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

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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 125I-labeled DNA probes (Gen-Probe, Inc., San Diego, Calif.) (3, 10) and the BACTEC system (Becton Dickinson, 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 125I (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 125I-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. africana-num NIHJ1602), four strains of MAC standards (M. avium NIHJ1605, M. avium NIAH1106, M. intracellulare NIHJ1618, 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 NIHJ1617, 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; pigmentaion; 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)
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. Acidinium esters bound to the hybridized DNA probes were hydrolyzed by the addition of 200 μl of 0.1% H2O2 solution (detecting reagent I) and 200 μl of 1 M NaOH (detecting reagent II), and the amount of light emitted from the acidinium (chemiluminescence) was measured quantitatively in a Leader luminometer (Gen-Probe, Inc.). The result was expressed as relative light units (RLUs), which were defined arbitrarily according to the sensitivity of the instrument.

125I-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 125I-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 × 10^3 versus 3 × 10^4 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 × 10^4 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 125I-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 125I-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 125I-labeled DNA probes (Table 2). The RLUs obtained with the AE-DNA probe for M. tuberculosis (represented by mean ± standard deviation

<p>| TABLE 1. RLUs of the AE-DNA probes for strains of different types of mycobacteria |
|---------------------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Type (no. of strains)</th>
<th>Mean ± SD RLUs (range) of AE-DNA probes fora:</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis complex (3)</td>
<td>422,742 ± 70,423 (283,930-501,575)</td>
</tr>
<tr>
<td>MAC (4)b</td>
<td>2,827 ± 610 (2,160-4,282)</td>
</tr>
<tr>
<td>Non-MAC (9)c</td>
<td>3,285 ± 1,225 (2,056-6,938)</td>
</tr>
</tbody>
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* The M. tuberculosis complex consisted of M. tuberculosis NIHJ1613, M. bovis NIHJ1607, and M. africanum NIHJ1602.  
* MAC isolates consisted of M. avium NIHJ1605, M. avium NIHJ1610, M. intracellulare NIHJ1618, and M. intracellulare NIHJ1613.  
* Non-MAC isolates consisted of M. kansasi NIHJ1619, M. marinum NIHJ1620, M. simiae NIHJ1627, M. scrofulaceum NIHJ1626, M. gordonae NIHJ1617, M. xenopi NIHJ1638, M. gastri NIHJ1616, M. fortuitum NIHJ1615, and M. chelonel NIJH1611.

Fig. 1. Correlation between the detection limits of the AE-DNA and 125I-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 125I-labeled DNA probe was greater than 10%. The assays were performed in duplicate, and representative results were plotted.
TABLE 2. Comparison of the AE-DNA and ¹²⁵I-labeled DNA probes with the conventional tests for 107 clinical isolates of mycobacteria

<table>
<thead>
<tr>
<th>Conventional test result</th>
<th>No. of isolates</th>
<th>AE-DNA probes for:</th>
<th>¹²⁵I-labeled DNA probes for:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>MTBb</td>
<td>MACc</td>
</tr>
<tr>
<td>Positive</td>
<td>47</td>
<td>0</td>
<td>35 (7)</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>60 (7)</td>
<td>0 (7)</td>
</tr>
</tbody>
</table>

| Positive                 | 9 (7)          | 0                 | 0 (7)                       | 9   | 0   | 0 (7) |

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.

One isolate identified as M. avium by the conventional tests did not react with either the AE-DNA probe for MAC or the ¹²⁵I-labeled DNA probe for M. avium.

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 ¹²⁵I-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 ¹²⁵I, 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 ¹²⁵I-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 ¹²⁵I-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 RNA 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 10⁶ 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.

ACKNOWLEDGMENTS

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REFERENCES


DISCUSSION

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 ¹²⁵I-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 ¹²⁵I, 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 ¹²⁵I-labeled probe has a shelf life of only 1 month. In contrast to these