JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1996, p. 689–694 Vol. 34, No. 3
0095-1137/96/$04.00+0


# Characteristics of *Escherichia coli* Strains Belonging to Enteropathogenic *E. coli* Serogroups Isolated in Italy from Children with Diarrhea

ANNA GIAMMANCO,[1]* MARGHERITA MAGGIO,[1] GIOVANNI GIAMMANCO,[1] ROBERTO MORELLI,[2] FABIO MINELLI,[2] FLEMMING SCHEUTZ,[3] AND ALFREDO CAPRIOLI[2]

*Dipartimento di Igiene e Microbiologia, Universitá di Palermo, 90127 Palermo,[1] and Laboratorio di Ultrastrutture, Istituto Superiore di Sanitá, 00161 Rome,[2] Italy, and Department of Clinical Microbiology, The International Escherichia and Klebsiella Centre, Statens Seruminstitut, DK-2300 Copenhagen, Denmark[3]*

Received 11 July 1995/Returned for modification 14 September 1995/Accepted 14 December 1995

**Fifty-five *Escherichia coli* strains belonging to enteropathogenic *E. coli* (EPEC) serogroups were examined for phenotypic and genetic factors associated with virulence. The strains were isolated in Italy from children with diarrhea and identified as EPEC by clinical laboratories using commercially available antisera. O:H serotyping showed that 35 strains (27 of O26, O111, and O128 serogroups) belonged to 11 serotypes considered to be classical EPEC O:H serotypes. The other 20 isolates were classified as 15 nonclassical EPEC O:H serotypes. All the potential EPEC virulence factors associated with bacterial adhesion (localized adherence, fluorescent-actin staining test positivity, presence of the attaching and effacing [*eaeA*] gene), the production of verotoxin, and the positivity with the enterohemorrhagic *E. coli* probe were significantly more frequent among isolates belonging to classical than nonclassical serotypes. Strains displaying an aggregative adhesion and hybridizing with the enteroaggregative DNA probe were found in serogroups O86, O111, and O126. Verotoxin-producing isolates belonged to serogroups O26, O111, and O128. Only one of the isolates hybridized with the EPEC adherence factor (EAF) probe, but 33 strains gave positive results with the *eae* probe, confirming that the former is more suitable in epidemiological studies in European countries. These results indicate that up to 75% of strains identified as EPEC by commercial antisera may possess potential virulence properties and/or belong to classical EPEC O:H serotypes and suggest that O grouping is still a useful diagnostic tool for presumptive identification of diarrheagenic *E. coli* in clinical laboratories.**

Enteropathogenic *Escherichia coli* (EPEC) strains are diarrheagenic *E. coli* belonging to specific O:H serotypes historically associated with outbreaks of infantile diarrhea, particularly during the 1940s and 1950s (7, 21). EPEC do not produce enterotoxins and do not show the shigella-like invasion of epithelial cells (21). Characteristically, they attach in large numbers to the small intestine, causing a localized effacement of the microvilli (7, 21). These attaching and effacing (A/E) lesions are characterized by close adhesion of bacteria to the enterocyte membrane, and they have been observed both in humans with naturally occurring EPEC infections and in experimental infections in rabbits and piglets (7, 21, 40) and, more recently, in human volunteers (8).

The adhesive properties of EPEC have been examined *in vitro* with human duodenal biopsy material (18) and cell cultures (17, 29). In HEp-2 cells, a localized adherence (LA) pattern was clearly differentiated from two other patterns, diffuse adherence (DA) and aggregative adherence (AA) (29). The LA pattern has been observed in many EPEC and enterohemorrhagic *E. coli* (EHEC) strains (40). In cell cultures, bacteria produce a lesion which is very similar to the A/E lesion of enterocytes. In both cases, EPEC and EHEC cause an accumulation of depolymerized actin filaments in apical cytoplasm beneath the sites of adhesion (17, 18, 40). This cytoskeletal alteration can be seen on the fluorescent-actin staining (FAS) test with fluorescein-labeled phalloidin, which has been proposed as a diagnostic test for EPEC and EHEC identification (17).

Large plasmids, referred to as EPEC adherence factor (EAF) plasmids, seem to be necessary for the full expression of LA and the ability of EPEC to cause diarrhea in volunteer feeding experiments (22). A 1-kb DNA probe has been cloned from one of these plasmids and used for the detection of EPEC (28). Also, many EHEC carry large plasmids, and a 3.4-kb fragment has been cloned from an O157:H7 strain and used as a probe to detect plasmids in EHEC (24).

