Detection of enteroadherent *Escherichia coli* associated with diarrhoea in Italy

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**Summary.** One hundred and sixty-eight isolates of *Escherichia coli* obtained in Italy from 112 children with diarrhoea and 56 age-matched controls were examined by the HEp-2 cell adhesion assay. Sixteen strains showed localised adherence (LA), 29 showed diffuse adherence (DA) and eight strains showed aggregative adherence (AA). No adhesion pattern was significantly associated with disease. Strains that showed LA or AA were further characterised by serotyping, fluorescent actin staining (FAS) test and hybridisation with the EPEC adherence factor (EAF), *E. coli* attaching and effacing (eae) and enteroaggregative (EAgg) DNA probes. Strains that showed poor LA were FAS-negative, did not belong to EPEC serotypes and did not hybridise with EPEC probes. Conversely, the two strains that showed a good LA pattern belonged to serotype O128:H2, were FAS positive and hybridised with the eae probe. No isolate hybridised with the EAF probe. Only three of the eight strains with the AA pattern hybridised with the EAgg probe. Probe positivity correlated with the ability to produce clumps at the surface of the liquid culture and to agglutinate rat erythrocytes. In two of these EAgg probe-positive strains, electronmicroscopy revealed the presence of fibrillar bundles which seem to mediate bacterial aggregation.

**Introduction**

The property of some *Escherichia coli* strains to adhere to HEp-2 or HeLa cells has been used to identify some categories of *E. coli* associated with diarrhoea. Localised (LA), diffuse (DA) and aggregative (AA) patterns of adherence have been described. Localised adherence has been associated with strains that belong to the characteristic entero-pathogenic *E. coli* serotypes (EPEC). This pattern is characterised by tight clusters of adherent bacteria on the surface of cells. LA-positive *E. coli* strains interact with both the intestinal mucosa and cultured cells, producing either *in vivo* or *in vitro* a characteristic cytoskeletal lesion referred to as “attaching and effacing” (A/E). High concentrations of filamentous actin are seen in the host cell beneath the site of bacterial attachment, a characteristic which has been utilised in the fluorescent-actin staining (FAS) assay to reveal the presence of A/E lesions in tissue culture cells.

In some strains, a particular 60-MDa plasmid was shown to be necessary for the full expression of LA. A 1-kb probe, the “EPEC adherence factor” (EAF) probe, has been obtained from this plasmid and used for epidemiological studies. The pathogenicity of LA-positive strains has been demonstrated in human volunteer studies and their role in childhood diarrhoea was confirmed in several epidemiological surveys in Asia and South America. *E. coli* strains that show DA (DAEC) bind to the entire available surface of cells as well separated, distinct bacteria and do not belong to particular serotypes. Their pathogenicity and epidemiological significance are still controversial.

The AA pattern is characterised *in vitro* by clumps of bacteria with a “stacked brick” appearance that are attached to the cell monolayer and to exposed areas of the glass slide. Recently, fimbrial structures (aggregative adherence fimbriae I, AAF/I) have been involved in this kind of adhesion. Strains that show AA have been termed enteroaggregative *E. coli* (EAggEC). They usually carry a 60-MDa plasmid which confers AA and also encodes bundle-forming fimbriae and a heat-stable enterotoxin. A 1-kb fragment from this plasmid was found to be a highly specific DNA probe for identifying EAggEC. Recent epidemiological studies have implicated EAggEC strains as causative agents of persistent diarrhoea in...
infants and young children in South America and India. The involvement of adherent E. coli in childhood diarrhoea has been studied widely in developing countries. The characteristics of strains that belong to different EPEC serogroups isolated from epidemic outbreaks in the USA and sporadic cases in the UK have been reported, but the incidence and epidemiological features of the infections by the different groups of adherent E. coli in developed countries are still unknown.

This study reports the detection of enteroadherent E. coli in children with and without diarrhoea in Italy and their characterisation with adhesion-related DNA probes. Strains showing aggregative adhesion were further studied by electronmicroscopy.

Materials and methods

Study population

Patients consisted of 112 children, with a mean age of 23.9 (range 1–106) months, and 44 of them were infants < 1 year old. They were admitted consecutively because of diarrhoea to the paediatric wards of two hospitals in Northern and Central Italy. Cases were observed during 1 year, both as inpatients (73 children) and outpatients (39 children).

