

# Enter-adherent *Escherichia coli* is an important diarrhoeagenic agent in infants aged below 6 months in Calcutta, India

A. R. GHOSH, G. B. NAIR, T. N. NAIK, MOUSUMI PAUL, S. C. PAL and D. SEN\*

National Institute of Cholera and Enteric Diseases, P-33, CIT Scheme XM, Beliaghata, Calcutta-700 010, India

**Summary.** *Escherichia coli* adherent to HEp-2 and HeLa cells were isolated from the faeces of 43 (19.7%) of 218 hospitalised infants aged below 6 months with acute diarrhoea. No conventional virulence factors, including enterotoxin production—heat-labile (LT) or heat-stable (ST), the verotoxin (VT) or shiga-like toxin (SLT)—or the invasive phenotype (determined by the Sereny test) could be detected among these isolates. Out of the 43 isolates, 16 (37.2%) were of the known enteropathogenic O:K serogroups—enteropathogenic *E. coli* (EPEC). The remaining 27 (62.8%) isolates showed different types of adherence to HEp-2 and HeLa cells which was diffuse (40.7%), localised (37.0%), or both (22.3%); they were identified as enter-adherent *E. coli* (EAEC). The EAEC isolates adhered to HEp-2 and HeLa cells in the presence of mannose, lactose, fucose, galactose, and fetuin, indicating that adhesion was not specific for these sugars or glycoprotein. Haemagglutination and the salt aggregation test (SAT) did not correlate with patterns of adherence. The results of this study indicate that LA-EAEC is an important aetiological agent of acute diarrhoea in infants aged below 6 months in Calcutta.

## Introduction

Depending on the pattern of adherence, *Escherichia coli* of non-enteropathogenic serogroups that adhere to HEp-2 or HeLa tissue cells in culture have been referred to as entero-adherent *E. coli* (EAEC)<sup>1</sup> or entero-adherent aggregative *E. coli* (EA-AggEC).<sup>2</sup> Strains of EAEC that exhibit localised adherence (LA) form clusters or microcolonies of bacteria on the HEp-2 cell surface; strains that exhibit diffuse adherence (DA) are found evenly distributed over the surfaces of the cells.<sup>3</sup> In contrast, strains of *E. coli* classified as EA-AggEC adhere on the surfaces of the cultured cells as well as on the glass slide free from cells, assuming a characteristic stacked-brick pattern.<sup>2,4,5</sup>

The non-EPEC adherent groups of *E. coli* are emerging, and have been incriminated epidemiologically, as one of the conspicuous aetiological agents of diarrhoea amongst travellers<sup>1</sup> and children.<sup>2,6–10</sup> However, considering the controversy and debate on the aetiological role of EAEC and EA-AggEC and on the methodologies being used for the adherence test, the role of both the groups in causing diarrhoea in man needs to be investigated in greater detail.<sup>11</sup> The present study was initiated to determine the impor-

tance of adherent non-EPEC isolates of *E. coli* in causing acute diarrhoea in infants aged below 6 months in Calcutta.

## Materials and methods

### Faecal specimens

During the period from April 1986 to March 1988, 218 infants with diarrhoea aged below 6 months admitted to the Dr B. C. Roy Memorial Hospital for Children, Calcutta, Monday–Saturday, 0900–1300 h, were included in the study; 102 infants matched for age, gender and socio-economic status attending the out-patient department of the same hospital for immunisation or for reasons other than gastrointestinal illness, were also investigated concurrently and served as controls.

Faecal samples of the study population were examined for bacterial enteropathogens by standard procedures.<sup>12</sup> Stool samples were examined by microscopy for trophozoites and cysts of *Entamoeba histolytica* and *Giardia lamblia*. Oocysts of *Cryptosporidium* spp. were also sought by the acid-fast Zeil-Neelsen method.<sup>13</sup> All samples were examined for the presence of rotavirus antigen by the enzyme linked immunosorbent assay (ELISA)<sup>14</sup> (table I).

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\* Correspondence should be sent to Dr D. Sen.