Recent studies have shown that not all EPEC serotypes harbor EAF plasmids and that the EAF-negative EPEC strains also adhere to HEp-2 cells, even if in a weaker pattern, and cause A/E lesions and actin accumulation (19, 26, 35, 36). A chromosomal gene cluster necessary for the production of the A/E lesion has been identified recently and termed *eae* (stands for EPEC attaching and effacing) (7, 9, 16, 21, 40). One of the cluster's genes (*eaeA*) encodes for a 94-kDa outer membrane protein involved in the intimate adhesion of bacteria to the cell membrane and has been called intimin (15). The *eaeA* gene from the EPEC strain E2348/69 (O127:H6) has been sequenced (16), and a 1-kb fragment from within the *eaeA* open reading frame has been used as a DNA probe, showing that homologous sequences are present in other *E. coli* strains which are able to cause A/E lesions *in vitro* and *in vivo*, such as the rabbit pathogenic strain RDEC (4) and the EHEC strains of serotype O157:H7 (4, 7, 21, 40). The nucleotide sequence of the *eaeA* locus of EHEC O157 (44) is similar to that of the corresponding gene of the EPEC strain E2348/69 in the 5′ portion (97% homology in the first 2,200 bases), but it differs in the 3′ region (only 59% of homology in the last 800 bases).

* Corresponding author. Mailing address: Dipartimento di Igiene e Microbiologia, Universitá di Palermo, Via del Vespro 133, 90127 Palermo, Italy.

A considerable heterogeneity within the *eaeA* locus has been reported for EPEC and EHEC strains belonging to different serotypes (11, 25, 34).

Another virulence property that has been associated with EPEC is the production of verotoxin (VT), also known as Shiga-like toxin. Although strains from outbreaks usually did not produce VT, some strains, in particular of serogroups O26 and O111, from sporadic cases of diarrhea or hemolytic-uremic syndrome have been shown to possess this property (5, 6, 37, 38, 42) and are currently classified as EHEC. Therefore, although the mechanism by which EPEC strains evoke a fluid response in the intestine of the human host is still unclear, many virulence-associated factors have been identified in this group of organisms, and some of them have been proposed as possible markers for EPEC identification, in addition to or as replacement for the O serogrouping (17, 23). However, recent studies suggest that not all the virulence factors are evenly distributed in the wild-type EPEC strains circulating in different geographical areas (19, 23, 26, 35, 36, 38). The purpose of this study was to examine a sample of Italian isolates belonging to classical EPEC serogroups for virulence-associated properties. The strains were characterized by determination of their H antigens; hybridization with EAF, *eaeA*, EHEC, enteroaggregative (EAgg), VT1, and VT2 DNA probes; and ability to produce VT. Adherence to HEp-2 cells and the reaction in the FAS test were also examined.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* strains were isolated in Italy between 1987 and 1992 from the feces of infants and children with diarrhea. Five children developed hemolytic-uremic syndrome following a prodrome of bloody diarrhea. In all the other cases diarrhea was watery and lasted no more than 10 days. All strains but those from children with hemolytic-uremic syndrome were presumptively identified as EPEC by several clinical laboratories using commercially available antisera from different companies and sent to the Istituto Superiore di Sanitá for confirmation. The commercial antiserum sets included the following EPEC O groups: O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, and O142. The identification of the isolates was confirmed by the API 20E system (Bio Merieux Italia, Rome, Italy), and O:H serotyping was carried out by standard procedures (30). Cultures were kept frozen at −70°C in 1% (vol/vol) glycerol broth. The assays described below were performed by using working cultures maintained in Dorset egg medium.

**HEp-2 adhesion assay.** The LA, DA, and AA patterns were assessed as previously described (26) with HEp-2 monolayers grown on glass coverslips in 24-well tissue culture plates. The FAS test was performed according to the method of Knutton et al. (17). Monolayers were examined for the presence of fluorescent accumulations of filamentous actin beneath attached bacteria by consecutive incident light fluorescence and phase-contrast microscopy with the same specimen.

**Test for VT production.** Sterile culture supernatants of the bacterial strains grown in Trypticase soy broth were tested for the presence of VT by the Vero cell cytotoxicity assay (5). VTs were identified by seroneutralization assay as previously described (5), using neutralizing antisera to VT1 and VT2 raised in rabbits (5).

**Hybridization with DNA probes.** *E. coli* strains were tested by colony hybridization as described by Willshaw et al. (43). Probe-positive and -negative strains were included as controls on each membrane. The EAF, EHEC, EAgg, and *eaeA* probes were derived from plasmids pJPN16 (28), pCVD419 (24), pCVD432 (3), and pCVD434 (16), respectively (kindly provided by M. M. Levine and J. B. Kaper, Baltimore, Md.). The VT1 and VT2 probes were obtained from plasmids pACyC177.NTP705 and pACyC184.NTP707 (43), respectively (kindly provided by H. Smith, Colindale, London, United Kingdom. The EAgg *E. coli* (EAggEC) heat-stable enterotoxin 1 (EAST1) gene probe was composed of the complete toxin determinant (*astA*) generated by PCR as described by Savarino et al. (33). Plasmid pSS106 (kindly provided by S. J. Savarino, Bethesda, Md.) was used as the template. Probes were separated by gel electrophoresis, extracted from low-melting-point agarose, and labelled with deoxyadenosine 5′-α-($^{35}$P) thiotriphosphate by the random primer method (10). The EHEC probe was labelled with digoxigenin as described by the manufacturer (Boehringer, Mannheim, Germany).

