

## Genetic and Phenotypic Analysis of *Escherichia coli* with Enteropathogenic Characteristics Isolated from Seattle Children

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Coliform colonies from children whose stools were submitted for microbiologic analysis were studied prospectively to determine the frequency of shedding of enteropathogenic *Escherichia coli* (EPEC). In total, 2225 isolates from 445 patients were probed with *eaeA* (encoding intimin) and the EAF (EPEC adherence factor) probe, and adherence and actin-aggregating phenotypes were determined. Twenty-five patients (5.6%) shed non-O157:H7 *eaeA*<sup>+</sup> EAF<sup>-</sup> *E. coli*. Of these 25 patients, isolates from 5 produced Shiga toxins and from 3 possessed *bfpA* (encoding the bundle-forming pilus) sequences. Non-O157:H7 *eaeA*<sup>+</sup> *E. coli* from 21 (84%) of 25 patients adhered locally to and aggregated actin in HeLa cells. Four patients shed nonadherent EAF<sup>+</sup> *eaeA*<sup>-</sup> *E. coli*. Non-O157:H7 *eaeA*<sup>+</sup> and EAF<sup>+</sup> isolates belonged to diverse electrophoretic types and classical and nonclassical enteropathogenic serotypes. EPEC are relatively common in stools submitted for analysis in this North American pediatric hospital. Their etiologic role in childhood diarrhea warrants elucidation.

Enteropathogenic *Escherichia coli* (EPEC) have been postulated since the 1940s to be virulent for humans [1–3], but their nontoxic phenotype hampered acceptance of their pathogenicity [4]. Recently, however, identification of EPEC-specific phenotypes expanded our understanding of the role of these organisms in human diarrhea. First, the “attaching and effacing lesion,” consisting of microvilli destruction and accumulation of actin at the sites of intimate attachment of bacteria to the mucosa, was described as the cardinal lesion induced in animals by EPEC [5]. Intimate mucosal adherence of EPEC is also observed in infected humans [6, 7]. Second, epidemiologic studies correlated the localized adherence pattern of EPEC attachment to epithelial cells with diarrhea in the patients from whom the strains originated [8–11]. Third, the fluorescent actin stain (FAS) test permitted the rapid detection of actin aggregation (the in vitro correlate of the attaching and effacing lesion) in epithelial cells at the site of EPEC adherence [12].

Identification of genetic determinants of EPEC phenotypes has also facilitated investigations into the molecular basis of EPEC pathogenicity. These determinants include the EPEC adherence factor (EAF) plasmid [13]; *eaeA*, encoding intimin,

which mediates actin aggregation [14]; *eaeB*, encoding a protein that mediates signal transduction in epithelial cells [15]; and *bfpA*, encoding the bundle-forming pilus [16, 17].

The cloning of EPEC genetic loci associated with pathogenic phenotypes allows the detection of organisms containing these determinants by using nucleic acid hybridizations. Despite the availability of appropriate probes and our emerging knowledge of EPEC phenotypes, the frequency with which EPEC are present in children with diarrhea in the United States remains undetermined. Therefore, we conducted a 1-year prospective study to test the hypothesis that *E. coli* containing *eaeA* or EAF (or both) and expressing virulence phenotypes typical of EPEC are present in stools submitted for bacterial culture to the microbiology laboratory of a Seattle pediatric hospital and to determine the genetic relatedness and serotypes of any such organisms isolated.

### Materials and Methods

**Population studied.** Patients whose stools were submitted for bacterial culture to Children's Hospital and Medical Center between 15 January 1991 and 10 January 1992 were included in this study if at least 5 lactose-fermenting coliform colonies were present on the MacConkey's agar plate and the MacConkey's plate was available for processing on a weekday. Each specimen was analyzed for *Campylobacter* species using CVA *Campylobacter* agar and incubation at 42°C; *E. coli* O157:H7 using sorbitol–MacConkey's agar; *Salmonella* and *Shigella* species using Salmonella–Shigella agar, MacConkey's agar, and Hektoen enteric agar; and *Yersinia* using *Yersinia*-selective agar. Media were purchased from Prepared Media Laboratories (Tualatin, OR). All specimens submitted in a cup were examined for leukocytes by Gram's stain and wet mount.

**Microbiologic analysis.** Five random lactose-fermenting colonies of nonmucoid appearance were selected from the MacCon-

Received 30 September 1996; revised 9 January 1997.

Financial support: NIH (RR-05655, AI-24565, and AI-00964) and World Health Organization Health Manpower Development Program. P.I.T. was an AGA/Blackwell Scientific Publications Research Scholar during the performance of part of this work.