### Detection of toxin(s) and invasiveness in *E. coli* isolates

For each specimen examined, three characteristic lactose-fermenting colonies from the primary MacConkey plates, identified as *E. coli* by conventional procedures,<sup>12</sup> were screened for production of heat-labile toxin (LT) by the modified Elek test<sup>15</sup> and the GM<sub>1</sub>-ELISA,<sup>16</sup> for heat-stable toxin (ST) by the suckling mouse assay,<sup>17</sup> and for Vero toxin (VT) (shiga-like toxin; SLT) by the Vero cell assay<sup>18</sup> and by the recently developed sensitive VT1/VT2 bead-ELISA technique.<sup>19</sup> The strains were also tested for the invasive phenotype by the guinea-pig keratoconjunctivitis model.<sup>20</sup>

### Tests for adherence

Strains of *E. coli* which were non-invasive and non-producers of LT, ST or VT were tested for adherence to the HEP-2 and HeLa cultured cells by the procedure detailed by Cravioto *et al.*<sup>21</sup> Briefly, 20 µl (*c.* 10<sup>7</sup> cfu) of bacterial culture, grown overnight in tryptic soy broth with agitation at 37°C, was added to tissue-culture cells grown to *c.* 60% confluence on plastic petri dishes containing sterile glass cover slips. The inoculated tissue culture cells were incubated for 3 h at 37°C in CO<sub>2</sub> 5% in air. Adherence to mammalian cells was tested in the presence or absence of mannose, lactose, fucose or galactose (each at 1%) and the glycoprotein fetuin (0.1%). After incubation, the cover slips were washed with PBS (pH 7.2), dried in air, fixed in methanol for 2 min, stained with Giemsa 1% in methanol for 1 min and mounted on microscope slides with Canada balsam. Adherence of *E. coli* to

mammalian cells was examined under bright-field illumination. The pattern of bacterial adherence to HEP-2 and HeLa cells was evaluated according to the description of Nataro *et al.*<sup>2</sup> Although three isolates of *E. coli* were picked from each case, if one isolate from the case exhibited adherence, the other two isolates were not tested.

### Tests for EPEC serogroups, hydrophobicity and haemagglutination

*E. coli* strains that were adherent to HEP-2 and HeLa cells were tested for agglutination with commercially available EPEC (O:K) antisera (Wellcome Reagents). The entero-adherent non-enteropathogenic *E. coli* isolates were tested for hydrophobicity by a salt aggregation test (SAT)<sup>22</sup> with various molar concentrations of ammonium sulphate and for haemagglutination (mannose-sensitive and mannose-resistant)<sup>23</sup> with 3% suspensions of erythrocytes of human group A, bovine, chicken or guinea-pig.

### Results

Bacterial, viral and parasitic enteropathogens detected in the faeces of 167 (76.6%) infants with diarrhoea either singly (56.4%) or in association with other enteropathogens (20.2%) are shown in table I. In contrast, the frequency of detection of enteropathogens was 16.7% in the healthy controls. The three groups of diarrhoeagenic *E. coli*—EPEC, ETEC and EAEC—together accounted for 57.3% of the cases and were the major aetiologic agents; 81 of the

**Table I.** Prevalence of different enteropathogens among acute diarrhoeic and non-diarrhoeic infants aged below 6 months in Calcutta

Enteropathogen(s) detected	Number (%) of isolates from	
	cases (218)	controls (102)
<i>Sole pathogen</i>	123 (56.4)	14 (13.8)
Diarrhoeagenic <i>E. coli</i>	81 (37.2)	9 (8.9)
Enteropathogenic <i>E. coli</i> (EPEC)	46 (21.1)	7 (6.9)
Enterotoxigenic <i>E. coli</i> (ETEC)	17 (7.8)	0
Entero-adherent <i>E. coli</i> (EAEC)	18 (8.3)	2 (1.9)
Rotavirus	10 (4.6)	NT
<i>Salmonella typhimurium</i>	7 (3.2)	1 (1.0)
<i>Campylobacter jejuni</i>	7 (3.2)	1 (1.0)
<i>Aeromonas hydrophila</i>	7 (3.2)	3 (2.9)
<i>Shigella</i> spp.	4 (1.9)	0
<i>Cryptosporidium</i> spp.	6 (2.7)	0
<i>Entamoeba histolytica</i>	1 (0.5)	0
<i>Mixed pathogens</i> †	44 (20.2)	3 (2.9)
<b>Total*</b>	<b>167 (76.6)</b>	<b>17 (16.7)</b>

NT, not tested. \* $\chi^2 = 99.7186$ ;  $p < 0.0001$ .

† Forty-four diarrhoeagenic *E. coli* strains (26 EPEC, nine ETEC and nine EAEC) were associated with polymicrobial infections.

diarrhoeagenic *E. coli* were isolated as the sole pathogen and 44 were associated with polymicrobial infections. Other pathogens identified were rotavirus, *Salmonella typhimurium*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Shigella* spp., *Cryptosporidium* (oocyst) and cystic stages of *Ent. histolytica* (table I).