**Analysis of the *eaeA* genes.** Differences in the nucleotide sequence of the *eaeA* determinants were evaluated by PCR by the method of Schmidt et al. (34). Primers LP1 and LP2, based on the *eaeA* gene sequence of the EPEC strain E2348/69 (16), were used to amplify the whole EPEC *eaeA* gene (2,817 bp). Primer LP3, based on the *eaeA* gene sequence of the EHEC strain EDL933 (44), was used in combination with LP1 to amplify the entire EHEC *eaeA* gene (2,802 bp). Bacterial DNA was prepared by incubating 10 μl of bacterial suspension ($10^5$ cells) for 10 min at 95°C. PCR conditions were as described by Schmidt et al. (34). PCR products were examined by agarose gel electrophoresis and restriction enzyme analysis with *Pst*I according to the method of Schmidt et al. (34).

## RESULTS

The *E. coli* strains included in this study fell into 10 O groups, of which O26, O111, and O128 together accounted for 33 (60%) of the 55 isolates examined. Determination of flagellar antigens revealed a total of 26 O:H serotypes, of which 35 strains (27 of O26, O111, and O128 serogroups) belonged to 11 serotypes historically associated with infants with diarrhea and considered to be classical EPEC serotypes (21). The other 20 isolates represented nonclassical EPEC serotypes. Tables 1 and 2 show the classical and nonclassical serotypes found among our *E. coli* strains, according to the presence of the potential virulence properties considered in the study. In summary, 33 of 35 (94%) of the classical EPEC O:H serotypes and 9 of 20 (45%) of the nonclassical serotypes possessed at least one of the potential virulence factors considered in this study. Table 3 summarizes the prevalence of each virulence factor in the two groups of strains. Thirty-three strains hybridized with the *eaeA* probe, 11 hybridized with the EHEC probe, and only one isolate belonging to serotype O119:H$^-$ reacted with the EAF probe. The potential EPEC virulence factors associated with bacterial adhesion (LA, FAS positivity, and presence of the *eaeA* gene), the production of VT, and the positivity with the EHEC probe were significantly more frequent among isolates belonging to classical than nonclassical serotypes. Furthermore, strains without any of the considered virulence factors were significantly more frequent among nonclassical serotypes.

Isolates with the non-EPEC characters DA, AA, and hybridization with the EAgg probe were equally distributed between the two groups. However, 11 of the 35 (31%) classical EPEC isolates of O groups O26, O111, and O128 were positive with the EHEC probe. Eight of the 11 EHEC-positive isolates (73%) produced VT. Strains displaying an AA pattern, which has recently been associated with protracted diarrhea (2, 23, 29), were found in serogroups O86, O111, and O126, and four of five strains hybridized with the EAgg probe. Three of those strains hybridized with the gene probe specific for the heat-stable enterotoxin EAST1 (33). Isolates displaying DA or AA were negative by the FAS test. VTs were produced by 10 strains belonging to serogroups O26, O111, and O128; five of them were from children with hemolytic-uremic syndrome. Hybridization and cytotoxicity neutralization experiments with gene probes and antisera specific for VT1 and VT2 showed that six strains produced VT1, two strains produced VT2, and two strains produced both VT1 and VT2. All the VT-producing isolates hybridized with the *eaeA* probe, all but two hybridized with the EHEC probe, and six of them were also positive for LA and by the FAS test.

Table 4 summarizes the relationships observed between the HEp-2 cell adhesion phenotypes of the isolates and the isolates' reactivity with the related gene probes. All the 22 strains exhibiting a localized pattern of adherence to HEp-2 cells were positive with the *eaeA* probe, and all but two of these strains were also positive by the FAS test.

Interestingly, the *eaeA* gene was also present in 11 of 20 nonadherent bacterial strains but never in strains exhibiting other patterns of adhesion.

To further characterize the *eaeA* determinant of our isolates, *eaeA* probe-positive strains were subjected to PCR analysis

TABLE 1. Patterns of potential virulence properties among *E. coli* strains belonging to classical EPEC serotypes