Patients were enrolled in the study irrespective of the duration of diarrhoea or previous antibiotic treatment. Clinical details were ascertained from hospital clinical records and, in the case of outpatients, from questionnaires completed by their parents. Specimens were also collected from 56 children admitted to the same hospitals with other diseases, and without diarrhoea in the previous 2 weeks. One control child was enrolled for every two diarrhoeic patients. The age, sex and seasonal distribution of controls were approximately proportional to those of patients with diarrhoea.

Stool examination

Stool specimens were collected on admission and examined for common enteric pathogens as described previously. For E. coli isolation, faeces were streaked on to MacConkey agar plates and incubated overnight at 37°C. Ten lactose fermenting colonies tentatively recognised as E. coli were picked from each sample, pooled on to nutrient agar slants and examined by the HEp-2 cell adhesion assay. Positive cultures were repeated on each single colony. Strains that adhered to HEp-2 cells were identified by the API 20E system (Biomerieux, France). E. coli serogrouping was by standard techniques with rabbit antisera against the following EPEC serogroups: O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128 and O142. Further serotyping was performed at the International E. coli and Klebsiella Centre, Statens Seruminstitutet, Copenhagen, Denmark.

HEp-2 adhesion assay

The adherence patterns were assessed as described by Natario et al. with HEp-2 monolayers grown on glass coverslips. An overnight broth culture of bacteria (2 x 10^8 cfu) was added to the cell monolayer in the presence of D-mannose 1% w/v. After incubation for 3 h at 37°C with CO_2 5%, the monolayer was washed with phosphate buffered saline (PBS) to remove non-adherent bacteria; fresh tissue culture medium was added, and the cells were re-incubated for 3 h to improve the detection of adhesion patterns. LA was quantified according to the method of Knutton and co-workers by counting 500 cells on randomised microscopic fields, and recording the percentage of cells with adherent microcolonies. LA was defined as good when the percentage of cells with microcolonies was > 50%, and poor when the microcolonies were very small and the percentage was < 50%. The FAS test was performed according to Knutton et al. Positive controls of EPEC (LA+, FAS+), EAggEC and DAEC were included in each experiment.

Test for verotoxin (VT) production

Sterile culture supernates of the bacterial strains grown in trypticase soy broth (TSB) were tested for the presence of VT by the Vero cell cytotoxicity assay as described previously.

Bacterial clump formation and haemagglutination assay

EAggEC strains were grown in LB medium in static culture at 37°C overnight and the formation of bacterial clumps at the surface of the medium was judged with the naked eye.

The same cultures were tested for agglutination of rat erythrocytes in the presence of D-mannose 0.5%.

Transmission electronmicroscopy (TEM)

EAggEC isolates were observed by negative staining for the production of fimbriae. Bacterial cells, grown in LB medium, were washed and resuspended three times in distilled water. A drop of suspension was applied to a carbon-coated EM grid (400 mesh), and then stained with sodium-phosphotungstate (NaPT) 1%. The samples were examined in a Zeiss 902 electronmicroscope.

Scanning electronmicroscopy (SEM)

Infected HEp-2 cell monolayers were fixed in glutaraldehyde 3% in cacodylate buffer (0.1 M; pH 7.0) overnight at 4°C, washed twice in the same buffer and post-fixed in osmium tetroxide 1% for 1 h. The samples were dehydrated in ethanol before critical point drying and sputter coating with gold (thickness c. 20 nm) and then observed with a Cambridge 360 electronmicroscope.
Results

Neither the colony pools nor the isolated strains produced VT. The prevalence of adherent *E. coli* strains among patients and controls, as identified by the HEp-2 cell assay, is shown in table I. No statistically significant association between diarrhoea and the adhesive properties of isolated *E. coli* was demonstrated. In particular, DAEC and poor LA strains were frequently found in the control group.

<table>
<thead>
<tr>
<th>Pattern of adherence</th>
<th>Number (%) of strains isolated from</th>
<th>cases</th>
<th>controls</th>
<th>p†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localised (good)</td>
<td></td>
<td>2 (1.8)</td>
<td>0</td>
<td>0.45</td>
</tr>
<tr>
<td>Localised (poor)</td>
<td></td>
<td>10 (8.9)</td>
<td>4 (7.2)</td>
<td>0.48</td>
</tr>
<tr>
<td>Diffuse</td>
<td></td>
<td>22 (19.6)</td>
<td>7 (12.5)</td>
<td>0.39</td>
</tr>
<tr>
<td>Aggregative</td>
<td></td>
<td>7 (6.25)</td>
<td>1 (1.8)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*See Materials and methods. 
†Fisher’s exact test.

