J Diarrhoeal Dis Res 1993 Jun;11(2):101 - 104
0253 - 8768/93 $ 1.00 + 0.10

Short Report

Epithelial Cell Invasiveness of Non-enteropathogenic Serotypes of *Escherichia coli*

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ABSTRACT

Current evidence suggests that enteropathogenic *Escherichia coli* (EPEC) of traditional serotypes possess a three-stage pathogenesis: localised adherence (LA) to, attachment-effacement (AE) of, and penetration of, enterocytes, all of which can be reproduced in tissue culture models *in vitro*. Three *E. coli* isolates of non-traditional serotypes (02:H2, 02:H25 and 015:H2) isolated from children with diarrhoea were previously shown to be positive for LA and AE activities in laboratory models. In the present study, they were, in addition, shown to be positive for invasion of a HEp-2 cell monolayer. These findings further establish the pathogenicity of non-traditional serotypes of *E. coli* and their role in the causation of diarrhoea.

Key words: Non-enteropathogenic serotypes of *E. coli*; Pathogenesis; Invasion.

INTRODUCTION

Five categories of diarrhoeagenic *Escherichia coli* have been described so far. They are: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAggEC) (1,2). Each category has distinct virulence factors, serotypes, and epidemiological features and produces characteristic clinical symptoms (1). EPEC was the first group of diarrhoeagenic *E. coli* to be identified, and the World Health Organization's definition of EPEC includes the following serogroups: 026, 055, 086, 0111, 0114, 0119, 0125, 0127, 0128, 0142 and 0158 (3).

Recent studies have identified two virulence phenotypes for EPEC: localised adherence (LA) and attachment-effacement (AE). LA phenotype is characterised by adherence of bacteria in micro-colonies to localised regions in the intestines of affected animals and cultured mammalian cells such as HeLa and HEp-2. It is associated with an ~60 MDa plasmid called EPEC adherence factor (EAF) plasmid, the product of which (called EAF) mediates LA (4). A DNA-probe constructed from EAF plasmid identifies LA+EPEC with a high sensitivity and specificity (5). The most likely candidate for EAF seems to be the recently identified bundle-forming pilus (BFP) protein of approximately 19.8 kDa in molecular weight (6).

AE is the name given to the characteristic lesion produced by EPEC in the intestines of affected animals (7). In this lesion, bacteria destroy the brush-border of enterocytes and closely adhere to the apical surface causing cupping and pedestal formation by the cell membrane, and electron-dense fibrillar modification in the terminal web areas beneath the attached bacteria (7). The fibrillar modification presumably involves polymerisation of actin filaments which can be detected in cultured cells by a fluorescent actin staining (FAS) assay (8). The genes for the AE lesion are located in the bacterial chromosome and a locus for this lesion has been recently identified. This gene called *eae* (for *E. coli* AE) has a 2817 bp open reading frame which codes for an outer membrane protein of 94 kDa (9).

Although both LA (plasmid-borne) and AE (chromosomal) properties are required by EPEC for full expression of pathogenicity, EPEC unable to

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cause LA retains the ability to cause diarrhoea, albeit less effectively than strains which possess the LA phenotype (10). It appears that the plasmid associated EAF may facilitate initial contact of the bacteria with the mucosa, after which the chromosomally encoded factors produce the AE lesions (11). Another property, which was overlooked until recently, is the ability to penetrate the intestinal epithelial cells. Intracellular bacteria have been noted in the intestinal mucosa of affected animals and humans (12,13). This prompted investigators to quantify the invasiveness of EPEC *in vitro* in tissue culture systems, such as HEp-2 and Henle-407 monolayers, and this showed that EPEC displayed marked invasiveness (14,15). Thus, it appears that there is a three-stage process in the pathogenesis of EPEC diarrhoea: localised adherence to, attachment-effacement of, and invasion of, enterocytes (11).

Epidemiological studies have suggested that LA+EPEC serotypes are significantly associated with childhood diarrhoea (16,17). However, the role of LA+non-EPEC serotypes is not clearly defined (18), although in one study (19), they have been found in a significantly higher proportion of patients with diarrhoea than in controls. This prompted us to study the critical property of AE in three LA+non-EPEC serotypes isolated from children with diarrhoea in Bangladesh. These strains belonged to 02:H2, 02:H25 and 015:H2 serotypes, respectively. They were found to be positive for AE lesions in the FAS assay (20) *in vitro* and in the inoculated rabbit ileal loop on light microscopic examination and ultrastructural studies (20). Based on this observation, we concluded that LA and AE-positive non-EPEC serotypes should also be considered diarrhoeagenic (20).