| Serotype | No. of strains | Potential virulence property[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| O26:H11 | 1 | – | EHEC | *eae* | LA | FAS | VT1 | – | – | – | – |
| O26:H11 | 1 | – | EHEC | *eae* | LA | FAS | – | – | – | – | – |
| O26:H11 | 1 | – | EHEC | *eae* | LA | FAS | – | VT2 | – | – | – |
| O26:H11 | 1 | – | – | *eae* | – | – | – | – | – | – | – |
| O26:H$^-$ | 1 | – | EHEC | *eae* | LA | FAS | – | – | – | – | – |
| O26:H$^-$ | 1 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O26:H$^-$ | 1 | – | EHEC | *eae* | – | – | VT1 | – | – | – | – |
| O26:H$^-$ | 1 | – | EHEC | *eae* | – | – | – | – | – | – | – |
| O26:H$^-$ | 1 | – | – | – | – | – | – | – | – | – | – |
| O55:H7 | 2 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O55:H7 | 1 | – | – | *eae* | – | – | – | – | – | – | – |
| O111:H12 | 1 | – | – | – | – | – | – | – | AA | EAgg | – |
| O111:H12 | 2 | – | – | – | – | – | – | – | – | – | DA |
| O111:H$^-$ | 2 | – | EHEC | *eae* | LA | FAS | VT1 | VT2 | – | – | – |
| O111:H$^-$ | 1 | – | EHEC | *eae* | LA | FAS | VT1 | – | – | – | – |
| O111:H$^-$ | 1 | – | – | *eae* | LA | FAS | VT1 | – | – | – | – |
| O111:H$^-$ | 1 | – | EHEC | *eae* | – | – | VT1 | – | – | – | – |
| O111:H$^-$ | 1 | – | – | *eae* | – | – | VT1 | – | – | – | – |
| O111:H$^-$ | 1 | – | – | – | – | – | – | – | AA | EAgg | – |
| O119:H$^-$ | 1 | EAF | – | *eae* | LA | FAS | – | – | – | – | – |
| O119:H$^-$ | 1 | – | – | *eae* | – | – | – | – | – | – | – |
| O125:H6 | 1 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O125:H6 | 1 | – | – | *eae* | LA | – | – | – | – | – | – |
| O126:H27 | 1 | – | – | – | – | – | – | – | AA | EAgg | – |
| O128:H2 | 3 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O128:H2 | 1 | – | – | *eae* | LA | – | – | – | – | – | – |
| O128:H2 | 1 | – | EHEC | *eae* | – | – | – | VT2 | – | – | – |
| O128:H8 | 1 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O128:H$^-$ | 1 | – | – | *eae* | LA | FAS | – | – | – | – | – |
| O128:H$^-$ | 1 | – | – | – | – | – | – | – | – | – | – |

[a] EAF, EHEC, *eae*, VT1, VT2, EAgg, positive hybridization with the correlated probes. LA, AA, DA, adherence patterns displayed. FAS, positive by FAS test.

with the primer pairs LP1-LP2 and LP1-LP3, which are able to amplify the complete *eaeA* genes of the EPEC and EHEC reference strains E2348/69 (O127:H6) and EDL933 (O157:H7), respectively (34). Nineteen of the 33 *eaeA* probe-positive strains did not react at all with any of the primer pairs. The results of the 14 positive PCR analyses are reported in Table 5. The primer pair LP1-LP2 generated PCR products only with DNA from two isolates of serotype O125:H6. Primers LP1-LP3 produced amplicons of the expected size not only from four of six O111:H$^-$ EHEC strains but also from all the VT-negative isolates of serotypes O55:H7 (three strains), O127:H40 (three strains), O128:H8 (one strain), and O128:H$^-$ (one strain). Restriction enzyme analysis of the PCR products with *Pst*I showed four restriction fragment polymorphism profiles

TABLE 2. Patterns of potential virulence properties among *E. coli* strains belonging to nonclassical EPEC serotypes

| Serotype | No. of strains | Potential virulence property[a] | | | | | |
|---|---|---|---|---|---|---|---|
| O26:H8 | 1 | *eae* | LA | FAS | – | – | – |
| O26:H46 | 1 | – | – | – | – | – | – |
| O55:H21 | 1 | – | – | – | – | – | – |
| O55:H45 | 1 | – | – | – | – | – | DA |
| O86:H2 | 1 | – | – | – | AA | EAgg | – |
| O86:H4 | 1 | – | – | – | – | – | – |
| O86:H18 | 1 | – | – | – | AA | – | – |
| O111:H8 | 1 | – | – | – | – | – | – |
| O114:H4 | 1 | – | – | – | – | – | DA |
| O114:H4 | 1 | – | – | – | – | – | – |
| O125:H30 | 1 | – | – | – | – | – | – |
| O126:H21 | 1 | – | – | – | – | – | – |
| O126:H30 | 1 | – | – | – | – | – | – |
| O127:H21 | 1 | – | – | – | – | – | – |
| O127:H40 | 1 | *eae* | LA | FAS | – | – | – |
| O127:H40 | 2 | *eae* | – | – | – | – | – |
| O128:H35 | 1 | *eae* | – | – | – | – | – |
| O128:H35 | 2 | – | – | – | – | – | – |

[a] *eae*, LA, FAS, AA, EAgg, DA, as defined in Table 1.

TABLE 3. Prevalence of each potential virulence property among *E. coli* strains belonging to classical and nonclassical EPEC serotypes

| Property[a] | No. (%) of positive strains among: | | $P^b$ |
|---|---|---|---|
| | Classical EPEC ($n$ = 35) | Nonclassical EPEC ($n$ = 20) | |
| EAF | 1 (2.8) | 0 | 1.0 |
| EHEC | 11 (31.0) | 0 | 0.004 |
| *eae* | 28 (80.0) | 5 (25.0) | <0.001 |
| LA | 20 (57.1) | 2 (10.0) | <0.001 |
| FAS | 18 (51.4) | 2 (10.0) | 0.003 |
| VT | 10 (28.5) | 0 | 0.009 |
| DA | 2 (5.7) | 2 (10.0) | 0.616 |
| EAgg | 3 (8.7) | 1 (5.0) | 1.0 |
| AA | 3 (8.7) | 2 (10.0) | 1.0 |
| None | 2 (5.6) | 11 (55.0) | <0.001 |

[a] As defined in Table 1. VT, VT production.
[b] Fisher's exact test.

