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Evaluation of Acridinium-Ester-Labeled DNA Probes for Identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* Complex in Culture

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The detectability of mycobacteria in culture by the use of nonisotopic, chemiluminescent DNA probes for *Mycobacterium tuberculosis* and the *M. avium-M. intracellulare* complex (MAC) was evaluated and compared with that by the use of ^{125}I-labeled DNA probes for the same mycobacteria. In the assay, rRNA-directed DNA probes labeled with acridinium ester (AE-DNA probes) were used. Unhybridized probes were chemically degraded, and the esterified acridinium on the hybridized probes was hydrolyzed by the addition of alkaline hydrogen peroxide solution, resulting in the production of visible light which was measured with a luminometer. The detection limits of the AE-DNA probes were almost the same as those of the ^{125}I-labeled DNA probes. A total of 107 clinical isolates of mycobacteria (47 isolates of *M. tuberculosis*, 36 MAC, and 24 atypical mycobacteria other than MAC) were tested. The sensitivity and specificity of the AE-DNA probes for *M. tuberculosis* were 100% both for the conventional method and with the ^{125}I-labeled DNA probe. The sensitivity and specificity of the AE-DNA probes for MAC were 97.2 and 100%, respectively, for the conventional method and were both 100% with the ^{125}I-labeled DNA probes. Because the procedure is simple, reliable, rapid (it can be completed within an hour), and safe (it does not use radioisotopes), it can easily be performed in any clinical laboratory.

Infections caused by *Mycobacterium tuberculosis* are found worldwide (16), and those caused by *M. tuberculosis* and the *M. avium-M. intracellulare* complex (MAC) associated with AIDS have recently been found to be increasing (11). Therefore, infections caused by these mycobacteria have become a major clinical problem (6, 7). Rapid and accurate identification and discrimination between *M. tuberculosis* and MAC are important, since the treatment of each infection is significantly different (1, 14).

Conventional approaches to the laboratory diagnosis of infections caused by these organisms are based on acid-fast stains and the recovery of organisms from cultured clinical specimens; this is followed by a variety of tests. However, acid-fast stains cannot distinguish *M. tuberculosis* from MAC or other mycobacteria, and they are not sensitive enough in most instances. In addition, culture and biochemical testing require more than 6 to 8 weeks. Because of these disadvantages inherent in the conventional methods, several techniques, such as the use of ^{125}I-labeled DNA probes (Gen-Probe, Inc., San Diego, Calif.) (3, 10) and the BACTEC system (Becton Dikinson, Inc., Towson, Md.) (15), have been developed and are commercially available. However, both methods require radioisotope facilities, resulting in the limitation of their practical use in many clinical laboratories.

Acridinium-ester-labeled DNA (AE-DNA) probes have been made available as an alternative to DNA probes labeled with ^{125}I (5). By using AE-DNA probes, a new detection system, the so-called hybridization protection assay (2), which is based on the selective chemical degradation of acridinium on unhybridized probes, has been developed and has been applied to the use of AE-DNA probes for *M. tuberculosis* and MAC. The purpose of this study was to evaluate the accuracy and applicability of AE-DNA probes for the identification of *M. tuberculosis* and MAC by the conventional method and by using ^{125}I-labeled DNA probes as a reference.

MATERIALS AND METHODS

Mycobacterial cultures and isolates. The following mycobacterial reference strains were grown on Ogawa slants: three strains of *M. tuberculosis* complex standards (*M. tuberculosis* NIHJ1633, *M. bovis* NIHJ1607, and *M. africanum* NIHJ1602), four strains of MAC standards (*M. avium* NIHJ1605, *M. avium* NIAH1106, *M. intracellulare* NIHJ 1618, and *M. intracellulare* NIAH1132), and nine strains of atypical mycobacteria other than MAC (non-MAC) (*M. kansasii* NIHJ1619, *M. marinum* NIHJ1620, *M. simiae* NIHJ1627, *M. scrofulaceum* NIHJ1626, *M. gordonae* NIHJ 1617, *M. xenopi* NIHJ1638, *M. gastri* NIHJ1616, *M. fortuitum* NIHJ1615, and *M. chelonei* NIHJ1611). A total of 47 clinical isolates of *M. tuberculosis*, 9 clinical isolates of *M. intracellulare*, 27 clinical isolates of *M. avium*, and 24 clinical isolates of atypical non-MAC mycobacteria were obtained from the University of Tokyo Hospital. All clinical isolates were identified by conventional tests, which included growth rate; gross and microscopic colony morphology; pigmentation; and tests for niacin, catalase, nitrate reduction, and urease.