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The Journal of Infectious Diseases 1997;175:1382–9

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0022-1899/97/7506-0013\$01.00

key's agar plate of each stool culture and frozen in Luria Bertani (LB) broth [18] with 15% glycerol at  $-80^{\circ}\text{C}$  until analysis. An isolate was confirmed to be *E. coli* if it was oxidase-negative, produced indole, and was unable to use citrate as a sole source of carbon. Each isolate with homology to one or more probes was tested for the ability to ferment sorbitol following overnight incubation using sorbitol–MacConkey's agar plates. O and H serotypes were determined by microtiter agglutination using 173 standard World Health Organization O antisera, 16 OX (experimental) antisera, and 56 H antisera. Briefly, bacteria were cultured overnight on veal infusion yeast extract agar, suspended in formal-saline (6 mL of formaldehyde/L of normal saline), heated at  $100^{\circ}\text{C}$  for 2 h, and then tested for somatic antigen expression in 96-well microtiter trays using monovalent antisera. Agglutinating bacteria were tested further in microtitration assays to confirm the O serotype. Nonreactive organisms were reheated at  $121^{\circ}\text{C}$  for 1 h and retested; agglutination reactions were confirmed by titration. Each isolate was also propagated in motility media for at least 7 days. Motile isolates were cultured for 18 h in veal infusion yeast extract broth, diluted with formal saline, and analyzed by microtiter agglutination. Agglutinating bacteria were confirmed by microtitration against specific antisera to determine the H antigens.

Selected isolates were also characterized by their reactions in the API 20 E test (bioMérieux Vitek, Hazelwood, MO) and their susceptibility to amoxicillin–clavulanic acid, ampicillin, cefazolin, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, gentamicin, ticarcillin, tobramycin, and trimethoprim-sulfamethoxazole [19].

**Probes.** All colonies isolated were probed separately with DNA fragments specific for *eaeA*, the EAF plasmid, and a mix of *stx1* and *stx2*, which encode Shiga toxins 1 and 2. These fragments consisted of the 1-kb *Sall*–*StuI* fragment of pCVD434 [14], the 1-kb *Bam*HI–*Sall* fragment of pMAR2 [13], the 1.1-kb *TaqI*–*HincII* fragment of pJN37-19 [20], and the 0.8-kb *SmaI*–*PstI* fragment of pNN111-19 [20], respectively. Colonies demonstrating homology to *eaeA* or EAF were probed with the *bfpA* probe, consisting of the 0.85-kb *EcoRI* fragment of pMSD207, which became available after the initial screening of the bacteria [21]. All enzymes were purchased from Promega (Madison, WI).

**Colony hybridization.** Stored coliform isolates were cultured on LB agar. Resulting colonies were transferred to 541 paper (Whatman International, Maidstone, UK), steam-denatured, and neutralized [22]. Probes were radiolabeled using random hexanucleotide primers, the Klenow fragment of *E. coli* DNA polymerase I, and [ $^{32}\text{P}$ ]dCTP (NEN Research Products, Boston) [23]. Filters were prehybridized for 10 min at  $37^{\circ}\text{C}$  in  $5\times$  standard saline citrate (SSC), 0.1% SDS, 50% formamide, 1 mM EDTA, and 100  $\mu\text{g}/\text{mL}$  denatured calf thymus DNA before the addition of heat-denatured labeled probe ( $10^5$  dpm of each label/filter). After overnight incubation at  $37^{\circ}\text{C}$ , the filters were washed once in  $2\times$  SSC and 0.1% SDS at room temperature for 5 min, once in  $2\times$  SSC and 0.1% SDS at  $65^{\circ}\text{C}$  for 30 min, and once in  $0.5\times$  SSC and 0.1% SDS at  $65^{\circ}\text{C}$  for 30 min; then they were exposed to x-ray film overnight at  $-80^{\circ}\text{C}$  in the presence of intensifying screens.

To further characterize Shiga toxin–producing *E. coli* (STEC), colonies with homology to the mix of *stx* probes were hybridized separately with *stx1* and *stx2* under more stringent conditions to differentiate between these two alleles [20]. Positive controls consisted of EPEC strain B171 (*E. coli* O111:NM) for *eaeA* and *bfpA* [24], *E. coli* O26:H11 for *eaeA* and *stx1*, and *E. coli* O157:H7 86-

24 for *eaeA* and *stx2* [25]. *E. coli* HB101 [20] was the negative control.

**Adherence assay.** HeLa cells grown at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in MEM with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (100  $\mu\text{g}/\text{mL}$ ) in plastic culture flasks were used to assess adherence of organisms to epithelial cells [25]. The cells were trypsinized, added to glass chamber slides 2 days before the assay, and reincubated.

