Escherichia coli Strains Involved in Diarrhea in France: High Prevalence and Heterogeneity of Diffusely Adhering Strains

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Two hundred sixty-two strains of Escherichia coli isolated from diarrheal stool specimens from infants, children, and adults hospitalized in Clermont-Ferrand, France, were studied to classify them in the previously described pathogenic groups of E. coli involved in diarrheal diseases. A total of 1.5% of them belonged to the enterotoxigenic E. coli pathotype, but none belonged to the enteroinvasive E. coli, enterohemorrhagic E. coli, or enteropathogenic E. coli pathotypes. Seventeen strains (6.5%) exhibited an aggregative pattern of adhesion to HEp-2 cells (EAggEC pathotype), but of these, three (17.6%) did not hybridize with the EAggEC DNA probe. Most of the strains involved in diarrhea belonged to the diffusely adhering E. coli group; 100 strains (38.2%) exhibited a diffuse adhesion (DA) to HEp-2 cells. Only eight strains (8.9%) from controls diffusely adhered to HEp-2 cells. The highly significant difference (P < 0.0001) between DA strains from patients and from controls suggests that the diffusely adhering E. coli strains should be considered pathogens. Only 33 of them (33%) hybridized with the previously described DA DNA probe, and only 2 (2%) hybridized with the AIDA DNA probe. Four different major proteins were observed in the bacterial surface extracts of the 33 strains positive with the DA DNA probe. In addition, 16 strains that diffusely adhered to HEp-2 cells induced a cytotoxic effect on HEp-2 cells that was characterized by pyknosis and lysis of the cytoplasmic membrane. This cytotoxic effect was correlated with the synthesis of a hemolysin. The genes involved in diffuse adhesion to HEp-2 cells were located on conjugative R plasmids in strains that did not hybridize with the DA or AIDA DNA probes.

Escherichia coli strains associated with diarrheal disease have been divided into the following five major categories on the basis of pathogenic mechanisms: enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIEC), enterohemorrhagic E. coli (EHEC), and enteroaggregative E. coli (EAggEC) (for a review, see reference 24). Recently, a sixth category, termed diffusely adhering E. coli (DAEC), was identified as a putative cause of diarrhea in children in Mexico (15).

The EPEC, EAggEC, and DAEC strains are also characterized by their ability to produce distinct patterns of adherence to cultured epithelial cells in vitro. With HEp-2 or HeLa cells, three patterns of adherence can be discerned: localized, aggregative, and diffuse. Localized adherence (LA), which is often associated with the EPEC serotypes, involves clusters of bacteria that adhere to localized regions of HEp-2 or HeLa cells (8, 33). Enteroaggregative adherence is characterized by clumps of bacteria with a "stacked brick" appearance; these bacteria are attached to the surface of cultured epithelial cells and have exposed areas of the glass slide between epithelial cells. In diffuse adhesion (DA), E. coli organisms adhere over the entire surface of the cells.

The identification of diarrheagenic E. coli has been greatly facilitated in the past few years by the development of specific DNA probes. ETEC strains can be detected with two DNA probes specific for heat-labile (LT) and heat-stable (ST; STaII) enterotoxins (28, 35). For the identification of EIEC strains, an oligonucleotide probe specific for the invasive-associated locus (ial) was described by Frankel et al. (14). In EPEC strains, localized adhesion (LA) is correlated with the presence of an EPEC adherence factor (EAF) plasmid (1). A genetic locus associated with the EAF plasmid has yielded a specific DNA probe for the identification of localized adhering E. coli strains (29). Associated with the LA phenotype is the induction of the attaching and effacing lesions. A DNA probe derived from the chromosomal gene called eae (for E. coli attaching and effacing) has been developed and allows detection of EPEC and EHEC strains (19). EAggEC strains possess a plasmid of about 60 Mda, which is necessary for the expression of the aggregative phenotype. A 1-kb DNA fragment derived from this 60-MDa plasmid has been used as a probe to identify EAggEC strains (2). Recently, two DNA probes specific for DAEC strains have been described. Bilge et al. (4) characterized a 14.3-kDa chromosomally encoded adhesin, termed F1845, and developed an intragenic 1-kb DNA probe (4). Benz and Schmidt (3) described a plasmid-encoded outer membrane protein of 100 kDa, termed AIDA-I, which is involved in the DA phenotype. A cloned 6-kb DNA fragment from this plasmid, which was shown to be sufficient for the expression of DA phenotype, has been used as a specific DNA probe (3).

