Adherence of non-enteropathogenic *Escherichia coli* to HeLa cells

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Summary. Three hundred and nine strains of *Escherichia coli* isolated from infants and children with diarrhoea but not belonging to any recognised classes of diarrhoeagenic *E. coli* were investigated for their ability to adhere to HeLa cells in the presence of D-mannose. An enteroadherent-aggregative pattern (EAgg) was observed in 32·03%, localised adherence (LA) in 4·5%, diffuse adherence (DA) in 5·8%, and LA/DA and EAgg/LA in 1·9% and 1·2% of the isolates respectively. The results obtained with 100 control isolates were: EAgg 17%, LA 2%, DA 2%, LA/DA 2%, EAgg/LA 6% and DA/EAgg 1%. No adherence was manifested by 168 (54·36%) of 309 diarrhoeal isolates and 70% of the 100 control isolates. The results of this study showed that amongst non-enteropathogenic *E. coli*, strains exhibiting the EAgg pattern are significantly associated with diarrhoea ($p < 0·005$). Most of these strains showed a pattern of multiple drug resistance.

Introduction

Bacterial adherence to the intestinal mucosa has been identified as characteristic of *Escherichia coli* strains that cause diarrhoea in infants.¹ The observation that most enteropathogenic *E. coli* adhered to HEp-2 cells *in vitro* whereas non-pathogenic strains rarely did so led to the use of tissue-culture adherence assays to identify diarrhoeagenic *E. coli* strains.² Three patterns of adherence have been described: localised adherence (LA) in which microcolonies are formed at one or more places on the cells (HeLa or HEp-2), diffuse adherence (DA) where bacteria cover the cells uniformly³ and the enteroadherent-aggregative pattern (EAgg) in which the bacteria have a characteristic stacked brick appearance on the surface of the cells and on the glass slide free from the cells.⁴ Studies conducted so far have suggested that *E. coli* with an LA pattern and belonging to enteropathogenic serotypes (EPEC) are associated with diarrhoea.^{5,6} The role of *E. coli* showing a DA pattern is controversial^{5,7} and strains showing an EAgg pattern (EAggEC) have been incriminated as causing diarrhoea in children on the basis of epidemiological studies.^{8,9} The present study was undertaken to determine the importance of adherent non-EPEC and EAggEC strains among *E. coli* isolates from diarrhoeal stools.

Materials and methods

Bacterial strains

Three hundred and nine biochemically confirmed *E.*

coli strains isolated from infants and children with diarrhoea from whom no other bacterial pathogen had been isolated were studied and 100 strains from non-diarrhoeal age-matched infants and children attending the same hospital for vaccination purposes were used as controls. These strains were non-EPEC by slide agglutination with poly- and monovalent O antisera (Diagnostics Pasteur, France). They were non-toxicogenic in the infant mouse assay for heat-stable enterotoxin and the reversed passive latex agglutination test for heat-labile enterotoxin (Denka Seiken Co. Ltd, Japan). They failed to bind Congo red (Janssen Chimica, Belgium) and did not cause keratoconjunctivitis. These strains were also tested for their ability to ferment sorbitol with Sorbitol-MacConkey Agar (Difco).

Adherence assay

Strains were tested for adherence to HeLa cells in the presence of D-mannose (Merck, Germany) 1% by the procedure of Scaletsky *et al.*³ An overnight culture (50 µl) grown in Trypticase Soya Broth (Difco) was added to HeLa cell monolayers grown on coverslips in Leighton tubes. The inoculated cells were incubated at 37°C for 30 min (infection period), washed twice and incubated for a further 3 h (multiplication period). The cells were then washed twice, fixed in methanol:acetic acid (3:1), stained with Giemsa (Merck, Germany) and observed by light microscopy. The patterns of adherence were assessed according to Scaletsky *et al.*³ for LA and DA and Nataro *et al.*⁴ for EAgg (figure). *E. coli* strain K12 F⁻lac⁺Nal⁺ (a gift from Dr M. Katouli, Karolinska Institute, Stockholm, Sweden) was used as a non-adherent control and

Figure. Cultured HeLa cells incubated with strains of *E. coli* showing (A) LA, (B) DA, (C) EA.

strains showing LA, DA, and EA patterns were kindly provided by Dr J. B. Kaper, Center for Vaccine Development, University of Maryland, MD, USA.

Antibiotic sensitivity test

Adherent strains were tested for their sensitivity to various commonly used antibiotics by the disk diffusion method.¹⁰ Antibiotics tested were: ampicillin 10 µg, nalidixic acid 30 µg, cephalothin 30 µg, strep-

tomycin 10 µg, gentamicin 10 µg, tetracycline 30 µg, trimethoprim-sulphamethoxazole 1.25 + 23.75 µg, nitrofurantoin 30 µg, kanamycin 30 µg and chloramphenicol 30 µg. All disks were obtained from bioMérieux, France.