On the day of the assay, HeLa cells at  $\sim 80\%$  confluence were washed with sterile PBS and covered with 0.6 mL of incubation medium (MEM, 5% FCS, 2 mM L-glutamine, nonessential amino acids, and 0.5% D-mannose). Bacteria (20  $\mu\text{L}$ ) grown overnight with agitation at  $37^{\circ}\text{C}$  in LB broth were then added to the HeLa cells, and bacteria and cells were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 3 h (for nontoxigenic organisms and the negative control) or 1 h (for STEC and the positive control). They were then washed three times with sterile PBS, covered with 0.6 mL of incubation medium, and incubated again for 3 h. Nonadherent bacteria were removed by washing 10 times with PBS at the end of the assay. The cells were then fixed by adding 100% methanol to each chamber for 5 min.

Fixed cells and adherent bacteria were stained with Giemsa stain for 1 h, mounted, and examined. The positive control in all experiments consisted of EPEC B171 [24], which displays localized adherence. Nonadherent laboratory strain *E. coli* HB101 [18] was used as a negative control. The examiner did not know the identity of the isolates and controls.

**Actin aggregation.** The ability of adherent bacteria to induce actin aggregation was determined by FAS [12]. At the conclusion of the adherence assay, cells were fixed with 2% formalin in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 7.5 min, and stained in the dark with fluorescein isothiocyanate–conjugated phalloidin (Sigma, St. Louis; 5  $\mu\text{g}/\text{mL}$  in PBS) for 30 min. Phalloidin was removed by washing with PBS. The stained cells were mounted (fluorescent mounting medium or Glycergel; Dako, Carpinteria, CA) and examined using fluorescent microscopy by a reader unaware of which chambers contained the patient isolates and the controls. EPEC B171 was the positive control and *E. coli* HB101 was the negative control.

**Multilocus enzyme electrophoresis.** All isolates were characterized for allelic variation at 20 enzyme-encoding loci by multilocus enzyme electrophoresis [26, 27]. Electromorphs were equated with alleles at the corresponding gene locus, so that all bacteria were characterized by their multilocus genotypes (allele combinations) for the enzyme-encoding loci assayed [26]. Distinctive multilocus genotypes were designated electrophoretic types (ETs), which were numbered by their inferred relationships from a cluster analysis.

**Medical records.** The indications for stool culture were determined by review of the hospital records or interviews of the requesting physician for each patient whose stool contained colonies with homology to one or more of the probes. Diarrhea was considered acute if it was of  $<2$  weeks' duration when the culture was requested and chronic if it had lasted for  $\geq 2$  weeks. Associated symptoms (fever, vomiting, grossly bloody diarrhea) noted by the physician at the time of initial evaluation and the age of each patient were also recorded.

**Statistics.** Mean ages of children in each group were compared using the *t* test [27].

## Results

**Patients studied and bacterial pathogens recovered.** Coliform isolates from 445 patients were analyzed. *Campylobacter jejuni*, *E. coli* O157:H7, *Salmonella* organisms, *Shigella* organisms, and *Yersinia enterocolitica* were recovered from 11, 13, 15, 1, and 1 of these 445 patients, respectively.

**Colony hybridizations.** We probed 2225 colonies from the 445 study patients. Random coliform colonies from 34 patients (7.6%) contained DNA homologous to *eaeA*. The colonies detected by the *eaeA* probe from 9 patients were *E. coli* O157:H7 (4 additional patients were infected with *E. coli* O157:H7 that were detected on sorbitol–MacConkey's agar but not by probing). Group 1 comprised 20 patients with *eaeA*<sup>+</sup> *stx*<sup>-</sup> organisms (table 1); *E. coli* from 3 group 1 patients contained *bfpA* sequences. Group 2 consisted of 5 patients with non-O157:H7 *eaeA*<sup>+</sup> *stx*<sup>+</sup> *E. coli* colonies. The clinical characteristics and adherence phenotypes of group 2 isolates have been reported [25], but these organisms are included in this analysis to determine genetic relatedness among all bacteria recovered in this study. Non-O157:H7 *eaeA*<sup>+</sup> *E. coli* constituted the predominant fecal coliform flora sampled in 17 of the 25 group 1 and 2 patients. No group 1 or 2 *E. coli* contained EAF sequences. *E. coli* from 4 (0.9%) of the 445 stools studied hybridized to the EAF probe (group 3); group 3 *E. coli* did not contain *stx1*, *stx2*, *eaeA*, or *bfpA*.

**Sorbitol fermentation phenotypes of colonies with homology to one or more probes (excluding *E. coli* O157:H7).** Bacteria from 4 group 1 patients failed to ferment sorbitol. All other isolates studied were sorbitol fermenters.

**Coisolated pathogens.** Three group 1 patients were infected with *C. jejuni*, and 1 was infected with *E. coli* O157:H7. *E. coli* O157:H7 from patient 206 were detected only on the sorbitol–MacConkey's agar plate, whereas the more numerous sorbitol-fermenting *eaeA*<sup>+</sup> *E. coli* O26:HN were detected by hybridization analysis of the 5 randomly selected colonies.