(Fig. 1). Among the amplicons obtained with the primer pair LP1-LP3, the three O55:H7 EPEC isolates had the same pattern as that of the EHEC O157 control strain EDL933 (Fig. 1, lanes 2 and 4), while a different profile was shared by the VTEC O111:H$^-$ isolates and the VT-negative strains O127:H40, O128:H8 (Fig. 1, lanes 3, 5, and 6), and O128:H$^-$ (data not shown). The *Pst*I restriction pattern of the PCR products obtained with LP1-LP2 primers from the DNA of the two O125:H6 isolates was different from that of the EPEC control strain E2348/69 (Fig. 1, lanes 8 and 7, respectively).

## DISCUSSION

Historically, EPEC was defined as a category of *E. coli* belonging to certain serogroups that had been associated with outbreaks of infantile gastroenteritis. Several studies (19, 21, 26, 32, 35, 36, 38) have recently demonstrated that this group of organisms is actually quite heterogeneous in the possession of putative virulence properties. The hallmark of EPEC is considered the ability to cause A/E lesions in the intestine (7, 21), and the presence of the chromosomal *eaeA* gene cluster governing this property has been demonstrated in EPEC strains (7, 15, 16, 21). Another genetic locus associated with LA, the plasmid-encoded EAF, has been used as an epidemiological marker for classical class I EPEC strains associated with outbreaks (28), and it is of significant value in the detection of EPEC in developing countries (7, 23). However, many EPEC strains isolated in Europe that show LA do not possess the EAF marker (19, 26, 35, 36, 38). Similarly, some isolates within a given serogroup produce VT or hybridize with the EHEC probe, while others do not (24, 37, 38, 42). Finally, some strains of serogroups O44, O86, O111, and O126 (20, 26, 32, 36, 39) have been categorized as EAggEC by virtue of their characteristic pattern of adherence to cell cultures and/or their ability to hybridize with the corresponding DNA probe. The present study conducted with EPEC strains isolated in Italy is in broad agreement with the above-mentioned studies. The *eae* gene was indeed the potential virulence factor most frequently present in our strains, but only one of them hybridized with the EAF probe. This indicates that the circulation of EAF plasmids in Italy is as low as, and probably lower than, in the United Kingdom (19, 35, 36, 38) and confirms that the *eae* probe could be more suitable than the EAF probe for use in epidemiological studies in European countries.

According to other studies (5, 6, 24, 37, 38, 42), VT production and presence of EHEC plasmids were quite common among our isolates of serogroups O26, O111, and O128. Seventy-three percent of the EHEC probe-positive isolates produced VT, and only two VTEC strains did not hybridize with the EHEC probe. It has been suggested that the EHEC O157:H7 clonal lineage evolved from the closely related EPEC clone O55:H7 by acquisition of virulence factors such as the phage-encoded VTs or plasmid-encoded adhesins (41). In our study, the only discriminating factors between EPEC and EHEC strains within the O groups O26, O111, and O128 were indeed the ability to produce VT and/or the possession of virulence plasmids as indicated by the EHEC probe. This further supports the view that new pathogens such as the enterohemorrhagic VTEC strains may have emerged from EPEC progenitors already possessing the ability to colonize the human intestine. We also confirmed that some strains of O groups O86, O111, and O126 are EAggEc and showed that

TABLE 4. Relationship between adhesion phenotype and hybridization with related gene probes in *E. coli* strains

| Adhesion phenotype | No. of strains examined | No. of FAS-positive strains | No. of strains positive with probes for: | | | |
|---|---|---|---|---|---|---|
| | | | EAF | EHEC | *eae* | EAgg |
| LA | 22 | 20 | 1 | 7 | 22 | 0 |
| DA | 4 | 0 | 0 | 0 | 0 | 0 |
| AA | 5 | 0 | 0 | 0 | 0 | 4 |
| None | 24 | NT[a] | 0 | 4 | 11 | 0 |

[a] NT, not tested.

TABLE 5. Summary of *eaeA*-PCR positive results with primer pairs LP1-LP2 and LP1-LP3

| Serotype | VT production | PCR amplification of *eaeA* gene with: | | No. of positive strains/ no. tested |
|---|---|---|---|---|
| | | LP1-LP2 | LP1-LP3 | |
| O55:H7 | − | − | + | 3/3 |
| O111:H$^-$ | + | − | + | 4/6 |
| O125:H6 | − | + | − | 2/2 |
| O127:H40 | − | − | + | 3/3 |
| O128:H8 | − | − | + | 1/1 |
| O128:H$^-$ | − | − | + | 1/1 |