We studied 262 E. coli organisms isolated from 262 diarrheal stool specimens from infants, children, and adults hospitalized in Clermont-Ferrand, France. Our aim was to classify these strains into the pathogenic E. coli groups using specific DNA probes and tests of in vitro adhesion to epithelial cells. We found a high incidence of DAEC strains and observed that R plasmids were involved in the expression of the DA phenotype.

MATERIALS AND METHODS

Bacterial strains. During the year 1990, we selected 262 E. coli strains isolated from aqueous diarrheal stool specimens...
from 262 different patients; *E. coli* was the only diarrhea-causing agent isolated from the specimens. We excluded stool specimens containing the following other pathogens: bacteria (*Salmonella*, *Shigella*, and *Campylobacter* spp.) and parasites (*Giardia lamblia*, *Candida albicans*). The presence of rotaviruses and adenoviruses in the stools of children (less than 15 years old) was systematically determined, and positive samples were excluded from the present study.

We selected 90 *E. coli* strains from stool specimens from nonhospitalized healthy individuals as controls. These subjects, who were of European origin, belonged to the same age groups as the patients and lived in the same urban area, and their stool samples were obtained during the same period of time.

*E. coli* C1845, which produces the F1845 DA adhesin, was used as the reference strain (4). *E. coli* WAM589, which harbors the recombinant plasmid pWAM04, was used as a positive hemolytic control strain (39). The strains used as positive controls in the colony hybridization assay are listed in Table 1. *E. coli* K-12 C600 was used as the negative control strain in the colony hybridization assay and as the recipient strain in mating experiments.

**Serotyping.** For the detection of O serogroups, bacterial cells were heated to 100°C and were then tested for glass slide agglutination by standard methods (32). We used the following antisera: O55, O86, O114, O119, O125, O126, O127, O128, and O142, which are specific for *EPEC*, and O157, O26, and O111, which are specific for EHEC. All but one of the antiserum samples were purchased from Diagnostics Pasteur (Marnes-la-Coquette, France); O157 antiserum was purchased from Difco Laboratories.

**Hemagglutination tests.** The strains were tested for agglutination of human (type A +) and rat erythrocytes by the method of Evans et al. (12).

**Adhesion test to HEP-2 and Caco-2 cell lines.** Two cultured human cell lines were used. HEP-2 cells, derived from a human larynx carcinoma, were obtained from Flow Laboratories, Inc. (McLean, Va.). Caco-2 cells, from a human colon carcinoma (13), were kindly provided by A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Unité 178, Villejuif, France). The adhesion of *E. coli* to HEP-2 cells was examined by a modification of the HEP-2 cell assay of Cravioto et al. (8), and the adhesion to Caco-2 cells was performed as described previously (9). Briefly, monolayers of HEP-2 cells were grown to 50% confluence, whereas Caco-2 cells were used at postconfluence, after 15 days of culture. Before the adhesion test, the cells were washed with phosphate-buffered saline (PBS; pH 7.2). A suspension of about 10⁸ bacteria per ml in the cell line culture medium containing 1% (wt/vol) d-mannose was added to the tissue culture, and the culture was incubated for 3 h at 37°C. After three washes, the cells were fixed in methanol, stained with 20% Giemsa, and examined microscopically under oil immersion. An adhesion index representing the average number of bacteria per cell was determined by examining 100 cells.

**Hemolysin assay.** Columbia plates (Biomérieux, Marcy l’Étoile, France) containing 5% PBS-washed sheep erythrocytes were used to screen for bacterial hemolytic activity. Plates were seeded with bacterial strains and observed for lysis of erythrocytes after 24 h of growth at 37°C.