**Symptoms prompting stool culture and patient ages.** Indications for stool culture included acute and chronic nonbloody diarrhea, acute bloody diarrhea, and chronic gastrointestinal blood loss. The medical records also indicated that 6 patients were febrile, and 5 patients experienced vomiting. Group 3 patients (mean age, 9.5 months) were significantly younger than patients in group 1 or 2 (mean ages, 28.2 and 67.6 months, respectively;  $P = .005$  and  $.04$ , respectively). The difference in the mean ages of the children in groups 1 and 2 was not statistically significant ( $P = .1$ ).

**Fecal leukocytes.** Stools were submitted as cup specimens from 27 (93%) of the 29 patients. The 3 stools in which fecal leukocytes were detected also contained STEC.

**Adherence phenotypes of *E. coli* recovered.** *E. coli* from 16 (80%) of the 20 group 1 patients, all 5 of the group 2 patients, and none of the 4 group 3 patients adhered to HeLa cells in a localized pattern. *bfpA*<sup>-</sup> isolates adhered as prominently as *bfpA*<sup>+</sup> isolates. Representative adherence of *eaeA*<sup>+</sup>

*stx*<sup>-</sup> *bfpA*<sup>-</sup> isolate TB 216 B to HeLa cells is shown in figure 1A. Two of the 4 patients whose stools contained nonadherent non-O157:H7 *eaeA*<sup>+</sup> *E. coli* were also infected with *C. jejuni*.

**Actin-aggregating phenotypes of adherent isolates.** Each adherent isolate in groups 1 and 2 aggregated actin except isolates TB 227 A and B. A representative FAS assay demonstrating actin aggregation in HeLa cells to which isolate TB 216 B adhered is shown in figure 1D.

**Genetic diversity and clonal relationships of isolates.** Of the 20 enzymes studied in each isolate, 17 (85%) were polymorphic (3.8 alleles/locus, on average). The multilocus arrays defined 27 distinct ETs and serotypes (figure 2, table 1). The average single-locus diversity was  $0.41 \pm 0.07$  SD. Multiple colonies from 22 of the 29 patients hybridized to at least one probe. Discordant ETs and serotypes were present among the isolates from patients TB 96 and TB 227.

ET 7 isolates were recovered from 2 patients and were each *eaeA*<sup>+</sup>, EAF<sup>-</sup>, *stx*<sup>-</sup>, and serotype O55:H7. In 3 patients, ET 14 isolates were *stx1*<sup>+</sup> *E. coli*; 2 of these isolates expressed O antigen 26. In an additional patient, 4 *eaeA*<sup>+</sup> *stx*<sup>-</sup> ET 14 isolates were serotype O118:H8. ET 15 *E. coli* were recovered from 2 patients and were of serotype O103:H6 (1 patient) and serotypes O25:H2 and O128:H2 (1 patient). However, on API testing, the *E. coli* O128:H2 from this latter patient did not produce ornithine decarboxylase, whereas the O25:H2 isolates did. Furthermore, the *E. coli* O128:H2 was susceptible to all antibiotics tested, while the *E. coli* O25:H2 isolates were resistant to ampicillin, amoxicillin–clavulanic acid, and cefazolin.

**Genetic relationships among clones.** We identified 6 major groups of ETs (designated clone complexes A–F) among the 27 ETs that clustered mainly at genetic distances between 0.30 and 0.35 (figure 2). Complex A was the most diverse, with a clustering distance of 0.58, and was highly divergent from the other *E. coli* clusters. *eaeA*<sup>+</sup> organisms occurred in all of the major complexes; EAF<sup>+</sup> bacteria also occurred in four divergent lineages. The 5 *E. coli* with *stx* genes (group 2) were limited to clone complexes D and E.

**Comparison with diarrheogenic *E. coli* (DEC) clones.** Of the 27 ETs displayed by bacteria described here, 4 ETs were indistinguishable from the allele profiles of specific DEC clones that together account for >70% of organisms examined among isolates of five serogroups associated with EPEC and STEC infections [28]. ETs 7, 12, 13, and 18 were identical to DEC clones 5, 8, 9, and 13, respectively.

The patients whose isolates were in clone complexes D and E had common clinical features: 5 of 6 cluster D patients had acute diarrhea and 3 of 4 cluster E patients had chronic diarrhea. Otherwise, there was no association between allele profiles of isolates and the syndromes in the patients from whom the bacteria were recovered.

