Comparison of DNA Probes and the Sereny Test for Identification of Invasive *Shigella* and *Escherichia coli* Strains

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Forty-two *Shigella* and 29 *Escherichia coli* strains were screened for invasiveness in the Sereny test and for hybridization with two recently described DNA probes for the invasiveness plasmid. Both probes produced identical results. All Sereny-positive strains hybridized with both DNA probes. Three Sereny-negative strains also hybridized with the probes, suggesting that there are strains containing the invasiveness plasmid that are not pathogenic in animal models.

Shigella spp. are an important cause of gastrointestinal illness in the United States, with an average of over 12,000 isolates reported to the Centers for Disease Control, Atlanta, Ga., each year (3). Virulence is dependent on the ability of the organisms to invade intestinal epithelial cells, multiply within these enterocytes, and cause an inflammatory response. Sereny was able to demonstrate these properties by inoculating the conjunctival sac of a guinea pig with pathogenic strains, producing a purulent keratoconjunctivitis associated with invasion of corneal epithelial cells and infiltration with polymorphonuclear leukocytes similar to that seen in intestinal lesions caused by shigellae (9, 17). This same response has been shown to occur with strains of *Escherichia coli* associated with outbreaks of dysentery (4). Whereas the Sereny test has been instrumental in identifying pathogenic shigellae and enteroinvasive *E. coli*, the test is not without problems. Guinea pigs are expensive, animal facilities are required, and the test is subject to variability. The availability of a good screening assay for invasive isolates would facilitate further epidemiological studies of both *Shigella* spp. and enteroinvasive *E. coli* (22).

Invasive strains of *Shigella* (6, 14, 15, 19) and enteroinvasive *E. coli* (6, 7) have been shown to carry a 120- to 140-megadalton plasmid (the invasiveness plasmid) that encodes genes necessary for virulence. Based on identification of critical regions on this plasmid, Boileau et al. (2) and Small and Falkow (20) have independently developed DNA probes to identify potentially invasive isolates. There have been no direct comparisons between results obtained with the two probes, and only limited data are available comparing DNA probe results with those obtained by the Sereny test. This study was designed to provide data on the sensitivities and specificities of these probes compared with the Sereny test and to permit comparison of the two probes under identical laboratory conditions.

Forty-two clinical *Shigella* (18 *Shigella sonnei*, 19 *S. flexneri*, 3 *S. boydii*, 2 *S. dysenteriae*) and 29 *E. coli* strains were selected for study (Table 1). *Shigella* strains included isolates provided by the Maryland Department of Health and Mental Hygiene; strains isolated in Baltimore, Md., Chile,

and Pakistan by University of Maryland investigators; and strains isolated in Sao Paulo, Brazil, by the Escola Paulista de Medicina. The *E. coli* isolates included 20 strains from Sao Paulo previously identified by the Sereny test as enteroinvasive. Also tested were four enteropathogenic (HEp-2 adherent, with the 60-megadalton plasmid encoding HEp-2 adherence) (8, 12), three enterotoxigenic (heat-labile and -stable enterotoxin producing), and two Vero toxin-producing *E. coli* strains (A. D. O'Brian, T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702, 1983). All isolates were stored in tryptic soy broth (Difco Laboratories, Detroit, Mich.) with 15% glycerol at -70°C on receipt by our laboratory.

For the Sereny test, Hartley guinea pigs (circa 200 g) were used. *Shigella* strains were inoculated into veal infusion broth (Difco) and incubated overnight at 37°C with shaking (250 rpm). Cells were washed three times and suspended in phosphate-buffered saline (Dulbecco modified formula; Flow Laboratories, Inc., McLean, Va.) at a final concentration of approximately 5×10^{12} CFU/ml. *E. coli* strains were inoculated into veal infusion broth, and 0.1 ml of an overnight (37°C) culture was plated in a lawn on L agar. After overnight incubation at 37°C, cells were harvested and suspended in phosphate-buffered saline at approximately 5×10^{12} CFU/ml. Inocula were standardized based on optical density at 650 nm, with plate counts performed for selected strains. Bacterial suspensions (0.025 ml) were inoculated into the right eyes of guinea pigs, and sterile phosphate-buffered saline was placed in the left eyes as a control. The test was considered positive if there was evidence of conjunctivitis, ulceration, or opacity of the inoculated eyes during 5 days of observation. Exudates from the eyes of animals with evidence of keratoconjunctivitis were cultured to confirm the presence of either shigellae or *E. coli*. A minimum of two guinea pigs were inoculated for each strain.

