Comparison of Alkaline Phosphatase-Conjugated Oligonucleotide DNA Probe with the Sereny Test for Identification of Shigella Strains

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We compared an alkaline phosphatase-conjugated oligonucleotide DNA probe with the Sereny test to determine the sensitivity and specificity of the probe in detecting virulent Shigella strains. The probe hybridized with all 52 Sereny-test-positive strains (sensitivity, 100%) and 4 of 21 Sereny-test-negative strains (specificity, 81%). The probe did not hybridize with any of the Sereny-test-negative S. dysenteriae type 1 strains. This nonradioactive, synthetic probe provides a simple, rapid way to test a large number of strains simultaneously in a field setting, which will contribute to an improved understanding of the epidemiologic patterns of shigellosis in developing countries.

As worldwide efforts to control mortality from diarrhea improve through better use of oral rehydration solutions, the proportion of severe diarrhea cases due to invasive organisms such as Shigella spp. may rise. The recent, sudden, large epidemics of multiple-drug-resistant Shigella dysenteriae type 1 infections in south and Southeast Asia and central Africa (1, 6, 8, 9, 15) and the sudden increase of such infections in the United States in 1988 (4) demonstrate the importance of improved understanding of the epidemiologic patterns of shigellosis in the global efforts to control diarrheal diseases.

The capacity to process a large number of samples simultaneously is an important feature of a laboratory procedure used in epidemiologic investigations. Recently developed suitable methods that offer this advantage include the enzyme-linked immunosorbent assay to detect Shigella spp. and enteroinvasive Escherichia coli strains that express the so-called virulence marker antigens (10) and the DNA hybridization method based on the knowledge that invasive strains of Shigella (and enteroinvasive E. coli) carry a 120- to 140-megadalton plasmid (invasiveness plasmid) that encodes genes responsible for virulence (12).

The ability of bacteria to produce dysentery is associated with their ability to produce keratoconjunctivitis in guinea pigs (Sereny test) (13), and new tests to detect Shigella are often validated by comparison to this test. The 32P-labeled 17-kilobase probe described by Boileau et al. (3) and the 32P-labeled 2.5-kilobase probe of Small and Falkow (14) were found to have a sensitivity of 100% and specificity of 91% compared with the Sereny test in the detection of Shigella spp. (16). However, in developing countries, where the problem of shigellosis is most acute, the requirements for radioisotopes and cold storage of these plasmid-derived probes make their use unfeasible. In this report, we compare an alkaline phosphatase-conjugated oligonucleotide DNA probe (5a, 7) with the Sereny test to determine the sensitivity and specificity of the probe in the detection of Shigella spp. under conditions that mimic those of a peripheral laboratory in a developing country.

Seventy-three strains of Shigella (13 of S. dysenteriae type 1, 54 of Shigella flexneri, 4 of Shigella sonnei, and 2 of Shigella boydii) isolated from patients with diarrhea or dysentery who were admitted to two hospitals in New Delhi, India, from 1986 to 1989 were studied at the Department of Microbiology, Lady Hardinge Medical College, New Delhi, India. The strains were identified as Shigella spp. on the basis of biochemical tests and serotyped by the method recommended by Edwards and Ewing (5). The strains had been stored in Trypticase soy broth with 15% glycerol at −20°C prior to testing.

Each strain was tested for virulence in the eyes of adult albino guinea pigs by a previously described method (17). Sterile saline was placed in the left eyes as a negative control. Strains which produced keratoconjunctivitis, ulceration, or opacity of the inoculated eyes within 72 h were considered to be virulent.

The Shigella strains were examined for the presence of high-molecular-weight plasmids by a method modified from the technique of Birnboim and Doly (2). Two strains of E. coli, E11 and V517, with plasmids of known molecular weights (E11 strain provided by S. Falkow, Stanford University, Stanford, Calif.; strain V517 provided by S. C. Pal, WHO Collaborating Centre for Diarrhoeal Research, Cuttputa, India) were included on each electrophoresis gel.

The DNA hybridization procedure was conducted at the National Institute of Communicable Diseases, Delhi, India. The alkaline phosphatase-conjugated DNA probe to detect Shigella spp. (kindly supplied by Molecular Biosystems, San Diego, Calif.) is a 21-base oligonucleotide DNA (S'-CCA-TCT-ATT-AGA-ATA-CCT-GTG-3') (5a) synthesized from a sequenced segment of the 2.5-kb probe of Small and Falkow (14). Bacteria grown overnight in Trypticase soy broth (100 µl) were filtered through a nylon membrane (GeneScreen; Dupont, NEN Research Products, Boston, Mass.) on a hybridization manifold. The membrane was then placed with the bacterial cell side up on Whatman 3MM filter paper saturated with lysing buffer inside a covered plastic petri dish and left at 50°C in a water bath for 30 min. The lysing

