

A study on some phenotypic virulence markers of enteropathogenic *Escherichia coli*

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Background & objectives: The problem of enteropathogenic *Escherichia coli* (EPEC) causing diarrhoea in infants exists in India. But often the enteropathogenic status is not based on adequate characterization. Hence there is a need for evaluating the serotyping being used to identify EPEC for its validity in the light of recent knowledge on phenotypic markers of virulence. This study was done to evaluate the EPEC isolates for two potential virulence factors namely entero-adhesiveness with subsequent actin accumulation and verotoxin production.

Methods: Fifty consecutive EPEC strains identified by serotyping from stool samples of children with diarrhoea during January 1997 to June 1999 were studied for HEp-2 cell adherence, the fluorescent actin staining (FAS) characteristics of Hep-2 cells and vero cytotoxin production.

Results: Serotypes O55, O125 and O126 accounted for most of the isolates. In the Hep-2 assay, 72 per cent of the strains showed localised pattern of adherence and 22 per cent showed a mixed pattern of localised and diffuse adherence. In the FAS test 96 per cent strains showed typical staining while none of the strains produced verotoxin.

Interpretation & conclusion: 'O' serogrouping appears to be still the simplest and an useful test for presumptive identification of EPEC. The FAS test for confirmation of EPEC was found to be very consistent in indicating EPEC.

Key words Adherence - EPEC - phenotypic virulence markers - verotoxin

Enteropathogenic *Escherichia coli* (EPEC) is defined as a category of *Esch. coli* belonging to certain serogroups that have been consistently associated with outbreaks of infantile diarrhoea¹, this appears to be more so in the developing world². There have been recent reports from India also to this effect^{3,4}. The observation that many strains belonging to the EPEC serogroups are actually devoid of any, as yet known, virulence factors and that only particular H types within an 'O' serogroup

are associated with diarrhoea have led investigators to consider 'O' serogrouping as an outmoded method for identifying EPEC⁵.

Studies have demonstrated that this group of organisms is actually quite heterogenous in the possession of putative virulence properties^{1,6}. The hallmark of EPEC is its ability to adhere intimately to the mucosa of the bowel and efface microvilli producing attaching/effacing (A/E) lesions⁷. This

could be reproduced in cell culture. In cell culture EPEC are seen to adhere in a localised pattern, with bacteria adhering in dense clusters or micro colonies.

The initial observation by Knutton *et al*⁸ that the A/E lesion was found to contain a high concentration of polymerised filamentous actin led to the development of the fluorescent actin staining (FAS) test. Prior to this, the A/E histopathology could be detected only by electron microscopy. This test uses fluorescein labelled phallotoxin that binds to filamentous actin⁸.

The possibility that a cytotoxin may play a role in the pathogenesis of EPEC infection had been suggested and had been looked for. Subsequently production of a toxin that was cytotoxic to Vero cells by some strains of EPEC was demonstrated⁹.

This was called the verocytotoxin. It was shown that the verocytotoxin and the shiga toxin were the same^{5,9}. These strains were subsequently classified as enterohaemorrhagic *Esch. coli*. This study was done to evaluate our EPEC isolates for two potential virulence factors namely entero-adhesiveness with the subsequent actin accumulation and the production of verotoxin. This was required as laboratories in India resort to serotyping to identify EPEC and the extent of its association with virulence markers among Indian strains is not known.

Material & Methods

Bacterial strains: *Esch. coli* isolated between January 1997 – June 1999 from stool samples of infants and children under 5 yr with diarrhoea were tested by slide agglutination using EPEC antisera (polyclonal raised in rabbits in the laboratory)¹⁰. Multiple colonies from each culture were screened. The EPEC antisera used included the following 'O' serogroups: O127, O26, O55, O86, O128, O126, O125, O124, O111, O119 which were raised in rabbits in our laboratory using standard methods¹⁰. The phenotypic virulence markers of EPEC were investigated for 50 consecutive strains, which were isolated from cases of diarrhoea. All assays included non EPEC *Esch. coli* as negative controls.