## Discussion

Without comparison with an appropriate control group, we cannot assign a definite pathogenic role to the non-O157:H7

**Table 1.** Characteristics of bacteria (EPEC) isolated and the patients from whom they were recovered.

Patient, isolate	Patient characteristics						Notes
	Age (months)	Syndrome	ET	Serotype	Localized adherence*	FAS	
Group 1 ( <i>eaeA</i> <sup>+</sup> <i>stx</i> <sup>-</sup> EAF <sup>-</sup> )							
TB 85 A, B, C, D, E	27	CD	23	O131:HN	+	+	<i>bfpA</i> <sup>+</sup> , <i>C. jejuni</i> also isolated, SNI
TB 96 A	24	BD	10	O75:HN	-	-	
B, C, D, E			20	O156:H8	+	+	
TB 135 A	38	CD	15	O128:H2	+	+	
B, C, D, E			15	O25:H2	+	+	
TB 156 A, B, C, D, E	51	CD	7	O55:H7	+	+	
TB 171 A, B, C, D, E	1	D, V	5	O125:H6	+	+	SNI
TB 182 A, B, C, D, E	14	CD	7	O55:H7	+	+	
TB 183 A, B, C, D, E,	26	D	8	O127:H40	+	+	
TB 204 A, C	22	D, V, F	1	O96:HN	-	-	<i>C. jejuni</i> also isolated
TB 206 A, B, C, D, E	113	BD	13	O26:HN	+	+	FL, <i>E. coli</i> O157:H7 also isolated
TB 209 E	21	CD	27	O15:HN	+	+	
TB 216 A, B, C, E	19	D	14	O118:H8	+	+	SNI
TB 220 B, D	13	CD	18	O153:HN	-	-	<i>C. jejuni</i> also isolated
TB 227 A	10	CD	22	O8:H41	+	-	
B			17	ON:HN	+	-	
C, D, E			24	O86:H34	+	+	<i>bfpA</i> <sup>+</sup>
TB 269 C, D	12	D, F	6	O145:HN	-	-	SNI
TB 280 A, B, C, D, E	19	CD	21	O66:HN	+	+	<i>bfpA</i> <sup>+</sup>
TB 309 A, B, C, D, E	79	CD	16	O128:H2	+	+	
TB 320 A, B, C, D, E	19	BD, V	3	ON:HN	+	+	
TB 342 C	20	CD	11	O115:NM	+	+	
TB 353 E	16	D	19	ON:HN	+	+	
TB 425 B	20	CD	2	ON:HN	-	-	
Group 2 ( <i>eaeA</i> <sup>+</sup> <i>stx</i> <sup>+</sup> EAF <sup>-</sup> )							
TB 154 A, B, C, D	74	D, MA	15	O103:H6	+	+	Stx1, FL
TB 226 A, B, C, D, E	37	BD, F, V	12	O111:HN	+	+	Stx1, Stx2, FL
TB 285 A, C, D, E	135	D	14	O26:H2	+	+	Stx1
TB 334 C	19	D	14	O85:NM	+	+	Stx1
TB 352 A, B, C, D, E	73	CD	14	O26:NM	+	+	Stx1
Group 3 ( <i>eaeA</i> <sup>-</sup> <i>stx</i> <sup>-</sup> EAF <sup>+</sup> )							
TB 278 E	10	CGIB	4	O18:HN	-	-	
TB 311 A, B, C, D	11	D, F	26	O23:H15	-	-	
TB 349 A, C, D, E	11	D, F	9	O12:NM	-	-	
TB 382 B	7	CD, F, V	25	O46:HN	-	-	

NOTE. BD, bloody diarrhea; CD, chronic diarrhea; CGIB, chronic gastrointestinal blood loss anemia; D, acute diarrhea; ET, electrophoretic type; F, fever; FAS, fluorescent actin stain; FL, fecal leukocytes; HN, motile but flagellar antigen-nontypeable; MA, mesenteric adenitis; NM, nonmotile; ON, O antigen-nontypeable; SNI, sorbitol-nonfermenting isolate; Stx, Shiga toxin; V, vomiting.