**DNA probes and colony hybridization.** The specific DNA probes, either fragments extracted from recombinant plasmids or oligonucleotides, used in the present study are listed in Table 1. Colony hybridization was performed with 50% formamide as described by Mass (26). Optimal hybridization temperatures were determined for each probe by using positive and negative controls. The heat-denatured probes were used at a concentration of 10⁶ dpm/ml and were incubated overnight with gentle agitation. After washings with twice-concentrated 1× SSC (1× SSC is 0.15% NaCl plus 15 mM sodium citrate), the filters were exposed to X-Omat-AR X-ray film (Kodak, Rochester, N.Y.) for 12 h at −80°C. The films were developed according to the manufacturer’s instructions.

**Preparation of plasmid DNA and agarose gel electrophoresis.** Plasmid DNAs were extracted from wild-type strains and from their transconjugants by the rapid procedure of Kado

### Table 1. Sources and relevant characteristics of DNA probes

<table>
<thead>
<tr>
<th>E. coli group</th>
<th>DNA probe specific for:</th>
<th>E. coli strain used as a positive control</th>
<th>Plasmid</th>
<th>Restriction endonuclease</th>
<th>Probe and probe size</th>
<th>Hybridization temp (°C)</th>
<th>Washing temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td>Enterotoxin LT</td>
<td>H10407</td>
<td>pEW2D99</td>
<td>HindIII</td>
<td>850 bp</td>
<td>42</td>
<td>42</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Enterotoxin STaI</td>
<td>H10407</td>
<td></td>
<td>Synthetic StNII probe (30 bases)</td>
<td></td>
<td>65</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>EPEC</td>
<td>eae gene</td>
<td>2348169</td>
<td>pCDV434</td>
<td>SalI-KpnI</td>
<td>1,000 bp</td>
<td>37</td>
<td>65</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>EAF plasmid</td>
<td>K12 C600/ pJPN16</td>
<td></td>
<td>BamHI-SalI</td>
<td>1,000 bp</td>
<td>42</td>
<td>42</td>
<td>29</td>
</tr>
<tr>
<td>EAggEC</td>
<td>Enteragggregative adhesin</td>
<td>17-2</td>
<td>pCDV432</td>
<td>EcoRI-PstI</td>
<td>1,000 bp</td>
<td>42</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td>EIEC</td>
<td>Invasive associated locus</td>
<td>Hb101/ pH54108</td>
<td></td>
<td>Synthetic iai probe (21 bases)</td>
<td></td>
<td>42</td>
<td>42</td>
<td>14</td>
</tr>
<tr>
<td>DAEC</td>
<td>F1845 daac gene</td>
<td>C1845</td>
<td>pSLM852</td>
<td>Psrl</td>
<td>390 bp</td>
<td>37</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>DAEC</td>
<td>AIDA-1 adhesin</td>
<td>K12 C600/ pIB6</td>
<td>pIB6</td>
<td>EcoRI</td>
<td>450 bp</td>
<td>42</td>
<td>50</td>
<td>3</td>
</tr>
</tbody>
</table>
and Liu (20) and were electrophoresed in a 0.7% agarose gel with reference plasmid DNA of known molecular size.

**Transfer of R plasmids.** Conjugation experiments were carried out as described previously (23). The plasmids from wild-type strains were transferred to mutants of *E. coli* K-12 C600 resistant to nalidixic acid. Transconjugants were selected on Mueller-Hinton agar containing nalidixic acid (150 mg/liter) and ampicillin (20 mg/liter).

**Extraction of bacterial surface proteins.** The extraction of bacterial surface proteins was performed essentially as described by Stirm et al. (36).

**Electrophoretic study.** The molecular weights of bacterial surface proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (22), in 15% acrylamide gels against standard molecular size markers (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analysis. The data were analyzed by the chi-square test unless the variables needed a two-tailed Fisher exact test. A *P* value of less than or equal to 0.05 was considered statistically significant (40).

**RESULTS**

The abilities of the *E. coli* strains to adhere to HEp-2 and Caco-2 cells and the relationship between hybridization with DNA probes and adhesion patterns are given in Tables 2 and 3, respectively. The optimal hybridization conditions were determined for each DNA probe and are listed in Table 1.