AE-DNA probes. The ACCUPROBE (Gen-Probe, Inc.) for *M. tuberculosis* and MAC (probes for both *M. avium* and *M. intracellulare* were included) was used in this study. All components for the test reagents were provided by the manufacturer. This hybridization protection assay consists of the following four basic steps: (i) sample preparation, (ii) hybridization, (iii) selective chemical degradation, and (iv)

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TABLE 1. RLUs of the AE-DNA probes for strains of different types of mycobacteria

Type (no. of strains)	Mean ± SD RLUs (range) of AE-DNA probes for[a]:	
	M. tuberculosis	MAC
M. tuberculosis complex (3)[a]	422,742 ± 70,423 (283,930–501,575)	3,364 ± 663 (2,722–4,282)
MAC (4)[b]	2,827 ± 610 (2,160–4,282)	297,844 ± 132,849 ((178,171–604,721)
Non-MAC (9)[c]	3,285 ± 1,255 (2,056–6,938)	3,464 ± 843 (2,082–4,933)

[a] The *M. tuberculosis* complex consisted of *M. tuberculosis* NIHJ1633, *M. bovis* NIHJ1607, and *M. africanum* NIHJ1602.
[b] MAC isolates consisted of *M. avium* NIHJ1605, *M. avium* NIAH1106, *M. intracellulare* NIHJ1618, and *M. intracellulare* NIAH1132.
[c] Non-MAC isolates consisted of *M. kansasii* NIHJ1619, *M. marinum* NIHJ1620, *M. simiae* NIHJ1627, *M. scrofulaceum* NIHJ1626, *M. gordonae* NIHJ1617, *M. xenopi* NIHJ1638, *M. gastri* NIHJ1616, *M. fortuitum* NIHJ1615, and *M. chelonei* NIHJ1611.

chemiluminescence measurement. The assay was performed according to the instructions of the manufacturer. Briefly, 0.1 ml of the bacterial suspensions, which were adjusted to a MacFarland no. 1 standard by mixing the colonies grown on Ogawa slant with distilled water, were sonicated in tubes containing glass beads and lysing reagents in a sonication bath (BRANSONIC B-1200R-4; Branson Cleaning Equipment Co., Shelton, Conn.) at 60°C for 15 min and were subsequently heated at 95°C for 10 min. The lysates were then transferred to tubes coated with each of the DNA probes and were incubated at 60°C for 15 min.

A total of 300 μl of 200 mM sodium tetraborate solution containing 1% Triton X-100 (selective reagent) was subsequently added to each tube to chemically degrade any unhybridized probe. The tube was mixed well, further incubated at 60°C for 5 min, and kept at room temperature for 5 min. Acridinium esters bound to the hybridized DNA probes were hydrolyzed by the addition of 200 μl of 0.1% H_2O_2 solution (detecting reagent I) and 200 μl of 1 M NaOH (detecting reagent II), and the amount of light emitted from the acridinium (chemiluminescence) was measured quantitatively in a Leader I luminometer (Gen-Probe, Inc.). The result was expressed as relative light units (RLUs), which were defined arbitrarily according to the sensitivity of the instrument.

^{125}I-labeled DNA probe. The commercial Gen-Probe Rapid Diagnostic systems for *M. tuberculosis*, *M. avium*, and *M. intracellulare* were used according to the instructions of the manufacturer. This separation assay consists of the following four steps: (i) sample preparation, (ii) hybridization with the ^{125}I-labeled probes, (iii) separation of unhybridized probes with hydroxyapatite, and (iv) measurement of radioactivity.

Statistical analysis. All assays were performed in duplicate. The statistical parameters of sensitivity and specificity were calculated by standard methods (12).

RESULTS

The accuracy of the detection system with the AE-DNA probes was evaluated by testing various strains of the different types of mycobacteria (Table 1). The average RLUs for strains of the *M. tuberculosis* complex obtained by using the *M. tuberculosis* probe were over 100 times higher than those for any mycobacteria other than the *M. tuberculosis* complex (4×10^5 versus 3×10^3 RLUs). The average RLUs for strains of MAC obtained by using the MAC probe were 100 times higher than those for the *M. tuberculosis* complex and non-MAC strains (3×10^5 versus 3×10^3 RLUs). These results indicated the good discriminatory power of the assay system with the AE-DNA probes. Accordingly, it would be reasonable to define the cutoff value of the assay as 3×10^4 RLUs; a specimen was considered positive if a luminescence of greater than 3×10^4 RLUs was obtained.

