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# Comparison of Three Assay Systems for Detection of Enterotoxigenic *Escherichia coli* Heat-Stable Enterotoxin

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**In this study, a commercial DNA-DNA hybridization kit for the detection of *Escherichia coli* heat-stable enterotoxin is compared with a competitive enzyme-linked immunosorbent assay (ELISA) and the suckling mouse bioassay. Taking the suckling mouse assay as the "gold standard," the gene probe was the more specific and the ELISA was the more sensitive of the assays. The ELISA and the suckling mouse test are semiquantitative. The ELISA was the most rapid method, most amenable to automation, and most suitable for the examination of large numbers of specimens. The gene probe is particularly applicable in relatively primitive laboratory conditions. The suckling mouse assay was the least suitable system for the examination of large numbers of specimens.**

Enterotoxigenic *Escherichia coli* is among the commonest causes of gastroenteritis in the developing world and has been implicated in traveler's diarrhea (14, 18, 21, 23). It has also been associated with diarrheal illness in the developed world, and many outbreaks have been described (2, 7, 13, 19). One or both of two enterotoxins may be produced by enterotoxigenic *E. coli* (16). Detection assays for the heat-labile enterotoxin have been described (6, 22), and their performance is within the scope of most clinical microbiology laboratories (6, 22).

The heat-stable enterotoxin most closely associated with human disease is STa (4). The suckling mouse bioassay is the standard STa detection method (3). Its characteristics make it unsuitable for use in large-scale studies. In recent years, recombinant DNA and synthetic oligonucleotide probes which hybridize with complementary sequences necessary for the production of STa and heat-labile enterotoxin have been produced (4, 5, 15, 25). Enzyme immunoassays have also been applied to the detection of STa (2, 18). In this paper, a commercially available oligonucleotide gene probe system and a competitive enzyme-linked immunosorbent assay (ELISA), developed by me, are compared with the infant mouse bioassay.

The culture media used in this study, MacConkey agar, blood agar, MacConkey broth, brain heart infusion, peptone water, and Simmon citrate medium, were all supplied by Oxoid Ltd., Basingstoke, England, and were reconstituted as recommended. The Casamino Acids-yeast extract medium contained 30 mg of Casamino Acids (Difco Laboratories, Detroit, Mich.) and 6 mg of yeast extract per liter. It was made up and trace elements were added as described by Lallier et al. in 1982 (8).

Specimens were selected from the stools of 183 children under 3 years of age who suffered from diarrhea and from whom no other enteric pathogen was isolated. Possible *E. coli* strains were selected on the basis of their appearance on MacConkey agar and examined biochemically. Organisms which produced acid and gas in MacConkey broth at 44°C, did not utilize citrate, and produced indole were selected. They were stored on glass beads (26). One colony per patient was selected.

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Prior to assay for enterotoxin production, an isolate was recovered from storage. Three colonies were added to 20 ml of Casamino Acids-yeast extract medium in a sterile 250-ml conical flask. The flask was incubated at 37°C for 18 h with agitation. The bacteria were precipitated by centrifugation, and the supernatant was passed through a 0.2-μm filter (Gelman Sciences, Inc., Ann Arbor, Mich.). Filtrates were tested for STa immediately or stored at –76°C.

The suckling mouse assay described by Dean et al. in 1972 was used as the standard STa detection method (3). The competitive ELISA was based on the method described by Rönnberg et al. and has been described previously (2, 18). The SNAP hybridization system for detecting *E. coli* STa (Du Pont Co., Wilmington, Del.) was used. Organisms to be tested were suspended in 0.5 ml of peptone water and applied to a sterilized nitrocellulose membrane which overlaid a plate of MacConkey agar. After overnight incubation at 37°C, the bacterial DNA was lysed and immobilized on the nitrocellulose by the method of Moseley et al. (15) or the method of Maas (11). The prepared membranes were then hybridized according to the manufacturer's recommendations.

The organisms were stored over a 3-year period. The mouse assay was done at intervals during this time.

The ELISA and gene probe test were performed at the end of this period. When discordant results occurred, all three assays were repeated to ensure that errors due to plasmid loss during storage did not occur.