**Occurrence of ETEC, EIEC, and EHEC strains.** LT and STaII DNA probes were used for the detection of ETEC strains in colony hybridization assays. None of the 90 *E. coli* control strains hybridized with these probes, and of the 262 strains from diarrheal stools, only 4 hybridized: 3 (1.1%) with the LT probe and 1 (0.4%) with the STaII probe (Fig. 1). No strain from our collection hybridized with the *ial* probe specific for EIEC. The occurrence of EHEC was determined by hybridization with the *eae* probe (19) and by slide agglutination with O157, O26, and O111 antisera. Nine strains (3.4%) hybridized with the *eae* probe, but none was agglutinated by the three anti-serum samples tested.

**Occurrence of EAggEC strains.** Fifteen of the 262 strains tested (5.7%) and 1 of the 90 strains from controls (1.1%) hybridized with the EAggEC DNA probe. An aggregative pattern of adhesion to HEp-2 cells was found with 17 *E. coli* strains, including 14 that hybridized with the EAggEC probe (Table 3). Three distinct aggregative patterns were observed: bacterial adhesion to the cells only but not to the glass slide, to the glass only, or to both the cells and the glass. The enterogaugregative phenotype always corresponded to an aggregation of 10 to 50 bacteria per clump, and the adhesions of the 17 strains were uniformly distributed among the three adhesion patterns.

A high level of correlation between adhesion to HEp-2 cells and to Caco-2 cells was observed for EAggEC strains. Fourteen of the 17 EAggEC strains also adhered to Caco-2 cells (Table 2). However, the aggregative adhesion pattern was difficult to determine with Caco-2 cells because the confluency was too large after 15 days of culture.

One nonadherent strain gave a positive result in the hybridization assay with both the EAggEC probe and the *eae* probe specific for EPEC and EHEC strains (Table 3). Another strain hybridized with both the EAggEC and DA probes.

Mannose-resistant hemagglutination of both human and rat erythrocytes was observed with 10 strains; 5 strains agglutinated only human A* erythrocytes and no strain agglutinated rat erythrocytes only (Table 2).

**Analysis of the strains giving localized adhesion and/or hybridizing with the *eae* probe.** Localized adhesion was observed with both HEp-2 and Caco-2 cells for 12 (4.6%) of the 262 strains tested (Table 2) but for none from controls. Differences in the levels of adhesion were observed in these 12 LA strains. Ten strains adhered strongly (more than 40% of HEp-2 cells presented clumps of at least 20 to 50 bacteria), and two adhered moderately (from 20 to 30% of HEp-2 cells

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**TABLE 2. Adhesion abilities of different pathotypes to HEp-2 and Caco-2 cells and erythrocytes**

<table>
<thead>
<tr>
<th><em>E. coli</em> pathotype or adhesion pattern</th>
<th>No. of strains adherent to:</th>
<th>HEp-2</th>
<th>Caco-2</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td></td>
<td>1a</td>
<td>3b</td>
<td>0</td>
<td>NDc</td>
</tr>
<tr>
<td>EAggEC</td>
<td></td>
<td>17d</td>
<td>14</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>LA</td>
<td></td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>100f</td>
<td>80</td>
<td>24</td>
<td>ND</td>
</tr>
</tbody>
</table>

* This ETEC strain hybridized only with the LT probe.
* Two strains were positive with the LT probe and one was positive with the STaII probe.
* ND, not determined.
* Five strains adhered to HEp-2 cells with both an aggregative and a DA pattern.

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**TABLE 3. Relationship between hybridization with DNA probes and adhesion pattern on HEp-2 cells of diarrheagenic *E. coli***

<table>
<thead>
<tr>
<th>Adhesion pattern (no. of strains)</th>
<th><em>eae</em></th>
<th>EAF</th>
<th>EAggEC</th>
<th>DA</th>
<th>AIDA</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized (12)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Aggregative (17)</td>
<td>0</td>
<td>0</td>
<td>14a</td>
<td>5e</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse (100)</td>
<td>1b</td>
<td>0</td>
<td>5e</td>
<td>33a,b</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>Nonadherent (133)</td>
<td>6c</td>
<td>0</td>
<td>1f</td>
<td>10</td>
<td>1</td>
<td>116</td>
</tr>
</tbody>
</table>

* Five *E. coli* strains hybridized with both EAggEC and DA probes and presented both EAgg and DA patterns of adhesion to HEp-2 cells.
* One strain hybridized with both the *eae* and DA DNA probes.
* One strain hybridized with both the *eae* and EAggEC DNA probes.