HEp-2 adherence assay: The method used in the adherence assay was as described earlier¹¹. Monolayers of HEp-2 cells were grown in 10 spot multitest slide (ICN Biomedicals, USA). 40 μ l of overnight bacterial culture was added to 0.5 ml of minimum essential medium (MEM) (without antibiotics) containing 2 per cent foetal calf serum (FCS) and D-mannose 1 per cent w/v; these diluted bacterial cultures were added to the monolayer, and incubated at 37°C for three and six hours (2 sets). These were then washed with MEM (without antibiotics), fixed with methanol and stained with 10 per cent Giemsa for 30 min. The slides were then viewed under an oil immersion objective of a light microscope for adherence.

FAS test: The test was performed according to the method described previously⁶. Overnight bacterial culture was incubated with HEp-2 cells as for the adherence assay. After completion of the incubation period monolayer was fixed and stained with fluorescein isothiocyanate (FITC) labelled phallotoxin (Sigma, USA).

Monolayers were examined for the presence of punctate spots of intense fluorescence using a Leitz Ortholux II fluorescence microscope (Germany) and subsequently by phase contrast microscopy (Leitz Biomed, Leitz, Portugal).

Test for verotoxin production was carried out as previously described⁹ using the Vero cell cytotoxicity assay, in which bacteria free culture filtrates were grown in trypticase soy broth, added to monolayers of Vero cells in 96 well tissue culture plates. Plates were incubated at 37°C in a 5 per cent CO₂ incubator, and examined after 1 day and on the fourth day of incubation for rounding and detachment of the monolayer.

Results & Discussion

Of the 50 strains studied, the most common serotype was O55, which accounted for 11 of the 50, followed by serotypes O125 (9) and O126 (8). The majority of the strains studied belonged to the classical EPEC serogroups and were O55, O25, O119, O125, O126, O127, O128.

The HEp-2 adherence assay showed that 36 (72%) strains adhered in a localised pattern, and 11 (22%) adhered in a mixed pattern of localised and diffuse. One strain adhered in an aggregative pattern showing bacteria in a characteristic 'stacked brick' pattern and two strains were non adherent at the end of the 6 h incubation period. Eleven strains showed a combined pattern of adherence *i.e.*, localised with either diffuse or aggregative adherence (Table). The strain that showed an aggregative pattern of adherence belonged to serogroup O126. It has been reported that strains displaying an aggregative pattern of adherence are most often seen within serogroups O86, O111 and O126 and would probably belong to the category of enteroaggregative *Esch. coli*⁸.

The association between the HEp-2 adherence assay and the FAS test is shown in the Table. The ability of EPEC to adhere to the cells and produce A/E lesions has been attributed to the genes encoded on the pathogenicity island the 'locus of enterocyte effacement'. This includes the *Esch. coli* attaching and effacing gene (*eae* gene). Until the development

of the FAS test the A/E lesion could only be demonstrated by electron microscopy of intestinal biopsy sections or in experimental animals. The FAS test is a sensitive and specific test for detecting A/E histopathology produced by EPEC¹².

As seen in the Table, 48 of the 50 strains gave a positive FAS test while two were negative. Of these two, one showed an aggregative pattern of adherence and belonged to serogroup O126 and the other strain showed a mixed pattern of diffuse and aggregative adherence. The results obtained in our study are in accordance with previous reports^{5,8}. Diffuse and aggregative adherent strains are negative in the FAS test. The two strains that were negative by the HEp-2 adherence assay were positive by the FAS test, even after repeat Hep-2 adherence assay and FAS test. *Esch. coli* isolates that belong to EPEC serogroups that are FAS positive but EPEC adherence factor (EAF) probe negative and adhere poorly to HEp-2 cells have been reported¹⁶.