TABLE 1. DNA hybridization and sample preparation

| Isolate no. | Suckling mouse assay result | Result at indicated hybridization temp by: | | | |
|---|---|---|---|---|---|
| | | Method of Moseley | | Method of Maas | |
| | | 50°C | 60°C | 50°C | 60°C |
| 35 | + | + | + | + | + |
| 55 | + | + | − | + | + |
| 56 | + | + | + | + | + |
| 57 | + | − | − | + | − |
| 58 | + | − | − | + | + |
| 61 | + | + | + | + | + |
| 62 | + | + | − | + | + |

TABLE 2. Comparison of ELISA and DNA-DNA hybridization assay with the infant mouse assay as the reference method (total number of samples, 183)

| Method | No. of results | | | | % | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Sensitivity | Specificity | Predictive value | |
| | True positive | True negative | False positive | False negative | | | Positive | Negative |
| ELISA | 20 | 151 | 10 | 2 | 90.9 | 93.7 | 66 | 98.7 |
| DNA-DNA hybridization | 19 | 161 | 1 | 2 | 90.4 | 99.0 | 95 | 98.7 |

In the initial assessment of the kit, a panel of 7 suckling mouse assay-positive and 11 suckling mouse assay-negative organisms were assayed using hybridization temperatures of either 60 or 50°C and lysing and immobilizing the DNA by either of the methods mentioned above. Each test was performed in triplicate. All of the bioassay-negative organisms were also negative by the gene probe method, regardless of the test parameters. Since the gene probe and mouse bioassay concurred when the lysing-and-immobilizing method of Maas (11) and a hybridization temperature of 50°C were used (Table 1), these test conditions were then applied to the examination of 183 clinical isolates.

In Table 2, the characteristics of the ELISA and gene probe as applied to 183 organisms are outlined; the mouse test is considered the reference method to which the other tests are compared. The ELISA was the more sensitive and the gene probe was the more specific of the tests. Applying McNemar's test to these results, a $P$ value of $<0.002$ was obtained. Ten false-positive results were recorded, ten by ELISA and one by the probe. Two false-negative results were recorded by the two assay systems, with the same two isolates in each case. In each instance, the mouse assay was repeated to ensure that the toxigenic plasmids were not lost during storage.

The suckling mouse bioassay is a relatively straightforward assay. It detects STa in amounts as low as 2.7 to 8.3 μg/liter (1). No expensive equipment is necessary. However, results are affected by the medium used to grow the test organism and by incubation parameters (20). The major defects of this system are the logistic difficulties experienced in providing sufficient numbers of mice for a large-scale study. In the present study, more than 700 mice were used. All but one of the *E. coli* strains positive for STa by the bioassay were labile toxin producers, as determined by tissue culture and Bicken plate methods (2).

The gene probe in the Du Pont SNAP kit detects as few as 1.37 copies of cDNA when used with a nitrocellulose membrane (12). This particular system has been used by other researchers (16, 17). In both of these papers, a hybridization temperature of 60°C was used. Nishibuchi et al. noted that the oligonucleotide probe reacted only with the STh variant of STa at this temperature (16). Reducing the temperature reduces the stringency of hybridization. The improved result obtained by hybridizing at 50°C may be due to both genetic forms of STa being detected under less stringent hybridization conditions. Using the colony blotting technique described here, the method was not technically demanding and took 24 h. No elaborate or expensive equipment was required, and results were visually readable. The alkaline phosphatase label avoided the hazards associated with radioactive labels.

Compared with the mouse assay, the SNAP kit had a sensitivity of 99% and a specificity of 90.4%. It has two major advantages over the other methods: the results are not affected by the incubation parameters of the test organisms, and once fixed on the nitrocellulose membrane the colony blots were noninfectious and quite stable provided they were kept dry.

The ELISA was the most technically demanding method. Test parameters had to be carefully controlled, and positive and negative controls had to be included in each plate. A spectrophotometer was necessary to read the results. Positive results were obtained with as little as 1 μg of STa per liter, approximately one third the minimal amount of STa detected by the mouse test. This ELISA was not as sensitive as the competitive ELISA of Lockwood and Robertson, which detected 0.166 μg of STa per liter (10). Their assay used a biotin-avidin label, which is probably more sensitive than the horseradish peroxidase system used in the method described in this paper. A monoclonal anti-STa-based ELISA described by Svennerholm et al. was also more sensitive, detecting 0.5 μg of STa per liter (24).

Compared to the mouse method, the ELISA had a sensitivity of 90.9% and a specificity of 93.7%. Some of the suckling mouse assay-negative, ELISA-positive results may just reflect the ability of the ELISA to detect lower amounts of STa than the mouse test does. Therefore, its true specificity may be higher than that obtained by comparison with the suckling mouse bioassay. The major advantage of the ELISA was the ease with which it could be scaled up to deal with large numbers of specimens.

Both methods described in this paper have advantages over the suckling mouse method. The gene probe is the most convenient method in less-developed areas. Direct application of gene probes to stool blots would simplify matters further. Unfortunately, very low STa detection rates have been experienced with this method (9). The low minimum detectable dose of STa by the ELISA may allow it to be used to detect STa in fecal filtrates. In this study, stored isolates were used and such studies were not possible. The ease with which the ELISA can be scaled up to deal with large numbers of specimens and its sensitivity make it an ideal system for large-scale epidemiological studies.

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