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Heat-Stable-Enterotoxin-Producing *Escherichia coli* Strains Isolated from Dogs

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Five strains of hemolytic *Escherichia coli* isolated from dogs suffering from diarrhea were shown by radioactive and enzyme-labeled oligonucleotide probes to possess genes coding for heat-stable enterotoxin (STIa). Four of the strains were shown by immunoassay (enzyme-linked immunosorbent assay) and bioassay (infant mouse test) to produce STI in vitro. All five strains, however, were able to induce fluid accumulation in ligated dog intestinal loops. The four STI-producing strains all possessed the K99 fimbrial antigen (F5) and belonged to serotype O42:H37. In these strains, genes encoding STI were located on a 98-megadalton plasmid. In the fifth strain, which produced STI in vitro only after several subcultivations, the STI gene was located on an 80-megadalton plasmid. This strain was nontypable.

Enterotoxin-producing *Escherichia coli* (ETEC) strains are important causes of acute diarrhea in humans and several other animal species. These ETEC strains produce one or two enterotoxins classified according to their temperature stability (16, 21). Most of the strains also possess fimbrial antigens, of which some seem to be host specific. The F antigens F4 and F6 (K88 and 987p) (18) are found in porcine strains only, while the F5 antigen is observed in strains of porcine, ovine, and bovine origin as well. It is generally accepted that the genes encoding enterotoxins and adhesion fimbriae are located on conjugatable plasmids (5). The ETEC strains isolated from animals seem to be limited to a relatively small number of serogroups.

Dogs have been reported to suffer from ETEC-induced diarrhea, though the bacterial isolates have not been characterized as thoroughly as, for example, porcine strains. Olson et al. (13, 14) found heat-labile-enterotoxin (LT)- or heat-stable-enterotoxin (ST)-producing *E. coli* strains in 6 of 148 dogs with diarrhea. The strains belonged to O groups 4, 17, and 20 and possessed fimbriae which failed to react with antisera to the F4 or F5 antigens.

This report describes the characterization of ETEC strains isolated from dogs, using oligonucleotides as probes to detect toxin-encoding genes and immunoassays and various bioassays to detect produced toxin.

MATERIALS AND METHODS

Strains. Hemolytic *E. coli* strains in pure culture were isolated from dogs with diarrhea. The clinical and pathological findings will be published elsewhere. Five strains from four different dogs were examined extensively.

Colony blot hybridization. Colony blots were hybridized with two oligonucleotide probes coding for parts of the ST (STIa, formerly STp) and the LT (LTp and LTh) genes. These probes were supplied by Molecular Biosystems, San Diego, Calif. The probes were labeled with [^{32}P]ATP, using T4 polynucleotide kinase. Hybridization conditions, reagents, and development of signal were performed as described by Hill et al. (6).

Serology and antimicrobial susceptibility testing. The strains were examined for O and H antigens by the standard methods of Edwards and Ewing (3). Testing of antimicrobial susceptibility to streptomycin, neomycin, tetracycline, ampicillin, penicillin, chloramphenicol, sulfonamides, and trimethoprim was performed with Neo-sensitabs (Rosco, Taastrup, Denmark).

Adhesion fimbrial antigen. The strains were cultivated on blood agar or Minca medium plates overnight and tested for adhesion fimbriae (F4 [K88], F5 [K99], F6 [987p], F2 [colonizing fimbrial antigen I], and F3 [colonizing fimbrial antigen II]), using specific polyclonal serum in a slide agglutination test.

Immunoassay. STI were assayed by using an enzyme-linked immunosorbent assay (ELISA) procedure. The reagents and the performance of the assay were as described previously (10). A similar ELISA system was used for detection of the LT (15).

Bioassay. The infant mouse assay was used to test for the presence of STI in supernatants from overnight broth (2). The dog loop assay (jejunal Thiry-Vella loops) was carried out as described by Sack et al. (19).

Plasmid profiles. Plasmid DNA was isolated by using a method described by Kado and Liu (9). The plasmids were then separated by electrophoresis in a vertical agarose gel and stained with ethidium bromide, and pictures were taken as described previously (16). Reference *E. coli* strains with plasmids of known molecular weight were used as controls.