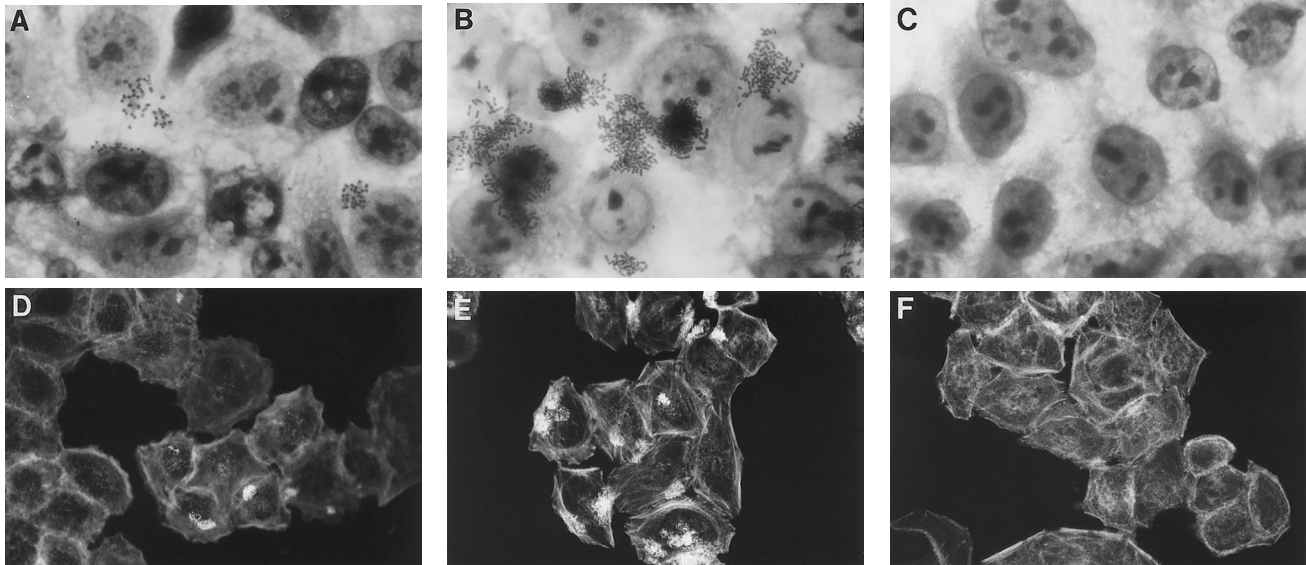
\* Microcolonies of ≥5 bacteria/cluster (+).

*E. coli* identified by probing in this study. Nonetheless, when present, EPEC (with localized adherence to epithelial cells and induction of actin aggregation in the cells to which they adhere) usually constituted the predominant fecal coliform flora, suggesting that such organisms are quite possibly pathogens in Seattle children. Furthermore, many isolates displayed ETs identical to those of classic diarrheogenic EPEC [28].

Of interest, *C. jejuni* or *E. coli* O157:H7 was also identified in the stools of 4 group 1 patients. *C. jejuni* can be shed without symptoms [29], and we do not know if the *C. jejuni* or the simultaneously recovered *eaeA*<sup>+</sup> *E. coli* caused the symptoms

that prompted the stool culture in the patients from whom *C. jejuni* was also recovered. None of the patients excreting *C. jejuni* had bloody diarrhea, unlike the situation in most symptomatic North American *Campylobacter* infections [30], a comparison that raises questions about the significance of the isolation of *C. jejuni* from these patients.

EAF<sup>-</sup> *E. coli* displaying localized adherence, a phenotype of most of the group 1 and 2 bacteria, have been described [11, 31]. Furthermore, EPEC E2348/69 cured of the EAF plasmid retains some ability to cause diarrhea in adult volunteers [32]. It is likely that these organisms possess colonization mechanisms



**Figure 1.** A, *eaeA*<sup>+</sup> *bfpA*<sup>-</sup> *E. coli* isolate TB 216 B adheres to HeLa cells in localized microcolonies. EPEC B171 (B) and *E. coli* HB101 (C) are used as positive and negative controls, respectively. Fluorescent actin stains corresponding to these adherence assays are demonstrated in panels D, E, and F, respectively. Magnifications:  $\times 475$  (A–C),  $\times 395$  (D–F).

separate from the bundle-forming pilus. The pathogenic potential of the nonadherent EAF<sup>+</sup> bacteria remains speculative.

Only 10 group 1 and 2 patients shed organisms expressing “classical” EPEC O antigens (O groups 26, 44, 55, 86, 111, 114, 119, 125, 127, 128, 142, and 158) [32]. This is consistent with other reports that serology neither sensitively nor specifically identifies EPEC [33, 34].

Genetic analysis strongly supports the hypothesis that EPEC belong to a limited number of clones [28, 35], so that similarities among independent isolates, such as O:H serotype, biotype, and major outer membrane protein patterns, result from recent descent from a common ancestral cell [36, 37]. However, EPEC are not all closely related and they do not represent a single evolutionary lineage or clade. To this end, we have demonstrated that EAF and *eaeA* are in a wide variety of chromosomal backgrounds, as reflected in the high diversity among ETs, as was observed in Brazilian *E. coli* O111 isolates [38]. Furthermore, clones separated by genetic distances of only 0.10 can differ in pathogenic genotypes, suggesting that the rate of turnover of EPEC virulence factors, such as the EAF plasmid, exceeds the rate of mutations in genes analyzed in multilocus enzyme electrophoresis.