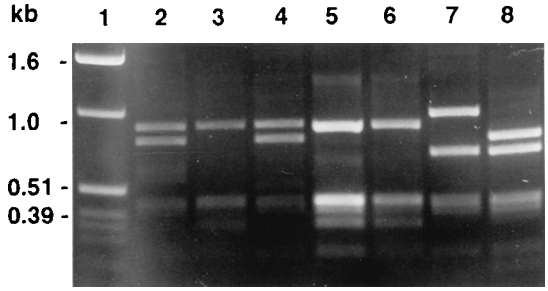


FIG. 1. *Pst*I restriction profiles of the *eaeA* PCR products obtained with DNA from representative *E. coli* strains of different serotypes. The *eaeA* determinants were amplified with primers LP1-LP3 (lanes 2 to 6), which are homologous to the *eaeA* from the EHEC reference strain EDL933 (O157:H7), and LP1-LP2 (lanes 7 and 8), which are homologous to the *eaeA* from EPEC reference strain E2348/69 (O127:H6). These primers generate a ca. 2.8-kb fragment in PCR experiments. The *Pst*I profiles refer to serotypes O157:H7 (lane 2, strain EDL933), O111:H$^-$ (lane 3), O55:H7 (lane 4), O127:H40 (lane 5), O128:H8 (lane 6), O127:H6 (lane 7, strain E2348/69), and O125:H6 (lane 8). Lane 1 contained molecular size markers.

most of them harbor the determinant of the recently described heat-stable enterotoxin EAST1 (33).

The *eae* gene was present in all the LA-, FAS-positive strains but also in 11 isolates that did not attach to HEp-2 cells. It is well known that the EPEC pathogenesis is a multifactorial event requiring an initial stage of bacterial adherence before the intimate adherence to the intestinal mucosa (attaching) and the cytoskeletal disruption (microvillus effacement) can be achieved (7, 21, 40). An important role of EAF plasmids in the initial adhesion of EPEC to intestinal mucosal surface and tissue culture cells has been suggested by several studies. Jerse and coworkers (14, 15) showed that these plasmids display a positive regulatory role in the *eae* gene cluster expression and in the development of both LA and A/E lesions. EAF plasmids also carry the determinants for the inducible bundle-forming pili described by Giron et al. (12), and two additional morphological types of fimbriae expressed by EAF-positive EPEC strains have been recently described (13). However, Scotland et al. (35) showed that the adhesion to HEp-2 cells of LA-positive, EAF-negative strains of *E. coli* O128 was mediated by a large plasmid that was thought to have functions similar to those of the EAF plasmid. Moreover, Cantey and Moseley (4), confirming that the proximity of the bacterial cell to the target cell is necessary for the expression of the *eae* gene cluster, have also shown that any adhesive function which enables cell-to-cell contact is sufficient for *eae* expression. They also hypothesized that while the EAF-mediated adhesin(s) may work in vivo as well as in tissue culture assays, in other *eae*-positive strains such as the rabbit pathogen RDEC-1 or the EHEC O157:H7, the putative adhesin which functions *in vivo* may not function in tissue culture cells. In light of these observations, our *eaeA*-positive, nonadherent strains could possess adhesins capable of recognizing the intestinal mucosal surface but not the cultured cells used in our laboratory assay for adhesion. On the other hand, it is also possible that plasmid-mediated adherence factors, such as the EAF, were lost during the storage or the passages of our strains.

PCR and restriction analysis of the *eaeA* determinants of our EPEC and EHEC strains confirmed that considerable heterogeneity exists within the *eaeA* locus of *E. coli* (11, 25, 34). The gene from the reference EPEC strain E2348/69 (O127:H6) was indeed different from those of strains belonging to most of the classical EPEC serotypes. Similarly, we observed homology between the *eaeA* determinant of the EHEC O157 strain EDL933 and those of four EHEC O111:$H^-$ strains but not with the genes from two other O111:$H^-$ and four O26 EHEC strains. The primer pair based on the sequence of EDL933 generated PCR products also with DNA from VT-negative strains of serotypes O55:H7, O127:H40, O128:H8, and O128:$H^-$. Homologies between the *eaeA* genes of EHEC O157:H7 and EPEC O55:H7 have also been reported by Gannon et al. (11) and Louie et al. (25) and seem to support the hypothesis that these strains share some common evolutionary origin (41).

The present study showed that potential virulence factors (i.e., LA pattern, FAS positivity, presence of the *eaeA* gene, positivity with the EHEC probe, and production of VT) were more common among classical than nonclassical EPEC serotypes. However, we found a few isolates with the *eae* gene also among the nonclassical serotypes. In particular, three of these strains belonged to serotype O127:H40, which was previously reported among strains from children with diarrhea (19, 38). Several authors have discussed the possibility of including new serotypes in the list of EPEC serotypes on the basis of their virulence properties (1, 31). *E. coli* O127:H40 seems to be a candidate EPEC serotype if further studies confirm its involvement in childhood diarrhea.