Localization of genes encoding STI. The plasmid DNA in the agarose gels from plasmid profiling was transferred to a nylon membrane, using the Southern blot procedure. The nylon membrane (Gene Screen plus; Dupont, NEN Research Products, Boston, Mass.) was first prehybridized for 10 min at 50°C in hybridization solution without the probe (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate). The alkaline-phosphatase-labeled oligonucleotide probe (detecting both STIa and STIb; 100 ng) (Dupont, NEN Research Products) (7) was then added to 1 ml of hybridization solution and hybridization occurred at 50°C for 10 min. The membrane was washed by using three different procedures: first, for two 5-min periods in SSC with 1% sodium dodecyl sulfate at

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TABLE 1. Characterization of ETEC strains isolated from dogs

Strain[a]	Enterotoxins						Adhesion factor	Serotype	Plasmids (mol wt, 10^6)[b]	Antibiotic resistance[c]
	LT		ST							
	Probe	ELISA	Probe	ELISA	Mouse	Dog loop				
B9430	−	−	+	+	+	+	K99	O42:H37	**98**, 63, 4.1, 3.9, 3.2	Strep, Neo
B9429	−	−	+	+	+	+	K99	O42:H37	**98**, 63, 4.1, 3.9, 3.2	Strep, Neo
B9431	−	−	+	+	+	+	K99	O42:H37	**98**, 63, 4.1, 3.9, 3.2	Strep, Neo
B9432	−	−	+	+	+	+	K99	O42:H37	**98**, 60, 40, 3.9, 2.6	Strep, Neo
B9435	−	−	+	−/+[d]	−/+[d]	+		ONT:HNM[e]	**80**, 48, 25, 5.1, 3.7, 2.9	Strep, Neo

[a] Centers for Disease Control reference number.
[b] Plasmid in boldface type carried the STI genes.
[c] Strep, Streptomycin; Neo, neomycin.
[d] Converted after several subcultivations in the laboratory.
[e] ONT, O nontypable; HNM, H nonmotile.

room temperature; second, by two 5-min periods in SSC with 1% Triton X-100 at 50°C; and third, in SSC only at room temperature. The membrane was then incubated with the substrate (Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, both supplied by Molecular Biosystems) in the dark at room temperature overnight (12).

Restriction endonuclease digests. The plasmids were cut with restriction enzyme *Eco*RI (Amersham, Little Chalfont, England) following the producer's recommendation. The fragments were then separated by electrophoresis in 0.7% agarose at 70 mA for 2 h.

RESULTS

Five strains that were found to possess the STIa genes after colony blot hybridization were studied further (Table 1). None of the strains was positive with the LT probe. Four of the five STIa probe-positive strains produced STI in vitro as demonstrated by ELISA and the infant mouse test when tested just after identification. All five strains did, however, induce fluid accumulation when introduced into ligated dog intestinal loops. The four strains that were phenotypically positive for STI in vitro were all of serotype O42:H37 and possessed the F5 (K99) antigen. The fifth strain was nontypable, nonmotile, and negative for F2, F3, F4, F5, and F6. This strain produced STI in vitro only after several subcultivations in the laboratory. The STI gene in this strain was located on an 80-megadalton (MDa) plasmid, while in the other four strains the STI gene was located on a 98-MDa plasmid (Fig. 1). Three of the O42:H37 strains revealed an identical plasmid profile, one had only the 98-MDa plasmid in common with the others, and the plasmid profile of the nonmotile strain was totally different from the other four. The plasmids of the three strains with an identical plasmid profile also showed an identical DNA restriction enzyme fragment pattern when cut with endonuclease *Eco*RI. All five strains were resistant to streptomycin and neomycin.

DISCUSSION

The five strains investigated in this work were selected because they were found to possess the STIa genes as demonstrated by hybridization on colony blots with radiolabeled oligonucleotide probes. The strains were also examined for the LT genes, but proved to be negative. Such probes have been used in several investigations and have been shown to be specific, sensitive, and simple to use to test for ETEC strains (4, 6, 20).

Only four of the strains were found to produce STI in vivo when first tested. The fifth strain eventually also produced STI in vitro. The test for toxin was performed by utilizing an ELISA. The toxin from these strains were also tested in the infant mouse assay, all inducing fluid accumulation in this bioassay.