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buffer consisted of 10% sodium dodecyl sulfate (SDS) in 1× SSC solution (1× SSC is 0.015 M sodium citrate and 0.15 M sodium chloride in deionized water). The membrane was quickly washed in distilled water and placed for 15 min atop Whatman 3MM filter paper presoaked with a denaturation solution containing 0.5 M sodium hydroxide and 1.5 M sodium chloride. The membrane was washed again in water and then neutralized for 5 min atop Whatman 3MM filter paper saturated with 2 M ammonium acetate solution. The membrane was then placed in a sealed plastic bag containing 5 ml of prehybridization buffer (0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone [average molecular weight, 40,000], and 1% SDS in 5× SSC) for 15 min at 41°C. The filter membrane was hybridized for 20 min at 41°C in hybridization buffer (2 μl of the probe in 1 ml of prehybridization buffer). Then the membrane was washed in 0.5× SSC–2× SSC at room temperature for 15 min, in 0.1% SDS–1× SSC at room temperature for 15 min, in 0.5% Triton X-100–1× SSC at room temperature for 15 min, and in 0.5× SSC at room temperature for 5 min. Finally, the membrane was placed between two pieces of Whatman 3MM filter paper saturated with a color development solution (3 mg of Nitro Blue Tetrozolium in 100 μl of 70% [vol/vol] dimethylformamide and 1.5 mg of 5-bromo-4-chloro-3-indolyl phosphate [BCIP] in 100 μl of dimethylformamide added to 10 ml of a solution containing 0.1 M sodium hydrogen carbonate and 0.001 M magnesium chloride in distilled water, with pH adjusted to 9.8 with 10 N sodium hydroxide). The membrane was exposed to this solution in the dark for 2 to 4 h until the positive control developed color (Fig. 1). The control strains included the enteroinvasive E. coli strain (E11) as a positive control and a strain each of enteropathogenic E. coli, enterotoxigenic E. coli, and E. coli K-12 and an avirulent Shigella dysenteriae type 1 strain as negative controls. The reaction was stopped by placing the membrane in distilled water.

Fifty-two of 73 Shigella strains (9 of S. dysenteriae type 1, 41 of S. flexneri, and 2 of S. sonnei) caused keratoconjunctivitis in guinea pigs, harbored a 120- to 140-megadalton plasmid, and hybridized with the DNA probe (Table 1). All 52 strains that produced a positive reaction in the Sereny test hybridized with the probe (sensitivity, 100%). Seventeen of 21 Sereny test-negative strains did not hybridize with the probe (81% specificity). Only 1 of 4 DNA probe-positive, Sereny test-negative strains had a plasmid of 140 megadaltons.

Among 13 S. dysenteriae type 1 strains, the DNA probe hybridized with all 9 strains that produced a positive reaction in the Sereny test (sensitivity, 100%), and did not hybridize with the other 4 that were Sereny test negative.

In this study, the sensitivity of the alkaline phosphatase-conjugated oligonucleotide DNA probe in detecting virulent Shigella strains, compared with the Sereny test, was identical to that of the plasmid-derived, 32P-labeled probes of Small and Falkow (14) and Boileau et al. (3) (sensitivity, 100%). The specificity was lower (91% for the plasmid-derived probes) (16). However, the synthetic, nonradioactive probe detected all Sereny test-positive S. dysenteriae type 1 strains and none of the Sereny test-negative strains. Since a rapid diagnosis is most essential during S. dysenteriae type 1 outbreaks, this probe may be able to provide early diagnosis during such outbreaks to assist health authorities to initiate appropriate control measures.

The probe hybridized with one Sereny test-negative strain harboring the large plasmid. Sansonetti et al. (11) have shown that in addition to the plasmid, several loci in the chromosomal DNA are required for the full expression of virulence detected by the Sereny test. Hence, the probe may have specifically hybridized with this strain, but the strain may have lacked one or more of the chromosomal virulence genes.

The probe also hybridized with three S. flexneri strains that lacked the large plasmid and were Sereny test negative. These may represent nonspecific reactions. However, since it is known that the virulence plasmid in Shigella spp. often undergoes spontaneous deletions, the probe could have hybridized with smaller plasmids that had deletions in regions other than the region complementary to the probe. Although each of these three strains harbored plasmids smaller than the virulence plasmid, we did not perform hybridization with the electrophoretically resolved plasmids to determine whether such deletions had indeed occurred.

Although this study was designed specifically to examine the sensitivity and specificity of the new DNA hybridization test in comparison to the benchmark test for invasiveness

TABLE 1. Comparison of the Sereny test with the presence of 120- to 140-megadalton plasmids and detection by the alkaline phosphatase-conjugated oligonucleotide DNA probes of Shigella serotypes

<table>
<thead>
<tr>
<th>Species and serotypes (no. of strains)</th>
<th>Result of test&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sereny</th>
<th>120- to 140-megadalton plasmid</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. dysenteriae type 1 (9); S. flexneri types 1 (6), 2 (24), 3 (1), 4 (3), and 6 (7); and S. sonnei (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S. flexneri types 1 (1) and 2 (2)</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S. flexneri type 2 (1)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S. dysenteriae type 1 (4); S. flexneri type 2 (9); S. sonnei (2); and S. boydii types 3 (1) and 13 (1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
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<sup>a</sup> Number of positive strains detected: Sereny test, 52 (71%); 120- to 140-megadalton plasmid, 53 (72%); DNA probe, 56 (76%).
virulence (Sereny test), additional studies are necessary to assess the application of the probe in epidemiologic and clinical investigations. A recent study using the same probe to detect Shigella spp. in specimens from Mexico showed that the probe can be used to detect Shigella from a mixed stool culture (5a). This probe is unable to differentiate enteroinvasive E. coli from Shigella spp., nor can it distinguish the different serogroups or serotypes of Shigella spp. However, its future application may involve selection of suspected colonies from an overnight growth on a selective-medium plate of a stool or swab culture and testing them for hybridization with the probe; if the selected colonies are found to be positive, the other colonies from the same plate can be immediately tested for agglutination in appropriate group or type antisera for further differentiation. This and other applications of this probe must be further examined.

In this study, the laboratory was set up to mimic a peripheral health center laboratory in a developing country, and all reagents other than the probe and the filter paper were procured in New Delhi, the capital city of India. Hence, this probe offers a self-sufficient diagnostic capability in a peripheral health center of a developing country and an opportunity to develop a laboratory-based surveillance system for shigellosis and enteroinvasive E. coli diarrhea in a peripheral setting.

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LITERATURE CITED