The observation that many strains belonging to EPEC serogroups are actually devoid of any known virulence factor and that only particular H types within a serogroup are associated with diarrhea have led some investigators to consider O serogrouping an outmoded method for identifying EPEC (27). Most of the 55 strains included in this study were collected in clinical laboratories following a simple identification with EPEC commercial antisera. Nevertheless, 35 (60%) of them belonged to classical EPEC serotypes, and another 7 strains among nonclassical serotypes showed virulence properties such as the possession of the *eae* gene and AA. These results indicate that at least in the Italian settings there is a good probability (up to 75%) that an *E. coli* strain identified as an EPEC strain on the basis of the slide agglutination with commercially available antisera is actually a putative EPEC. So, in our opinion, O serogrouping is still a useful diagnostic tool and remains the simplest bacteriological test for presumptive identification of EPEC and other diarrheagenic *E. coli* in clinical laboratories. Subsequent confirmation of the identity of EPEC strains could be done at reference laboratories and should include H typing as a first choice. In our study, 94% of the classical EPEC O:H serotypes possessed putative virulence factors, compared with only 45% of the nonclassical serotypes. The second-choice assays seem to be examination for the *eaeA* gene or the cell adhesion assays, which in our hands were significantly associated with classical EPEC serotypes. Furthermore, if further characterization of presumptive EPEC isolates at reference laboratories includes VT production, this would greatly improve the surveillance of non-O157 VTEC infections, since in this study VTEC constituted 18% of the examined strains.

## ACKNOWLEDGMENTS

This work was partially supported by Consiglio Nazionale delle Ricerche, grants 91.00543.CT04, 93.00234.CT04, and 94.02706.CT04.

## REFERENCES

1. **Albert, M. J., K. Alam, M. Ansaruzzaman, J. Montanaro, M. Islam, S. M. Faruque, K. Haider, K. Bettelheim, and S. Tzipori.** 1991. Localized adherence and attaching-effacing properties of nonenteropathogenic serotypes of *Escherichia coli*. Infect. Immun. **59:**1864–1868.
2. **Baudry, B., S. J. Savarino, P. Vial, J. B. Kaper, and M. M. Levine.** 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrhoeal pathogen. J. Infect. Dis. **161:**1249–1251.
3. **Bhan, M. K., P. Ray, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal.** 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J. Infect. Dis. **159:**1061–1064.
4. **Cantey, J. R., and S. L. Moseley.** 1991. HeLa cell adherence, actin aggregation, and invasion by nonenteropathogenic *Escherichia coli* possessing the *eae* gene. Infect. Immun. **59:**3924–3929.
5. **Caprioli, A., I. Luzzi, F. Rosmini, P. Pasquini, R. Cirrincione, A. Gianviti, M. C. Matteucci, G. Rizzoni, and the HUS Italian Study Group.** 1992. Hemolytic-uremic syndrome and vero cytotoxin-producing *Escherichia coli* infection in Italy. J. Infect. Dis. **166:**154–158.
6. **Caprioli, A., I. Luzzi, F. Rosmini, C. Resti, A. Edefonti, F. Perfumo, C. Farina, A. Goglio, A. Gianviti, and G. Rizzoni.** 1994. Communitywide outbreak of hemolytic-uremic syndrome associated with non-O157 verocytotoxin-producing *Escherichia coli*. J. Infect. Dis. **169:**208–211.
7. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
8. **Donnenberg, M. S., C. O. Tacket, S. P. James, G. Losonsky, J. P. Nataro, S. S. Wasserman, J. B. Kaper, and M. M. Levine.** 1993. Role of eaeA gene in experimental enteropathogenic *Escherichia coli* infection. J. Clin. Invest. **92:**1412–1417.
9. **Donnenberg, M. S., J. Yu, and J. B. Kaper.** 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. **175:**4670–4680.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
11. **Gannon, V. P. J., M. Rashed, R. K. King, and E. J. Golsteyn Thomas.** 1993. Detection and characterization of the *eae* gene of Shiga-like toxin-producing