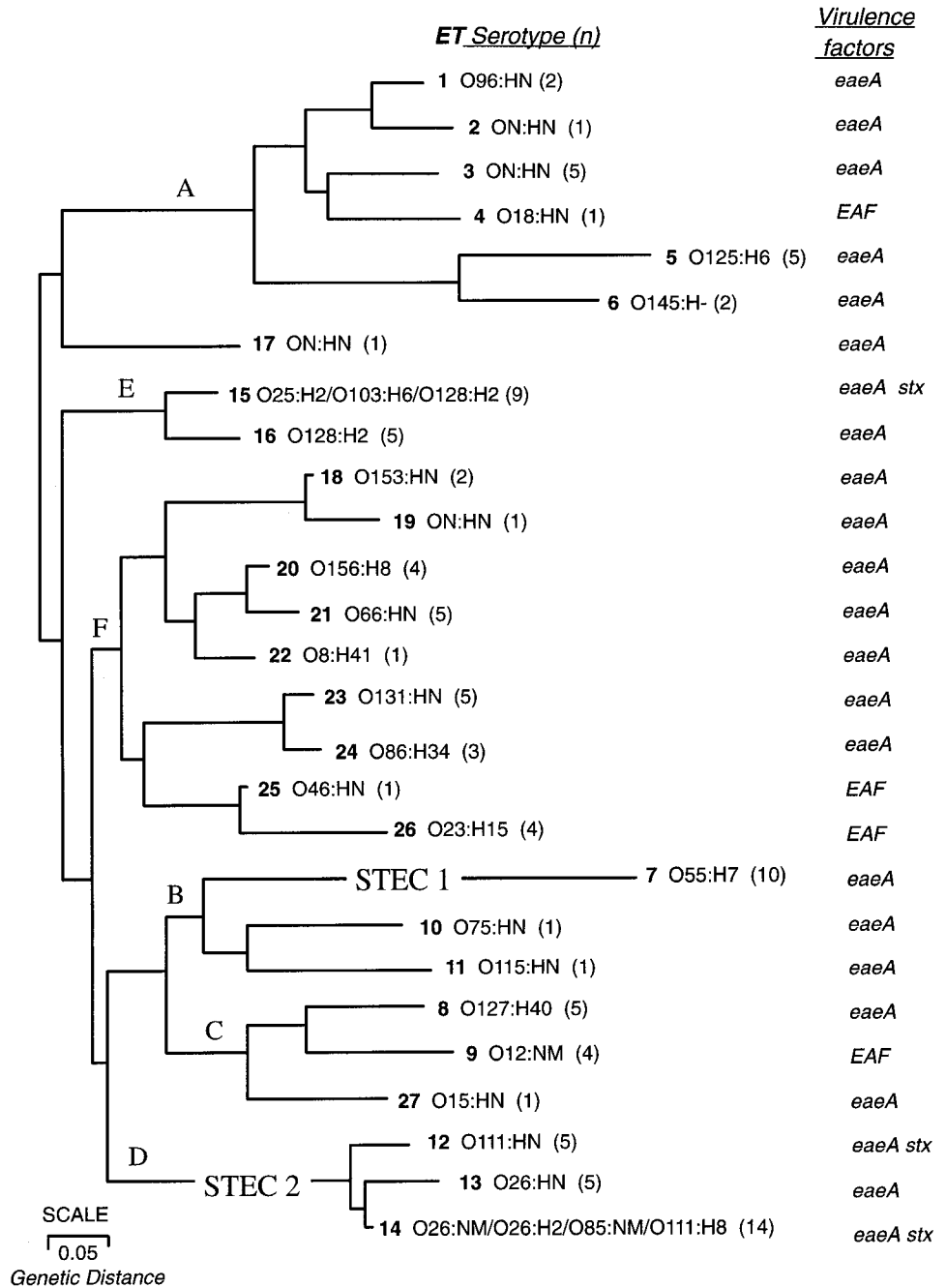
The diversity of lineages in which *eaeA* is found suggests that this gene has been acquired and lost by different clones under natural conditions. *eaeA* is in a large chromosomal segment in *E. coli* O157:H7, termed the locus of enterocyte effacement (LEE) [39]. LEE is integrated 16 bp downstream of *selC* in the *E. coli* O157:H7 and EPEC chromosomes, the site of integration of another large fragment that encodes uropathogenic *E. coli* virulence factors [40]. Furthermore, LEE has an atypically low (for *E. coli*) guanine plus cytosine content of

0.39. Therefore, *E. coli* LEE (and *eaeA*) may have been acquired from a species other than *E. coli* via an unknown mobile genetic element.