The detection limits of the assay obtained with AE-DNA probes were compared with those obtained with the ^{125}I-DNA probes by using serial 10-fold dilutions of six isolates of cultured mycobacteria (two isolates each of *M. tuberculosis*, *M. avium*, and *M. intracellulare*). As shown in Fig. 1, the results of the two methods correlated very closely. The cutoff value for the assay with ^{125}I-labeled DNA probes was previously defined as 10% hybridization, which was equivalent to a detectability of 10^6 CFU of bacteria per reaction tube (3). In good agreement with this, the detectability in the assay with the AE-DNA probe was also 10^6 CFU per reaction tube if the cutoff value of 3×10^4 RLUs was used.

A total of 107 clinical isolates of mycobacteria (47 *M. tuberculosis*, 27 *M. avium*, 9 *M. intracellulare*, and 24 atypical non-MAC mycobacteria) identified by the conventional method were tested with the AE-DNA probes. These results were then compared with those of the conventional method and the assay with ^{125}I-labeled DNA probes (Table 2). The RLUs obtained with the AE-DNA probe for *M. tuberculosis* (represented by mean ± standard deviation

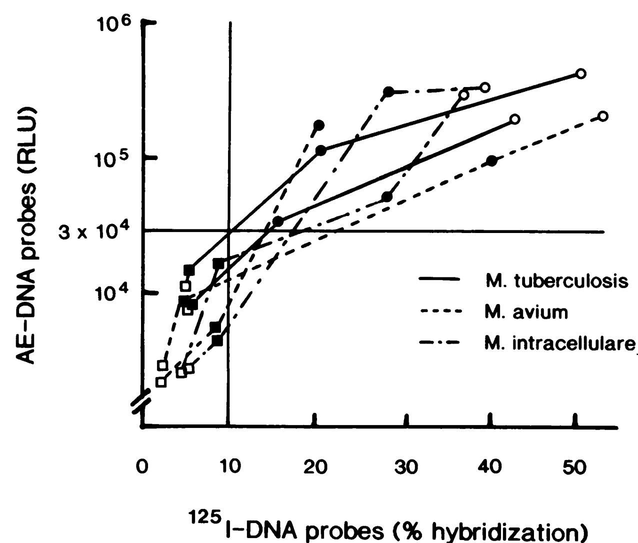

FIG. 1. Correlation between the detection limits of the AE-DNA and ^{125}I-labeled DNA probes for mycobacteria. Serial 10-fold dilutions of cultured mycobacteria (○, 10^7 CFU; ●, 10^6 CFU, ■, 10^5 CFU; □, 10^4 CFU) were tested by both methods. A sample was considered positive if the AE-DNA probe emitted greater than 3×10^4 RLUs or the proportion of hybridization by the ^{125}I-labeled DNA probe was greater than 10%. The assays were performed in duplicate, and representative results were plotted.

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TABLE 2. Comparison of the AE-DNA and ^{125}I-labeled DNA probes with the conventional tests for 107 clinical isolates of mycobacteria[a]

Conventional test result	No. of isolates									
	AE-DNA probes for:				^{125}I-labeled DNA probes for:					
	MTB[b]		MAC[c]		MTB		MA[d]		MI[e]	
	+	−	+	−	+	−	+	−	+	−
Positive	47	0	35	1[f]	47	0	26	1[f]	9	0
Negative	0	60	0	71	0	60	0	80	0	98

[a] A total of 107 clinical isolates of mycobacteria were identified by the conventional method as *M. tuberculosis* in 47 isolates, *M. avium* in 27 isolates, *M. intracellulare* in 9 isolates, and atypical non-MAC mycobacteria in 24 isolates.

[b] MTB, *M. tuberculosis*.

[c] MAC, *M. avium-M. intracellulare* complex.

[d] MA, *M. avium*.

[e] MI, *M. intracellulare*.

[f] One isolate identified as *M. avium* by the conventional tests did not react with either the AE-DNA probe for MAC or the ^{125}I-labeled DNA probe for *M. avium*.