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**FIG. 1. Percentage of *E. coli* strains isolated from patients' diarrheal stools and from controls that hybridized with DNA probes.** The different DNA probes used are listed in Table 1. Statistical analysis of the percentage of strains in patients and controls was as follows: hybridization with the STaII, LT, *ial*, EAF, *eae*, enterogaugregative, and AIDA probes, *P* > 0.05; hybridization with the DA probe, *P* < 0.001.
had clumps of about 15 bacteria each). None of the 12 LA strains was agglutinated by the EPEC O-serogroup antisera, none hybridized with the EAF probe, and only two hybridized with the eae probe (Table 3). In addition, seven strains without the LA phenotype hybridized with the eae probe.

**Analysis of strains giving diffuse adhesion. (i) Occurrence.** Diffuse adhesion to HEP-2 cells was observed for 100 strains (38.2%) isolated from diarrheal stool specimens (Table 2) and for 8 strains isolated from controls (8.9%). Statistical analysis of the occurrence of DA strains showed a highly significant difference (P < 0.0001) between cases and controls. Twenty-four of the 100 DAEC strains (24%) agglutinated the human A⁺ erythrocytes and 80 strains also adhered to Caco-2 cells (Table 2). Differences in the adhesion levels to HEP-2 cells were noted between *E. coli* strains isolated from diarrheal stool specimens and those from controls. The DA strains isolated from patients adhered to 40 to 80% of HEP-2 cells, with a mean number of 15 bacteria per cell. The eight strains isolated from controls showed a DA pattern, with a mean number of seven bacteria per cell adhering to 20 to 40% of cells.

Of the 100 strains that diffusely adhered to HEP-2 cells, only 33 (33%) hybridized with the DA probe and only 2 (2%) hybridized with the AIDA probe (Table 3). Therefore, 65 adhering strains (65%) did not hybridize with either of the two previously specific DNA probes. In contrast, 10 strains that hybridized with the DA probe and one strain that was positive with the AIDA probe were nonadherent. In addition, of the adherent strains, one was positive with both the DA and the eae probes (DA⁺ eae⁺) and five were positive with both the DA and the EAggEC probes (DA⁺ EAggEC⁺).

(ii) **Bacterial surface proteins.** Four different electrophoretic patterns were obtained when analyzing surface proteins extracted from the 33 adhering strains that were positive with the DA probe (Fig. 2). Eight strains harbored a major surface protein which had the same apparent molecular size as that of F1845, the major surface protein of *E. coli* C1845 (from which the DA probe was constructed). In our hands, the molecular size of the F1845 subunit was estimated to be 15.0 kDa instead of 14.3 kDa (4). Identification of the F1845 subunit in the cell surface extracts was confirmed by immunoblotting with a specific antiserum raised against F1845 (17). SDS-PAGE of the protein extracts of the 25 remaining strains displayed the following patterns: 4 strains harbored a major surface protein of 14.8 kDa, 13 strains harbored a major surface protein of 15.5 kDa, and 8 strains harbored a major surface protein of 16.0 kDa (Fig. 2). These major surface proteins were different from F1845, as determined by immunoblotting with antiserum raised against F1845 (17).

(iii) **Cytotoxic effect on HEP-2 cells.** A cytotoxic effect on the HEP-2 cell monolayer (Fig. 3) was observed after incubation with several DA strains, 14 from patients and 2 from controls. After a 3-h incubation, optical microscopy showed a pyknosis of the nucleus and a destruction of the cytoplasmic membranes. After 4 h of incubation, a 60 to 90% detachment of the monolayer was observed. All the strains responsible for these cellular modifications were hemolysin producers, as detected on sheep blood agar plates. When adhesion to HEP-2 cells was performed with *E. coli* WAM589, the *E. coli* strain harboring the recombinant plasmid pWAM04 encoding the *E. coli* hemolysin (39), the same cytotoxic effects were observed.