This study and our previous analysis [28] indicate that EPEC and pathogenic STEC represent diverse collections of distinct clonal lineages that can be organized into 6 major clusters characterized by similar multilocus enzyme arrays. Cluster A includes classical EPEC serotypes O55:H6 and O127:H6 (E2348/69) [32], which typically possess *eaeA* and the EAF plasmid. This cluster is the most divergent EPEC group. Pathogenic STEC fall into cluster B, which includes *E. coli* O157:H7 (STEC 1 in figure 2), as well as nontoxicogenic *E. coli* O55:H7, and cluster D, which comprises a second complex of pathogenic STEC strains (labeled STEC 2 in figure 2) that usually include serotypes O26:H11 or O111:H8 and display virulence traits similar to those of O157:H7 [41]. The occurrence of *eaeA* in a variety of genotypic contexts suggests that EPEC pathogenesis is determined by different combinations of virulence traits.

The different serotypes and ETs displayed by isolates recovered from patients 96 and 227 and the presence of both *E. coli* O157:H7 and *eaeA*<sup>+</sup> *E. coli* O26:HN in the stool of patient 206 demonstrate that patients can excrete *E. coli* of different lineages possessing the same virulence trait. To our knowledge, the simultaneous isolation from the same patient of *E. coli* of different serotypes with the same virulence traits has been described only twice, in cases in which non-O157:H7 STEC were recovered from stools that also contained *E. coli* O157:H7 [42, 43].

In 1 of our patients, *E. coli* expressing discordant O antigens possessed identical ETs. While multiple serotypes can be found



**Figure 2.** Dendrogram demonstrates inferred relations between each isolate listed in table 1 and probe that initially detected organisms in each electrophoretic type (*stx*<sup>+</sup> isolates are also *eaeA*<sup>+</sup>). Electrophoretic types are grouped into clusters A–F.

within clonally related *E. coli* [44], this is, to our knowledge, the first report of *E. coli* belonging to same ET, but expressing discordant serotypes, being recovered simultaneously from 1 person. The common *eaeA* sequences and identical ET and flagellar antigen suggest that the isolates of discordant O antigens share a common progenitor, raising the possibility of serotype conversion, a process noted in *Vibrio cholerae* [45, 46], *Shigella flexneri* [47], and *Salmonella enterica* serovar borreze [48]. However, the different API scores and antibiotic susceptibilities of the *E. coli* O25:H2 and O128:H2 isolates

argue that these organisms are not closely related, unless the loci responsible for the expression of the O25 antigen and ornithine decarboxylase and resistance to ampicillin, amoxicillin–clavulanic acid, and cefazolin, are linked on the same genetic element, and this element has been lost from the *E. coli* O128:H2 strain.

Our data suggest that *eaeA* (which encodes a critical EPEC virulence factor), and not the EAF probe (which comprises sequences of undetermined importance), should be used to detect putative EPEC. In our study, EAF<sup>+</sup> isolates were nonadher-

ent. In fact, the EAF probe failed to identify the 3 patients whose non-O157:H7 *eaeA*<sup>+</sup> *E. coli* contained *bfpA*, which is on the EAF plasmid.

Because we neither studied an appropriate control group nor administered standardized questionnaires to characterize symptoms, we can neither assign an etiologic role to the isolates reported nor confidently associate clinical syndromes with particular organisms. Nonetheless, we document for the first time that *eaeA*<sup>+</sup>, locally adherent, noncytotoxic *E. coli* that aggregate actin in epithelial cells to which they adhere (defining properties of EPEC) were found in 3.6% of specimens submitted to our microbiology laboratory. This frequency, which excludes the 4 patients with nonadherent *eaeA*<sup>+</sup> *E. coli* and the 5 patients with non-O157:H7 *eaeA*<sup>+</sup> STEC, exceeds the rate of recovery of *E. coli* O157:H7 or *Campylobacter*, *Salmonella*, *Shigella*, or *Yersinia* species. The possibility also exists that our analysis of only 5 random coliform colonies was not sufficiently sensitive to detect *eaeA*<sup>+</sup> organisms if they constituted <20% of the coliform population. In this regard, it should be noted that the *eaeA* probe detected only 9 (64.6%) of the 13 *eaeA*<sup>+</sup> *E. coli* O157:H7 identified by plating stool on sorbitol–MacConkey's agar.

Controlled studies are needed to confirm or refute the hypothesis that the *E. coli* described above are indeed pathogens. Our study should provide a frame of reference regarding ages and clinical syndromes observed in patients shedding such organisms in stool submitted for bacterial analysis. This work should also help delineate the genotypes and phenotypes likely to be of use in future prospective controlled analyses of diarrheal etiology in North America.

#### Acknowledgments

We thank Michael Donnenberg, Ann Jerse, James Kaper, Roger Neill, and John Newland for providing probes used in this study, Beth Bell for statistical advice, Christine Merrikin for secretarial assistance, the Medical Records staff of Children's Hospital and Medical Center for obtaining the pertinent records, and the physicians who requested the stool cultures for providing clinical information.

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