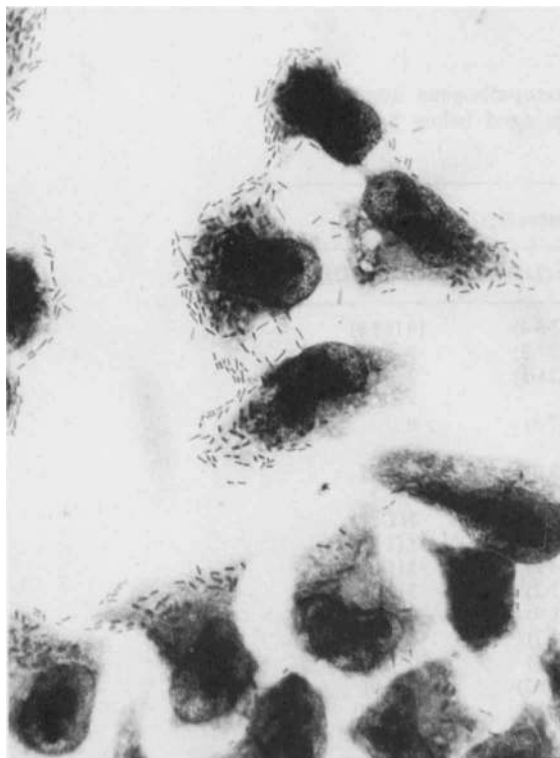
Among the diarrhoeagenic *E. coli*, EPEC dominated and was associated with 46 of the 218 cases as sole pathogen; EPEC were also found in seven of the 102 healthy controls. Of the isolates from the faeces of infants with diarrhoea, 43, including 13 from mixed infections and four isolates from healthy controls, were adherent to both HEP-2 and HeLa cells. Of the 43 adherent *E. coli* isolates, 16 (12 as sole pathogen and four from mixed infections) belonged to the conventional EPEC (O:K) serogroups. The distribution of the 16 EPEC serogroups were: O128:K67 (four strains); O127:K63, O119:K69, O44:K74 (three strains each); O55:K59, O126:K71, O114:K90 (one strain each). Twelve (all as sole pathogen) of the 16 EPEC isolates showed LA and two each (all from mixed infections) showed DA or both LA and DA (table II). The remaining 27 (18 as sole pathogen and nine from mixed infections) strains were non-EPEC and were classified as EAEC resulting in overall isolation rates of 12.4% in the 218 diarrhoeal infants investigated (including mixed infections) and 1.9% in the 102 healthy controls examined. Among the 27 EAEC strains, 11 (40.7%) showed DA and 10 (37%) showed LA; the other six (22.3%) exhibited both LA and DA (table II). The distribution of the 27 EAEC strains isolated as the sole pathogen and those associated with mixed infections among DA, LA and

**Table II.** Adherence patterns of EPEC and EAEC strains isolated from 218 infants with diarrhoea

Adherence patterns	Number (%) of isolates of	
	EPEC (16)	EAEC (27)
LA	12 (75.0)	10 (37.0)
DA	2 (12.5)	11 (40.7)
LA/DA	2 (12.5)	6 (22.3)

LA/DA were seven (sole) and four (mixed), eight and two, and three each, respectively. Four *E. coli* isolates from healthy controls exhibited DA; two belonged to the EPEC serogroups (O128:K67 and O114:K90) and two were EAEC. The DA type of adherence of a few strains of EAEC was not typical (fig. 1), but was classified as DA in this study. A photomicrograph of a representative EAEC strain exhibiting LA/DA is shown in fig. 2. The EAEC strains isolated from cases and controls were non-invasive and did not produce LT, ST or VT.

Adherence of the EAEC isolates was not inhibited by the four sugars tested or by fetuin. Screening of isolates for surface hydrophobicity by SAT revealed that 14 (51.8%) were hydrophobic and belonged either to SAT group I (precipitation at 0.01–0.09 M  $(\text{NH}_4)_2\text{SO}_4$  solution) or II (precipitation at 0.1–0.9 M  $(\text{NH}_4)_2\text{SO}_4$  solution). Furthermore, of the 14 hydrophobic isolates, eight showed mannose-sensitive haemagglutination (MSHA) with guinea-pig erythrocytes and six showed mannose-resistant haemagglutin-



**Fig. 1.** Photomicrograph of cultured HeLa cells incubated with a strain of EAEC showing atypical DA ( $\times 800$ ).



**Fig. 2.** Photomicrograph of cultured HeLa cells incubated with a strain of EAEC showing LA/DA ( $\times 800$ ).

ation (MRHA) with chicken erythrocytes. The remaining 13 non-hydrophobic and non-haemagglutinating EAEC isolates showed all the patterns of adherence to mammalian cells, indicating that adherence was independent of the bacterial cell-surface hydrophobicity and haemagglutination properties.