Table II. Characteristics of enteroaggregative *E. coli* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Hybridisation with probe</th>
<th>Serotype</th>
<th>Clump formation</th>
<th>Mannose-resistant haemagglutination</th>
<th>Fimbrial type*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EPEC adherence factor</td>
<td>enteroaggregative</td>
<td>eae</td>
<td>diffuse adherence</td>
<td></td>
</tr>
<tr>
<td>E-F 36</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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</tbody>
</table>

*Fimbrial type assessed by electronmicroscopy: R, rod; FB, fibrillar bundle. 
†Strain from an asymptomatic control.
Figure. Electronmicrographs showing the two distinct type of fimbriae produced by the EAggEC strains. a, One or a small number of fibrillar bundles were produced by EAgg probe-positive strains (bar = 0.5 μm); b, A typical bundle is seen extending between bacteria; rod-like fimbriae are also present. At high magnification (inset; bar = 25 nm), a regularly repeating periodicity was observed along the length of the fibrillar bundle (bar = 250 nm). c, At the scanning electronmicroscopy analysis, a filamentous network is seen forming EAggEC colonies (bar = 0.5 μm); d, filaments seem to connect bacteria together (bar = 125 nm).

the number of single filaments involved (figure a). In agreement with Knutton et al., a repeating periodicity of c. 5 nm was observed along the fibrillar bundles (figure b, inset).

When observed by SEM (figure c, d), the two strains positive for the fibrillar bundles showed the presence of a filamentous network that appeared to connect bacteria together and to the glass coverslip. Considering the gold layer thickness, the diameter of such filaments was consistent with that of the fibrillar bundles observed by TEM.

Discussion

The involvement of tissue culture adherent E. coli in childhood diarrhoea has been well established in several case-control studies in developing countries. EPEC strains showing strong LA and carrying the EAF gene have been found to be an important cause of diarrhoea in Mexico, Brazil and Chile. EAggEC strains have been associated with persistent diarrhoea in Mexico and India. Conversely, diffuse adhering strains have often been isolated with similar frequencies from patients with diarrhoea and from non-diarrhoeal controls.

In this study, strains showing DA or poor LA were found frequently among cases and controls. On the other hand, strains with good LA or AA also appeared not to be associated with disease, although the small numbers examined are inconclusive. The two characteristic EPEC strains isolated in this investigation belonged to serotype O128:H2. As in recent studies in the UK, they were FAS positive and hybridised with the eae probe, but not with the EAF probe.
The HEp-2 assay revealed several strains that showed poor LA, but none of them exhibited any of the features considered to be related to the ability to cause diarrhoea, namely positivity in the FAS test, hybridisation with the eae probe and typical EPEC serotype. These results suggest the opportunity to confirm, for strains from industrialised countries, the results of the HEp-2 cell assay with the FAS test or by hybridisation with the eae probe, or both. The EAgg probe recognised only three of these strains and, interestingly, only the probe-positive isolates showed characteristics reported for EAggEC in other studies,18,22,23 such as bacterial clump formation, haemagglutination and belonging to particular serogroups. In particular, one of the strains, which was judged by the Escherichia Centre in Copenhagen to be of serogroup 092 by Scotland18, was likely to be similar to strain 221, previously considered to be of serotype O78:H33,22 and then assigned to serogroup O92 by Scotland et al.23 As reported in other studies,17,18,23 strains with the aggregative phenotype did not hybridise with the EAgg probe. The existence of different categories of EAggEC has been proposed,17,18 however, we cannot exclude the possibility that the probe-negative isolates in this study were strains that showed a strong DA, which is sometimes difficult to distinguish from the Aag pattern. On the other hand, none of these aggregative strains hybridised with the DA probe.

Electronmicroscopy showed fibrillar bundles in two of the probe-positive strains similar to those described by Knutton et al.13 and Nataro et al.14 These fibrilae appeared to participate in the formation of EAggEC colonies linking bacteria together, as described previously.13,14 This hypothesis seems to be supported by SEM observations, that showed a filamentous network responsible for the bacterial aggregation.

Even though the numbers in this group of patients are small, the present study seems to exclude EAEC infections as a major cause of diarrhoea in Italian children.

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References
25. Scotland SM, Smith HR, Rowe B. Escherichia coli O128 strains from infants with diarrhea commonly show localized adhesion and positivity in the fluorescent-actin staining test.