(iv) **R plasmids are involved in expression of the DA phenotype.** Conjugal experiments were done on two *E. coli* strains, CF228 and CF266, which diffusely adhered to HEP-2 cells and which did not hybridize to either the DA or the AIDA probe. *E. coli* CF228 was resistant to β-lactams, chloramphenicol, and sulfonamides, and *E. coli* CF266 was resistant to β-lactams, streptomycin, and sulfonamides. They both carried a plasmid of about 110 kb. After mating between *E. coli* CF228 or CF266 and the recipient strain, *E. coli* K-12 C600, transconjugants were selected on agar medium containing ampicillin. All the transconjugants harbored a 110-kb plasmid; those obtained from *E. coli* CF266 showed the same resistance pattern as the original strain, and those obtained from *E. coli* CF228 were resistant only to β-lactams. In addition, all the transconjugants adhered to HEP-2 cells, as did the parental strains, but no major surface protein could be extracted from the bacterial surface.

**DISCUSSION**

The present study was performed to determine the predominant pathotype of diarrheagenic *E. coli* in hospitalized infants, children, and adults in Clermont-Ferrand, France. A total of 352 strains were studied: 262 wild-type strains isolated from diarrheal stools of patients in Clermont-Ferrand hospitals and 90 strains from healthy children and adults.

To classify them in the previously described pathogenic *E. coli* groups ETEC, EPEC, EIEC, EHEC, EAggEC, and DAEC, we used specific DNA probes, tests of in vitro adhesion to HEP-2 and Caco-2 cells, and agglutination with several O antisera. We identified only 1.5% of the strains as ETEC and none as EIEC or EHEC. These results confirm the findings of Bohnert et al. (5), who observed that these pathogenic *E. coli* strains are rarely observed in France. ETEC and EIEC strains are more common in developing...
countries and EHEC strains are more commonly observed in North America (24).

EPEC strains have had a particularly rich and controversial history, and it is increasingly difficult to classify pathogenic *E. coli* strains in this pathotype. In accordance with recent publications, we will consider as EPEC strains those exhibiting LA to HEp-2 cells and hybridizing with both EAF- and *eae*-specific probes (for a review, see reference 11). In our study, 12 strains adhered both to HEp-2 and Caco-2 cells with an LA pattern, but none of them hybridized with the EAF probe. On the basis of these criteria, no EPEC strain was found among the 262 strains tested. This confirms the low prevalence of EPEC strains in France (5).

Similar results were obtained in Great Britain, where Scotland et al. (34) reported that a majority (88%) of LA strains were EAF−. Two of the 12 LA+ EAF− strains hybridized with the *eae* probe but were not agglutinated by any of the three EHEC O antisera. These two strains might be EPEC strains without the EAF plasmid or EHEC strains not belonging to classic EHEC O serogroups. Moreover, six nonadhering strains (LA− EAF−) hybridized with the *eae* probe. They should be classified in another group, attaching-effacing *E. coli*, as suggested by Jerse et al. (18).

The rate of occurrence of EAEC is quite high, since 17 (6.5%) of the *E. coli* strains isolated from diarrheal stool specimens adhered to HEp-2 cells in an aggregative pattern. Fifteen of these 18 strains hybridized with the EAEC probe. Only one strain from controls (1.1%) hybridized with the EAEC probe, but it did not adhere. Previous studies have shown that some EAEC strains do not hybridize with the EAEC probe and that this group is heterogeneous (2, 25). Another way to identify EAEC strains is to determine their hemagglutination properties with human or rat erythrocytes in the presence of D-mannose (25, 34). Detection of mannose-resistant hemagglutination specifically with rat erythrocytes has also been suggested as a method of identification for these strains (34). In our study, 82% of EAEC strains agglutinated human erythrocytes in the presence of D-mannose, but only 55% agglutinated rat erythrocytes. Hence, the hemagglutination with rat erythrocytes was not a useful marker for the identification of EAEC strains.