## Discussion

Adherence of bacteria to intestinal mucosa appears to be a pre-requisite for infection, enabling organisms to resist expulsion by peristaltic clearing mechanisms, with subsequent proliferation and colonisation of the gut. Adherence can then be followed by toxin production or invasion. Several histopathological studies in infants and animals with EPEC infections have revealed that adherence of the bacteria to the small bowel mucosa is important in the induction of diarrhoea.<sup>24-28</sup>

Mathewson *et al.*<sup>1,6</sup> claimed that certain non-toxicogenic *E. coli* isolates, not belonging to the EPEC serogroups and exhibiting LA and DA to HEp-2, might be an important cause of travellers' and paediatric diarrhoea and termed the organisms entero-adherent *E. coli* (EAEC). Cravioto *et al.*<sup>7</sup> isolated non-EPEC strains showing LA to HEp-2 cells from faeces of Mexican children without diarrhoea and Scaletsky *et al.*<sup>29</sup> observed DA to HeLa cells among non-EPEC isolates.

Mathewson *et al.*<sup>6</sup> detected EAEC showing LA among 20.8% of the patients and 7.3% of the controls in Mexico. In the present study, EAEC was detected in the faeces of 12.4% of the 218 diarrhoeal infants investigated, including those with mixed infections; among the healthy controls, the isolation rate was 1.9%. Only DA-EAEC was detected from healthy controls whereas strains exhibiting LA, DA, and both LA and DA were found in infants with diarrhoea. Between the two modes of adherence, recent investigations<sup>2,8-10</sup> indicated that *E. coli* strains showing true DA were not associated with diarrhoea. Similarly, in this study, the difference in isolation rates of DA-EAEC between cases and controls was not statistically significant ( $\chi^2 = 0.997$ ;  $p > 0.3$ ) indicating

that strains of *E. coli* exhibiting DA may not be involved in the causation of diarrhoea.

No EA-AggEC, as described by Natato *et al.*,<sup>2</sup> were detected in this study. The assay used for determining entero-adherence in this study was that of Cravioto *et al.*<sup>21</sup> which permits the assessment of EA-AggEC and, therefore, the inability to detect EA-AggEC is not a reflection of inadequate methodology. However, it must be emphasised that the DA exhibited by certain strains of EAEC in this study was atypical and did not correspond with the original description of even distribution of bacteria over the surface of the tissue-culture cells. This atypical DA could not be classified as EA-AggEC, because of the absence of stacked-brick clumps of bacteria on the glass slides, unattached to HeLa cells, a feature which is characteristic of EA-AggEC. Thus, the results suggest that EAEC exhibiting LA play an important role in causing diarrhoea in infants aged below 6 months in Calcutta; those exhibiting DA need to be evaluated more critically.

Lack of enterotoxin (LT, ST) and cytotoxin (VT/SLT), and of the non-entero-invasive property, of the EAEC strains isolated in this study corroborates the findings of Mathewson *et al.*<sup>30</sup> and indicates that adherence to HEp-2 or HeLa cells, or both, is the only marker for detection of EAEC isolates. Results of hydrophobicity and haemagglutinating assays of EAEC strains suggest that adherence is independent of bacterial cell surface hydrophobicity and haemagglutination. The recent claim that plasmid-coded LA is fimbrial in nature<sup>31</sup> has not been accepted by several workers.<sup>32-34</sup> More detailed studies are needed to confirm the correlation between the possession of fimbriae and entero-adherence among EAEC isolates.

The duration of diarrhoea due to EAEC in the present study was 3-7 days. Practically no report is available regarding the clinical nature of the diarrhoea caused by this "newer" enteropathogen. The role of chemotherapy in the treatment of EAEC-associated diarrhoea is yet to be assessed. However, in-vitro antimicrobial susceptibility testing of the isolates in this study revealed that the EAEC are multi-resistant (unpublished observation). Further studies are needed to determine the significance of EAEC in diarrhoeal disease in man.

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