The DNA probe described by Boileau et al. (2) is a 17-kilobase *EcoRI* fragment of the invasiveness plasmid from an *S. flexneri* serotype 5 isolate ligated into pBR322 to form the plasmid pMR17 as described by Echeverria et al. (5) and Sethabutr et al. (18). The DNA probe described by Small and Falkow (20) is a 2.5-kilobase *HindIII* fragment of the invasiveness plasmid from an enteroinvasive *E. coli*

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TABLE 1. Genetic probe and Sereny test results by species tested

Species (no. of strains)	No. of strains hybridizing with DNA probes/no. that were:	
	Sereny positive	Sereny negative
<i>S. sonnei</i> (18)	3/3	1/15
<i>S. flexneri</i> (19)	13/13	1/6
<i>S. boydii</i> (3)	2/2	0/1
<i>S. dysenteriae</i> (2)	2/2	
<i>E. coli</i>		
Enteroinvasive (20) ^a	19/19	1/1
Enterotoxigenic (3) ^b		0/3
Enteropathogenic (4) ^c		0/4
Vero toxin producing (2)		0/2

^a Based on a positive Sereny test at the time of isolation in Brazil.^b Heat-labile and -stable enterotoxin producing.^c HEP-2 adherent, with the 60-megadalton plasmid encoding HEP-2 adherence.

strain. The probe is part of a 6-kilobase fragment ligated into pHCT9 to form plasmid pSF55. The DNA probes were isolated by digestion with appropriate restriction enzymes, preparative gel electrophoresis, and electroelution and were labeled by nick translation with [α -³²P]dATP (New England Nuclear Corp., Boston, Mass.) (10). For colony hybridization assays (11) with the DNA probes, the inocula used in the Sereny tests were spotted directly onto sterile nitrocellulose filters on L-agar plates. Filters were also prepared for an additional 61 gram-negative clinical isolates of 14 different bacterial species (Table 2). Filters were prepared and hybridizations were conducted under high-stringency conditions as described by Moseley et al. (11), with results visualized by autoradiography. Plasmids were extracted (1) from all *Shigella* and *E. coli* strains and visualized on a 0.7% agarose gel. Plasmid sizes were determined by using marker plasmids of known sizes.

The two DNA probes produced identical results. All 20 Sereny-positive *Shigella* strains hybridized with both DNA probes for a sensitivity of 100% (Table 1). Of the 22 Sereny-negative *Shigella* strains, 20 were also probe negative (91% specificity). All 19 Sereny-positive *E. coli* strains were probe positive; 9 of 10 Sereny-negative strains were probe negative. Of the 32 Sereny-negative strains, 5 had a plasmid of ≥ 120 megadaltons. The three strains that were

TABLE 2. Species other than shigellae and enteroinvasive *E. coli* tested with genetic probes^a

Species	No. of strains
<i>E. coli</i>	12
<i>Salmonella</i> spp. not <i>S. typhi</i>	10
<i>Pseudomonas aeruginosa</i>	9
<i>Aeromonas</i> spp.	5
<i>Plesiomonas shigelloides</i>	5
<i>Campylobacter jejuni</i>	5
<i>Klebsiella pneumoniae</i>	4
<i>Salmonella typhi</i>	4
<i>Proteus mirabilis</i>	2
<i>Proteus vulgaris</i>	1
<i>Providencia stuartii</i>	1
<i>Providencia alcalifaciens</i>	1
<i>Enterobacter cloacae</i>	1
<i>Acinetobacter calcoaceticus</i>	1

^a Neither probe hybridized with isolates of any of the species listed.

FIG. 1. Southern blots of DNA probes. (A) Agarose gel. Lanes: a, *Hind*III digest of Boileau probe; b, uncut Boileau probe; c, *Eco*RI digest of Small and Falkow probe; d, uncut Small and Falkow probe; e, λ *Hind*III digest. (B) Southern blot of gel hybridized with the Boileau probe showing hybridization with only lanes a and b. (C) Southern blot of gel hybridized with the Small and Falkow probe showing hybridization with only lanes c and d.

Sereny negative but probe positive were among these five; there was a highly significant association between the presence of a large plasmid and hybridization with the DNA probes among Sereny-negative *Shigella* and *E. coli* strains ($P = 0.002$; Fisher exact test). For selected strains we were able to demonstrate that loss of this large plasmid correlated with loss of a positive probe result. Neither probe hybridized with isolates of any of the other bacterial species tested or with *E. coli* strains other than those previously identified as enteroinvasive (Table 2).

The two probes did not hybridize with each other on Southern blots (21) (Fig. 1), suggesting that they identified distinct regions of the invasiveness plasmid. Both of the regions identified by the probes appear to be necessary for invasiveness, as all strains that were Sereny positive hybridized with both probes. However, there was a small group of large-plasmid-containing strains that hybridized with the probes but did not cause keratoconjunctivitis in guinea pigs. This may reflect false-negative Sereny test results, but every effort was made to standardize our assays, with multiple animals inoculated with each strain. It appears more likely that the DNA probes correctly identified an invasiveness plasmid in these strains but that strains were missing other critical plasmid or chromosomal loci necessary for virulence (13).

The one *E. coli* strain that was probe positive but negative in the Sereny test had been identified as Sereny positive when originally isolated in Sao Paulo; loss of invasive ability appears to have occurred between initial isolation and testing in our laboratory. In the one field study that has been reported with the probe described by Boileau et al., there was an exact correlation between probe and Sereny test results with fresh clinical isolates (18; T. Pal, P. Echeverria, D. N. Taylor, O. Sethabutr, and S. Hanchakay, Letter, Lancet ii:785, 1985). However, false-positive results were noted when laboratory-derived strains were tested (O'Brian et al., Lancet i:702). These data support the idea that during storage or passage strains undergo internal deletions or other changes that render them noninvasive (16). Whereas it should be recognized that these probes may give occasional false-positive results, they appear to be valuable tools for identifying invasive *Shigella* and *E. coli* strains based on

their sensitivity and their failure to hybridize with other bacterial species or other pathogenic *E. coli* strains.

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