex was 139 (SD, 187), with a range from 11 to 714.

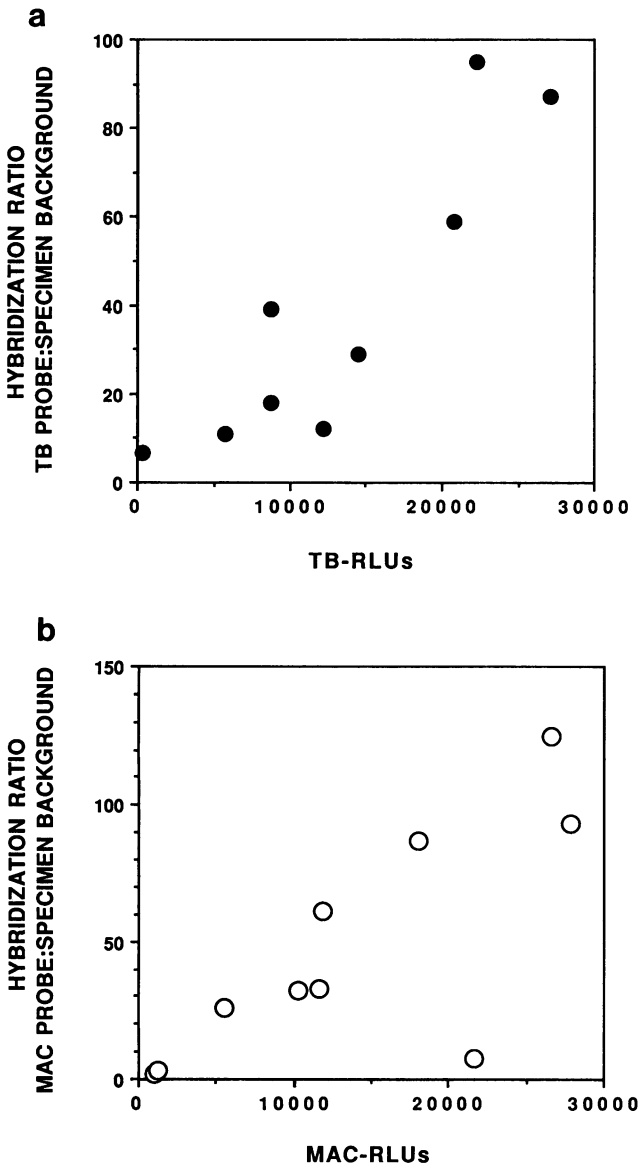

FIG. 3. BACTEC cultures which gave hybridizations of <30,000 RLUs for the relevant probe are shown. There were 9 cultures which grew the *M. tuberculosis* complex (a), and 10 from which the MAC was isolated (b). The graphs show both the RLUs for these specimens with the relevant probe and the ratio of the relevant probe RLUs to the specimen background RLUs.

If ≥30,000 RLUs is used as a criterion to determine whether BACTEC broth contains either the *M. tuberculosis* complex or a member of the MAC complex, then there were 19 false-negative BACTEC broths by the AccuProbe (Fig. 3). Of these, there were 9 that grew the *M. tuberculosis* complex and 10 that grew the MAC complex. If ≥30,000 RLUs is used as a criterion, testing of BACTEC broths would have a sensitivity of 47% (8 of 17) for those specimens which grew the *M. tuberculosis* complex and that of 90% (92 of 102) for those growing a MAC isolate.

There were 81 specimens for which the irrelevant probe was tested in addition to the relevant probe and the specimen blank. In all instances, the RLU values for the irrelevant probe were greater than those obtained with the specimen blank. The mean ratio of the irrelevant probe RLUs to the specimen RLUs for the 81 specimens was 3.7, with an SD of 3.1. Of the 81 irrelevant probe-to-background ratios, 2 were >10, with ratios of 11.6 and 26. Only the MAC was isolated from the latter culture, and the ratio of the MAC probe RLUs to the background RLUs was 785. The other culture, with a ratio of 11.6, grew *M. xenopi*, an organism for which there is no specific AccuProbe.

Therefore, if the specimens are reevaluated with a probe-to-specimen ratio of 30 used as the criterion for a positive hybridization result, this increases the sensitivity for the specimens containing the *M. tuberculosis* complex to 69% and that for those growing the MAC to 91%. As can be seen in Fig. 3, by using this criterion, four of the cultures which grew the *M. tuberculosis* complex but which had <30,000 RLUs for the relevant probe would now be considered positive, as would six of the MAC-containing cultures which gave <30,000 RLUs with the MAC probe. A ratio of 30 was chosen to achieve a specificity of 100%. However, if a ratio of 20 were taken as the cutoff value of a positive result, then the sensitivities of detecting and identifying *M. tuberculosis*- and MAC complex-positive cultures would be 77 and 96%, respectively. In this study, a ratio of ≥20 would include one false-positive hybridization result with the *M. tuberculosis* complex probe from a blood culture discussed above that gave a ratio of 26 for the irrelevant probe-to-specimen background and a ratio of 785 for the relevant probe-to-specimen background. Therefore, a ratio of ≥20 would give a specificity of 99%.