One strain, however, was negative in both the ELISA and the infant mouse assay right after identification; apparently it did not produce significant amounts of STI under the in vitro conditions provided. Nalin et al. (11) have reported similar observations as they found strains to be ST positive in the dog loop model, but not with the infant mouse assay. Genotypically positive, phenotypically negative STI *E. coli*

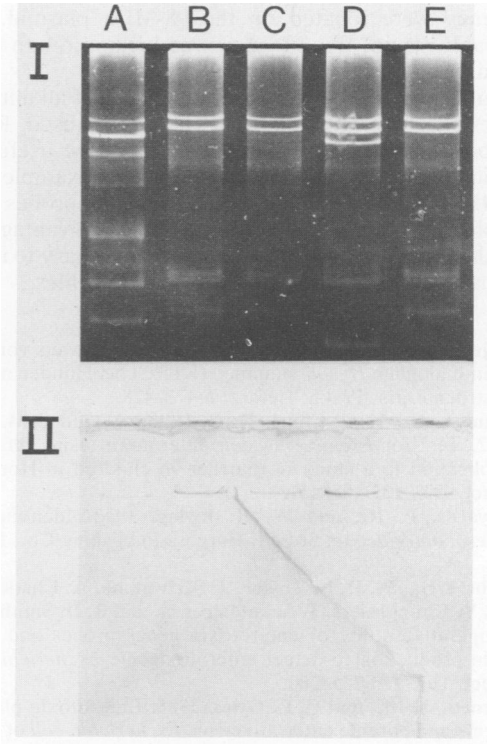

FIG. 1. Plasmid profiles of five *E. coli* strains isolated from dogs with diarrhea (I) and plasmids on which the genes coding for the STI were located (II). (I) Strain A (B9435) was O nontypable and nonmotile, while strains B (B9430), C (B9432), D (B9431), and E (B9429) were all O 42:H37 and possessed the F5 (K99) adhesion fimbrial antigen. (II) The profiles in section I were transferred to nylon membranes by Southern blot and then hybridized with an alkaline phosphatase-labeled synthetic oligonucleotide DNA probe encoding parts of the STI gene.

strains have been described previously. Oprandy et al. (17) observed that 33% of human isolates found to be STI positive with probes were negative in the infant mouse assay. Other authors have reported similar findings (4, 12). It should be noted, however, that some strains which are negative in the infant mouse test have been found to be positive for STI in the more sensitive ELISA (10). Our strain, like the other four, induced fluid secretion in ligated dog loops. *E. coli* was cultivated from the loops after the experiments, and it was confirmed that it possessed the same properties as the bacterium introduced into the particular loop.

The four in vitro STI-producing strains all possessed the F5 (K99) adhesion fimbrial antigen. This antigen has been shown to be an important virulence factor for enterotoxin-producing strains in that it gives them the ability to adhere to intestinal epithelial cells of pigs, calves, and lambs. Although other investigators have observed fimbrial structures on ETEC strains from dogs, the strains in question did not react with F5-specific antiserum (13). The strains possessing F5 all belonged to serotype O42:H37. O group 42 has previously been found in enterotoxigenic strains from dogs, but not combined with the F5 antigen. Other O groups observed in ETEC isolates from dogs are O groups 4, 5, 17, and 20 (1, 8, 22). It is well known that the genes coding for enterotoxins and adhesion fimbriae in *E. coli* are located on plasmids (5). Three of the four O42:H37 strains had identical plasmid patterns, and the restriction endonuclease digests of these plasmids were identical. This indicated that the plasmids had the same base sequence. In the four O42:H37 strains, the STI genes were located on the 98-MDa plasmid. In the nontypable strain, the ST gene was located on an 80-MDa plasmid.

In this study, both radioactive labeled and alkaline phosphatase-labeled synthetic DNA probes were used. Radioactive labeled probes have been reported to be useful in the screening of large samples of strains by, for example, colony blots (4). Only recently have enzyme-labeled probes become available (7, 12, 17), and they have several advantages. This study shows that they give results which are easy to interpret when used on Southern blots of plasmid profiles.

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