The Vero cell cytotoxicity assay showed that none of the strains studied produced cytopathic effect typical of verotoxin. The lack of verotoxin production was similar to other studies¹². One strain produced a cytopathic effect consisting of vacuolation and this strain was sent to the National Institute of Cholera and Enteric Diseases (NICED), Kolkata (India) for further characterisation. It was shown that this strain lacked the genetic potential for the production of verotoxin.

Of the 50 strains studied, 48 (96%) showed virulence properties associated with EPEC. These results indicate that in our laboratory there is a good probability that an *Esch. coli* strain identified on the basis of the slide agglutination with EPEC antiserum is actually a putative EPEC. So 'O' serogrouping is still an useful diagnostic test and remains the simplest bacteriological test for the presumptive identification of EPEC. However, identification of EPEC in the laboratory should not be based entirely on 'O' serogrouping but extended to serotyping. Demonstration of virulence markers either by phenotypic tests or by a genotypic test is essential. In this study, it was demonstrated by the FAS test that the most consistent phenotypic marker was the actin accumulation under the adherent bacteria.

Table. Association between findings of adherence assay and fluorescent actin staining (FAS) assay

Pattern of adherence	Adherence assay No. positive		FAS assay
	3 h	6 h	
LA	35	36*	Positive
DA	-	-	-
AA	1	1	Negative
LA + DA	6	6	Positive
LA + AA	4	4	Positive
AA + DA	1	1	Negative
NA	3*	2	Positive
Total	50	50	

NA, non adherent; LA, localised adherence; AA, aggregative adherence; DA, diffuse adherence

*One strain showed a localised pattern of adherence after the six hour incubation period

Hence, this is probably the single most useful test in the confirmation of EPEC.

References

1. Law D. Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. *Clin Microbiol Rev* 1994; 7: 152-73.
2. Gomes JAT, Rassi V, MacDonald KL, Ramos SRTS, Trabulsi LR, Vieira MAM, *et al*. Enteropathogens associated with acute diarrhoeal disease in urban infants in Sao Paulo, Brazil. *J Infect Dis* 1991; 164: 331-7.
3. Ghosh AR, Nair GB, Naik TN, Paul M, Pal SC, Sen D. Entero-adherent *Escherichia coli* is an important diarrhoeagenic agent in infants aged below 6 months in Calcutta, India. *J Med Microbiol* 1992; 36:264-8.
4. Varma M, Sachdeva V, Diwan N. Incidence of enteroadherence in diarrhoeagenic *Escherichia coli* in infants in Delhi. *J Commun Dis* 2000; 32: 161-8.
5. Giammanco A, Maggio M, Giammanco G, Morelli R, Minelli F, Scheutz F, *et al*. Characteristics of *Escherichia coli* strains belonging to enteropathogenic *E.coli* serogroups isolated in Italy from children with diarrhoea. *J Clin Microbiol* 1996; 34 : 689-94.
6. Knutton S, Phillips AD, Smith HR, Gross RJ, Shaw R, Watson P, *et al*. Screening for enteropathogenic *Escherichia coli* in infants with diarrhoea by the fluorescent actin staining test. *Infect Immun* 1991; 59:365-7.
7. Celli J, Deng W, Finlay BB. Enteropathogenic *Escherichia coli* (EPEC) attachment to epithelial cells: exploiting the host cell cytoskeleton from the outside. *Cell Microbiol* 2000; 2:1-9.
8. Knutton S, Baldwin T, Williams PH, McNeish AS. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Infect Immun* 1989; 57 : 1290-8.
9. Konowalchuk J, Speris JJ, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 1977; 18: 775-9.
10. Ewing WH. The Genus *Escherichia*. In : Edwards and Ewing's *identification of enterobacteriaceae*, 4th ed. New York: Elsevier Science Publishing Co; 1986 p. 93-134.
11. Kang G, Mathan MM, Mathan VI. Evaluation of a simplified HEp-2 cell adherence assay for *Escherichia coli* isolated from south Indian children with acute diarrhoea and controls. *J Clin Microbiol* 1995; 33 : 2204-5.
12. Nataro JP, Kaper JB. Diarrhoeagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; 11 : 142-201.

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