DISCUSSION

With the increase in mycobacterial infections over the past decade, there is a great need for rapid methods for detecting and identifying *Mycobacterium* spp. With the advent of AIDS and the concomitant rise in MAC and *M. tuberculosis* infections, it is extremely important from a treatment standpoint to differentiate those patients with *M. tuberculosis* from those with the MAC (4, 9, 11). In addition, multidrug-resistant *M. tuberculosis* strains are also increasing in number, making it important to identify patients with *M. tuberculosis* for the purposes of isolation and contact tracing (4). Recently, progress in the areas of more rapid detection and identification of *Mycobacterium* spp. has been made. The polymerase chain reaction has successfully identified *Mycobacterium* spp. directly from patient specimens (8, 14, 15). However, the polymerase chain reaction at present is not widely available and is costly, and it requires a considerable amount of technical expertise. Also, in our experience with *Mycobacterium* spp., it is still not as sensitive as culture (unpublished results). DNA probes have not yet achieved the level of sensitivity required to routinely detect *Mycobacterium* spp. from patient material. However, DNA probes in combination with conventional media or BACTEC broth have allowed for the rapid identification of *Mycobacterium* spp. from amplified primary cultures (12).

In a previous report in which isotopically labeled probes were used to identify mycobacteria directly from amplified BACTEC broths with a specificity of 100%, 83% of the *M. tuberculosis* isolates, 92% of the *M. avium* isolates, and 86% of the *M. intracellulare* isolates were directly detected and identified from BACTEC 12B broths which had GIs of ≥100 (12). In the present report, with a ratio of ≥20 for probe RLUs to the specimen background RLUs as a positive hybridization result, the acridinium-ester-labeled probes gave a specificity of 99% for both *M. tuberculosis* complex-

and MAC-positive cultures, with sensitivities of 77 and 96% for the *M. tuberculosis* complex and MAC isolates, respectively.

Although the sensitivity for the MAC probes was higher with the nonisotopic probe, the sensitivity of the *M. tuberculosis* probe was less than that achieved with the isotopically labeled probe. Possible explanations for this could be the relatively small number of *M. tuberculosis* complex isolates described in this study (17 versus 64 previously examined) and the multiple SDS-EDTA washes and centrifugations, in which organisms may have been lost, that were used to reduce the background contributed by the specimen. When evaluated by using hybridizations performed on colonies taken from solid media, the AccuProbe system was 97.2% sensitive and 100% specific for MAC isolates, and both the sensitivity and the specificity were 100% for *M. tuberculosis* isolates (5). Guruswamy and Welch (6) have recently described the identification of microcolonies of *Mycobacterium* spp. from solid media consisting of a thinly poured 7H11 plate. If enough organisms (10^6 CFU) are present in these microcolonies and if they appear before the BACTEC broth has a GI of ≥ 100, then this would avoid the problem of specimen nonspecific chemiluminescence and most likely show a greater sensitivity than hybridizations performed with the BACTEC pellet. In our experience, by using a conventional 7H11 plate and examining the plates frequently for small colonies, with approximately half of the cultures the colonies can be seen before the BACTEC GI has turned positive (3). Therefore, a combination of the BACTEC and frequent close examination of conventional media with DNA probes will give the optimum rapid system by using present methods that are commonly available.

In this study, a specimen blank was included with all hybridization determinations. This was felt to be necessary because of the nonspecific chemiluminescence seen with some specimens, mainly blood cultures. The cost of the additional blank was approximately $0.75 compared with more than $12 for the reagents for one hybridization with an AccuProbe. Frequently, we receive blood cultures from patients with disseminated MAC infections who are on experimental-treatment protocols. Upon holding these cultures beyond our normal incubation period of 8 weeks, we have detected positive MAC cultures, presumably due to the inhibitory effects of the antimicrobial agents used in treatment (unpublished results). However, the specimen background with these specimens, even with SDS-EDTA treatment, can go as high as 70,000 RLUs. Therefore, especially in the case of blood specimens, we feel that hybridization values from BACTEC broth with a specimen blank are necessary to avoid false-positive results. Davis and Fuller (1) also described blood interference when using the AccuProbe system to identify an *Enterococcus* sp., *Escherichia coli*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae* directly from amplified routine bacterial blood cultures. These authors used a combination of short centrifugations to eliminate blood cells and centrifugation at $9,600 \times g$ for 1 min to capture the bacteria.

In summary, although the AccuProbe system has the inherent problem of blood interference, it offers many advantages over the isotopically labeled probes. It has a longer shelf life; it reduces the documentation, monitoring, and record-keeping that are inherent in using a radioactive probe; and it is more rapid, with less hands-on time. The AccuProbe system is expensive and requires instrumentation for the reading of the assay. Therefore, there is still a great need for less expensive and more rapid and direct testing methods for the identification of *Mycobacterium* spp.

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