Of the 18 EAEC strains from our study, 5 hybridized with the DA probe and 1 hybridized with the *eae* probe; EAEC DA+ strains have already been reported (15). The *E. coli* strains classified in this pathotype may therefore have more than one virulence factor.

A high percentage of the *E. coli* strains tested showed a diffuse adhesion to HEp-2 cells. One hundred (38.2%) of the
262 strains isolated from patients with diarrhea had the DA pattern, whereas 8 (8.9%) of the 90 strains isolated from controls had the DA pattern. This highly significant difference ($P < 0.0001$) suggests that the DA strains were pathogens. The diffuse pattern of adhesion has been a source of discordant opinions; some investigators claim and others refute an association with diarrhea. In some reports there was no difference between patients and controls (16, 25, 30), while others showed a significant occurrence of DA strains in diarrheal stool specimens (15, 27). Our studies, which were performed with strains isolated in France, are in good agreement with the findings of the latter studies involving strains isolated in the United States and Mexico.

A high correlation between adhesion to HEp-2 and Caco-2 cells was observed for most of the diarrheagenic E. coli strains. An 80% correlation was found for the DA strains, and an 82.3% correlation was found for the EAggEC strains. Furthermore, a 100% correlation was observed for the LA strains, confirming the findings of Girón et al. (15).

Of the 100 DA strains, 33 hybridized with the DA probe and 2 hybridized with the AIDA probe. Thus, 65 strains (65%) with a DA phenotype adhered by means of an adhesin different from those described so far. A similar result was obtained by Girón et al. (15), who reported that 53.5% of DA strains did not hybridize with the DA probe.

SDS-PAGE analysis of bacterial surface extracts of the 33 strains that hybridized with the DA probe by SDS-PAGE showed four major surface proteins with different molecular sizes. Only eight strains produced the F1845 adhesin. The heterogeneity of the major surface proteins of the strains positive with the DA probe was not surprising since this probe is part of the daac accessory genes of the F1845 operon (4). F1845 is a member of a family of genetically related DA adhesins termed the Dr Family that includes Dr hemagglutinin, AFA I, and AFA III. Although they share related assembly and regulatory genes, their fimbrial subunit genes are divergent and encode proteins with distinct molecular sizes (21, 31, 37).

Conjugative transfer experiments performed with two DA strains that were negative with the DA probe (E. coli CF228 and CF266) revealed that R plasmids are involved in the expression of this phenotype. Further studies are under way to identify the strains of the DA strains and to determine their prevalence among diffusely adhering strains. The genes involved in this diffuse adhesion are located on a conjugative 110-kb plasmid, together with the genes encoding antibiotic resistance. Similar antibiotic resistance plasmids carrying virulence determinants have previously been reported in E. coli, Shigella spp., Salmonella spp., and Klebsiella pneumoniae (6, 10, 38). The use of antibiotics might therefore induce the emergence of strains with enhanced pathogenicities.

Sixteen DA strains showed a cytotoxic effect after a 3-h incubation with HEp-2 cells. The effect was characterized under an optical microscope by pyknosis of the nucleus and destruction of the cytoplasmic membrane and then detachment of the cells from the coverslips. The 16 strains were hemolysin producers, and control experiments showed that E. coli WAM589, which harbored the recombinant plasmid pWAM04 encoding the E. coli hemolysin, produced the same cytotoxic effect. This suggests that hemolysin production is responsible for HEp-2 cells lysis. Colonna et al. (7) showed that the hemolysin produced by lactose-negative diarrheagenic E. coli strains of serogroup O4 is responsible for a similar cytotoxic effect on HeLa cells.

In conclusion, the present study shows the difficulty in classifying diarrheagenic E. coli strains in one of the previ-ously determined pathotypes. EAggEC and DAEC strains and strains giving localized adhesion are not all recognized by the corresponding DNA-specific probes. Most of the strains isolated in our study belonged to the DAEC group, but 65% of them did not hybridize with any of the available DNA probes. Statistical analysis of E. coli strains isolated from diarrheal stool specimens and controls indicated that the strains that exhibited diffuse adhesion to HEp-2 cells should be considered potential pathogens.

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