Statistical analysis

To determine the statistical significance of the data, the chi-squared (χ^2) test was used.

Results

Of the 309 *E. coli* strains isolated from cases of diarrhoea studied for their adherence pattern on HeLa cells, 14 (4.5%) exhibited LA, 18 (5.8%) DA, six (1.9%) LA/DA, 99 (32.03%) EA and four (1.2%) EA/LA. In contrast, of the 100 isolates from control cases two (2%) demonstrated LA, two (2%) DA, two (2%) LA/DA, 17 (17%) EA, six (6%) EA/LA and one (1%) DA/EA patterns.

Overall, 45.63% of the isolates from cases of diarrhoea were adherent whereas adherence to HeLa cells was observed in only 30% of the control isolates (table I). Association of EA strains with diarrhoea among these non-EPEC isolates was statistically significant (32.03% in cases of diarrhoea and 17% in controls $p < 0.005$, $\chi^2 = 8.409$). On the other hand LA

Table I. Prevalence of adherent *E. coli* strains from cases of diarrhoea and controls

Adherence pattern	Strains from 309 cases of diarrhoea	Strains from 100 controls	p value
LA	14	2	< 0.3
DA	18	2	< 0.2
EA	99	17	< 0.005
LA/DA	6	2	NT
LA/EA	4	6	NT
DA/EA	0	1	NT
Total	141	30	—

$p < 0.05$ was considered significant.

NT, not tested.

Table II. Distribution of antibiotic resistance determinants amongst *E. coli* isolates with different adherence patterns

Adherence pattern	Number of resistance markers						Total
	Nil	1	2	3	4	5	
EA	5	3	0	4	14	73	99
DA	0	0	0	1	3	15	18
LA	1	0	0	0	0	13	14
LA/DA	0	0	0	1	0	5	6
LA/EA	0	0	0	0	0	4	4
Total	6	3	0	6	17	110	141

and DA were not found to be specific markers of these non-EPEC strains as far as diarrhoea was concerned ($p < 0.3$, $\chi^2 = 1.287$ and $p < 0.2$, $\chi^2 = 2.376$, respectively). Strains showing EAgg exhibited 39 different antibiotic resistance patterns and the majority carried more than two resistance markers. The isolates showed resistance to cephalothin, trimethoprim-sulphamethoxazole, tetracycline, ampicillin and streptomycin either singly or in combination (table II).

Discussion

The role of EAgg strains of *E. coli* in diarrhoea is still unclear. In this study, we have shown a close association with diarrhoea, similar to results reported from India and Chile.^{8,9} However, studies from Brazil and Thailand have failed to confirm such a conclusion.^{7,11} The LA pattern has been shown to be a specific marker for EPEC, whereas the role of strains with the DA pattern is still unclear.⁵⁻⁷ In the present study neither of these two patterns showed any significant association of non-EPEC strains with diarrhoea. Antibacterial sensitivity tests revealed that of the EAgg *E. coli* strains examined in this study, > 90% carried one or more resistance determinant. EAgg *E. coli* are not detected in any of the clinical laboratories in Iran due to the lack of availability of technical facilities for either tissue culture assays or use of specific DNA probes. Detection of this level of resistance to several antibiotics could indicate indiscriminate prescription of antibiotics to children in the absence of positive isolation of any recognised bacterial pathogen. In a recent publication¹² different

methods for detection of adherent *E. coli* were assessed and it was concluded that the modified UTH method in which the incubation period of bacteria with the HeLa cells was extended to either 3 or 4 h after the first washing step increased the rate of detection of EAgg. Our study was conducted with only the procedure of Scaletsky³ which could have resulted in underscoring the number of EAgg strains, especially in the case of four isolates exhibiting the LA/EA pattern and some of the strains showing DA pattern. However, the data obtained showed a statistically significant association between the EAgg pattern and diarrhoea amongst the *E. coli* isolates not belonging to any of the known classes of diarrhoeagenic *E. coli*, although more detailed epidemiological investigations in different parts of the country are required to fully determine the role of this class of *E. coli*.

In the present study, a correlation was observed between the EAgg pattern *in vitro* and surface clumping of bacteria in the broth culture. Adherence of EAgg *E. coli* to human or animal mucosal cells¹³ has also been found to be related to haemagglutinating ability (HA). This correlation was not observed in this study (unpublished data) nor in that reported from India¹⁴ which suggested independence of adherence from cell surface hydrophobicity and HA.

The presence of aggregative adherence fimbriae-1 (AAF-1) has been reported to be responsible for this pattern of adherence.¹⁵ Studies on the presence of a similar adherence factor in EAgg strains of *E. coli* and the existence of its possible analogue in EPEC isolates exhibiting the EAgg pattern is under investigation.

We are indebted to Mrs Z. S. Mirfatah, Mssrs M. Byramian, R. Jirsaraie and H. Soleimani for their technical help.

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