[range]) were 424,254 ± 71,445 (261,298 to 560,832) in the 47 isolates of *M. tuberculosis*, 2,305 ± 572 (1,312 to 4,045) in the 27 isolates of *M. avium*, 1,441 ± 549 (1,027 to 2,826) in the 9 isolates of *M. intracellulare*, and 2,967 ± 685 (1,691 to 5,299) in the 24 atypical non-MAC mycobacteria. The values obtained with the AE-DNA probe for MAC were 1,829 ± 900 (880 to 6,364) in *M. tuberculosis*, 342,418 ± 26,961 (291,051 to 382,118) in *M. avium*, 409,804 ± 42,008 (324,243 to 495,474) in *M. intracellulare*, and 2,841 ± 930 (1,097 to 5,751) in atypical non-MAC mycobacteria. The sensitivity and specificity of the detection system with the AE-DNA probe for *M. tuberculosis* in identifying *M. tuberculosis* were 100% for both the conventional method and the method with the ^{125}I-labeled DNA probe for *M. tuberculosis*. The sensitivity and specificity of the AE-DNA probe for MAC in identifying MAC isolates were 97.2 and 100%, respectively, for the conventional method, and were both 100% for the method with the ^{125}I-labeled DNA probes for either *M. avium* or *M. intracellulare*. One isolate identified as *M. avium* by the conventional method did not react with the AE-DNA probe for MAC. However, this isolate also did not react with the ^{125}I-labeled DNA probe for *M. avium*. If this isolate were tested by the SNAP system (Syngene, Inc., San Diego, Calif.) (9), it might be identified as *M. avium*. On the basis of the results of the detection limits, sensitivity, and specificity, the nonisotopic AE-DNA probes proved to have the same accuracy as the ^{125}I-labeled DNA probes.

DISCUSSION

The results of this study showed that the AE-DNA probes for *M. tuberculosis* and MAC can be used as a rapid and reliable diagnostic method in the clinical laboratory. Compared with ^{125}I-labeled DNA probes, nonisotopic probes had almost the same sensitivities, specificities, and detection limits. There are several assay formats for DNA probes. Commercially available DNA probes for *M. tuberculosis*, *M. avium*, and *M. intracellulare* are labeled with ^{125}I, which is hazardous to laboratory workers. Since washing steps are required to separate the hybridized and unhybridized probes in the assay, a large amount of isotopic waste must be kept while its radioactivity decays. Furthermore, the ^{125}I-labeled probe has a shelf life of only 1 month. In contrast to these limitations, the nonisotopic probe is safe and has a considerably longer shelf life; the AE-DNA probe can be stored for over 1 year if it is refrigerated. An acridinium ester has already been used to label the DNA probe for *Neisseria gonorrhoeae* (the prototype Gen-Probe PACE system) (5). However, in that assay, magnetic particles that specifically bind to hybridized probes are used, and washing must be repeated three times to remove unhybridized probes. In contrast, a new method, the so-called hybridization protection assay, was applied in the system described here. This method is based on the selective chemical degradation of the acridinium ester label so that the acridinium ester associated with the unhybridized probe is rapidly lost, whereas the acridinium ester associated with the hybridized probe is protected. Then, the chemiluminescence emitted by the acridinium ester released from the hybridized probe is measured (2). Thus, all the reactions are carried out in one tube and washing steps are not required. This one-step homogeneous format is one of the main advantages of the AE-DNA probe, and this makes it potentially automatable. Furthermore, all the procedures can be completed within 1 h.

The AE-DNA probe for MAC is a mixture of both the AE-DNA probe for *M. avium* and that for *M. intracellulare*. Using the ^{125}I-labeled probes which can distinguish *M. avium* from *M. intracellulare*, Saito et al. (13) reported a very interesting epidemiological study in which they elucidated the distributions of *M. avium* and *M. intracellulare* in various districts of Japan. However, in a clinical sense, the combined probe would be more convenient because differentiation of MAC isolates into *M. avium* and *M. intracellulare* is not necessary for determining optimal therapy.

Although the detection limits of this system are almost the same as those of the radioisotope probes, they are still not sensitive enough to directly detect mycobacterial rRNA in clinical specimens. There are several rRNA-directed DNA probes for other organisms. In some of them, such as those for *Legionella* spp. (4), *N. gonorrhoeae* (5), and *Chlamydia trachomatis* (8), organisms can be detected directly in clinical specimens by the DNA probes. The detection limits of the AE-DNA probes for mycobacteria achieved in this study were at the level of 10^6 CFU. One can increase the apparent detectability by lowering the cutoff value for the AE-DNA probes, but this will result in an increase in false-positive results. Therefore, to get reliable results, it is recommended that clinical specimens be cultured before they are tested with the AE-DNA probes used in this study. If a method could be developed to lyse the cell wall of the mycobacterium more efficiently, mycobacteria would be directly detectable by the DNA probe in clinical specimens.

In summary, the new method to detect *M. tuberculosis* and MAC with the AE-DNA probe is simple, rapid, and reliable and is a very practical diagnostic tool that can be used in any clinical laboratory.

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