*Escherichia coli* using polymerase chain reaction. J. Clin. Microbiol. **31:**1268–1274.
12. **Giron, J. A., A. S. Y. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
13. **Giron, J. A., A. S. Y. Ho, and G. K. Schoolnik.** 1993. Characterization of fimbriae produced by enteropathogenic *Escherichia coli*. J. Bacteriol. **175:** 7391–7403.
14. **Jerse, A. E., K. G. Gicquelais, and J. B. Kaper.** 1991. Plasmid and chromosomal elements involved in the pathogenesis of attaching and effacing *Escherichia coli*. Infect. Immun. **59:**3869–3875.
15. **Jerse, A. E., and J. B. Kaper.** 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. **59:**4302–4309.
16. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
17. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
18. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55:**69–77.
19. **Knutton, S., A. D. Phillips, H. R. Smith, R. J. Gross, R. Shaw, P. Watson, and E. Price.** 1991. Screening for enteropathogenic *Escherichia coli* in infants with diarrhea by the fluorescent-actin staining test. Infect. Immun. **59:**365–371.
20. **Knutton, S., R. K. Shaw, M. K. Bhan, H. R. Smith, M. M. McConnell, T. Cheasty, P. H. Williams, and T. J. Baldwin.** 1992. Ability of enteroaggregative *Escherichia coli* strains to adhere in vitro to human intestinal mucosa. Infect. Immun. **60:**2083–2091.
21. **Law, D.** 1994. Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. Clin. Microbiol. Rev. **7:**152–173.
22. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152:**550–559.
23. **Levine, M. M., V. Prado, R. Robins-Browne, H. Lior, J. B. Kaper, S. L. Moseley, K. Gicquelas, J. P. Nataro, P. Vial, and B. Tall.** 1988. Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. J. Infect. Dis. **158:**224–228.
24. **Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, J. Nataro, H. Karch, and I. K. Wachsmuth.** 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. **156:**175–182.
25. **Louie, M., J. De Azavedo, R. Clarke, A. Borczyk, H. Lior, M. Richter, and J. Brunton.** 1994. Sequence heterogeneity of the eae gene and detection of ve-rotoxin-producing *Escherichia coli* using serotype-specific primers. Epidemiol. Infect. **112:**449–461.
26. **Morelli, R., L. Baldassarri, V. Falbo, G. Donelli, and A. Caprioli.** 1994. Detection of enteroadherent *Escherichia coli* associated with diarrhoea in Italy. J. Med. Microbiol. **41:**399–404.
27. **Morris, K. J., and G. Gopal Rao.** 1992. Conventional screening for enteropathogenic *Escherichia coli* in the UK. Is it appropriate or necessary? J. Hosp. Infect. **21:**163–167.
28. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor in enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152:**560–565.
29. **Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. A. Vial, and M. M. Levine.** 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. J. Pediatr. Infect. Dis. **6:**829–831.
30. **Ørskov, F., and I. Ørskov.** 1984. Serotyping of *Escherichia coli*. p. 43–112. *In* T. Bergan (ed.), Methods in microbiology, vol. 14. Academic Press, Inc. Ltd., London.
31. **Pedroso, M. Z., E. Freymüller, L. R. Trabulsi, and T. A. T. Gomes.** 1993. Attaching-effacing lesions and intracellular penetration in HeLa cells and human duodenal mucosa by two *Escherichia coli* strains not belonging to the classical enteropathogenic *E. coli* serogroups. Infect. Immun. **61:**1152–1156.
32. **Robins-Browne, R. M., W. C. Yam, L. E. O'Gorman, and K. A. Bettelheim.** 1993. Examination of arche-typal strains of enteropathogenic *Escherichia coli* for properties associated with bacterial virulence. J. Med. Microbiol. **38:**222–226.
33. **Savarino, S. J., A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry.** 1993. Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. Proc. Natl. Acad. Sci. USA **90:**3093–3097.
34. **Schmidt, H., H. Russman, and H. Karch.** 1993. Virulence determinants in nontoxigenic *Escherichia coli* O157 strains that cause infantile diarrhea. Infect. Immun. **61:**4894–4898.
35. **Scotland, S. M., H. R. Smith, and B. Rowe.** 1991. *Escherichia coli* O128 strains from infants with diarrhea commonly show localized adhesion and positivity in the fluorescent-actin staining test but do not hybridize with an enteropathogenic *E. coli* adherence factor probe. Infect. Immun. **59:**1569–1571.
36. **Scotland, S. M., H. R. Smith, B. Said, G. A. Willshaw, T. Cheasty, and B. Rowe.** 1991. Identification of enteropathogenic *Escherichia coli* isolated in Britain as enteroaggregative or as members of a subclass of attaching-and-effacing *E. coli* not hybridising with the EPEC adherence-factor probe. J. Med. Microbiol. **35:**278–283.
37. **Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe.** 1990. Properties of strains of *Escherichia coli* O26:H11 in relation to their enteropathogenic or enterohemorrhagic classification. J. Infect. Dis. **162:**1069–1074.
38. **Smith, H. R., S. M. Scotland, N. Stokes, and B. Rowe.** 1990. Examination of strains belonging to enteropathogenic *Escherichia coli* serogroups for genes encoding EPEC adherence factor and vero cytotoxins. J. Med. Microbiol. **31:**235–240.
39. **Smith, H. R., S. M. Scotland, G. A. Willshaw, B. Rowe, A. Cravioto, and C. Eslava.** 1994. Isolates of *Escherichia coli* O44:H18 of diverse origin are enteroaggregative. J. Infect. Dis. **170:**1610–1613.
40. **Tesh, V. L., and A. D. O'Brien.** 1992. Adherence and colonization mechanisms of enteropathogenic and enterohaemorragic *Escherichia coli*. Microb. Pathog. **12:**245–254.
41. **Whittam, T. S., M. L. Wolfe, I. K. Wachsmuth, F. Ørskov, I. Ørskov, and R. A. Wilson.** 1993. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. Infect. Immun. **61:**1619–1629.
42. **Willshaw, G. A., S. M. Scotland, H. R. Smith, and B. Rowe.** 1992. Properties of vero cytotoxin-producing *Escherichia coli* of human origin of O serogroups other than O157. J. Infect. Dis. **166:**797–802.
43. **Willshaw, G. A., H. R. Smith, S. M. Scotland, A. M. Field, and B. Rowe.** 1987. Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probe. J. Gen. Microbiol. **133:**1309–1317.
44. **Yu, J., and J. B. Kaper.** 1992. Cloning and characterization of the eae gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **6:**411–417.