In the electron micrographs, we observed intracellular bacteria in the enterocytes of rabbit ileal loops inoculated with all three non-EPEC serotypes (20). Since carrying out this study, we have noticed that invasiveness has been considered an important virulence factor for EPEC serotypes (11,14,15). We were interested to find out whether the invasiveness of the three non-EPEC serotypes observed in ultrastructural sections of rabbit ileal loops can be reproduced and quantified *in vitro* in a tissue culture system using HEp-2 cells, as in the case of EPEC serotypes (11,14,15). The findings are reported in this communication.

MATERIALS AND METHODS

Bacteria. Three strains 21085, 5424, and 9254 used in a previous study (20) were tested. They belonged to the non-EPEC serotypes 02:H2, 02:H25, and 015:H2 respectively.

HEp-2 cell invasion assay. Epithelial cell penetration was quantified by using HEp-2 cells by the method of Small and Falkow (21) with some modifications (22). HEp-2 cells were maintained in minimum essential medium with Earle's salt and glutamine (MEM), supplemented with 10% foetal bovine serum and antibiotics (GIBCO Laboratories, Grand Island, NY, USA). Bacterial cells were grown as stationary cultures for 4 h in brain-heart infusion broth (DIFCO Laboratories, Detroit, MI, USA) at 37°C. They were then washed in phosphate-buffered saline (PBS, pH 7.4) and resuspended in MEM to approximately 2×10^7 organisms per ml. A 1-ml amount of this suspension was added to a HEp-2 cell monolayer (2×10^5 cells in a 6-ml vial [Kimble, Toledo, OH, USA] resulting in approximately 100 bacteria per cell) containing MEM, centrifuged at 200 X *g* for 10 min, and then incubated at 37°C for 2 h in a 5% CO_2 atmosphere. After washing the monolayer to remove non-adherent bacteria, it was incubated for 1 h in MEM containing 100 µg of gentamicin per ml to kill extracellular bacteria. After washing the monolayer, internalised bacteria were released by lysis of the monolayer with 1.0% Triton X-100 for 5 min and quantified by plate count. The positive and negative controls included were a strain of EPEC E2348/69 (serotype 0127:H6) (14) and a local derivative of original strain of *E. coli* K-12 (designated as *E. coli* K-12 EC101 [23]), respectively. Each strain was tested in triplicate thrice. The isolates were also exposed to gentamicin containing MEM (without HEp-2 cells) to ensure that they did not survive the antibiotic treatment.

Table. Invasion of HEp-2 cells by non-enteropathogenic serotypes of *E. coli* and control bacteria[a]

Bacteria	CFU of bacteria/ml: Inoculum[b]	CFU of bacteria/ml: Intracellular[c] (rage)	% of intracellular bacteria relative to inoculum (range)
Test bacteria			
E. coli 02:H2	2.0×10^7	2.0×10^4 ($1.8 \times 10^4 - 2.2 \times 10^4$)	0.1 (0.09 - 1.1)
E. coli 02:H25	2.0×10^7	4.0×10^4 ($3.7 \times 10^4 - 4.3 \times 10^4$)	0.2 (0.19 - 0.22)
E. coli 015:H2	2.0×10^7	3.0×10^2 ($2.5 \times 10^2 - 3.5 \times 10^2$)	0.002 (0.001 - 0.003)
Control bacteria			
E. coli E2348/69 (0127:H6)	2.0×10^7	2.4×10^4 ($2.2 \times 10^4 - 2.6 \times 10^4$)	0.12 (0.11 - 0.12)
E. coli K-12 EC101	2.0×10^7	0 (0 - 0)	0 (0 - 0)

[a] The data are based on triplicate samples from a typical experiment
[b] Bacteria inoculated into HEp-2 cell monolayers
[c] Bacteria surviving gentamicin treatment

RESULTS

The data on HEp-2 cell invasion of bacteria are shown in the table. It appeared that all the three

non-EPEC serotypes were invasive and the two isolates with 02 serogroups were as invasive as the positive control EPEC serotype strain E2348/69. The negative control *E. coli* K-12 EC101 did not invade the cells.

DISCUSSION

In EPEC diarrhoea, bacteria have been observed within enterocytes. This phenomenon *in vivo* has been reproduced *in vitro* using tissue culture systems and the intracellular bacteria have been quantified (14). However, the present report is the first report to quantify the invasiveness of non-EPEC serotypes using a tissue culture model *in vitro*. Thus, as in the EPEC diarrhoea (11), all three stages in the pathogenesis, viz: localised adherence to, attachment-effacement of, and invasion of, cells have also been demonstrated for non-EPEC serotypes. This further confirms our belief that strains not belonging to the EPEC serotypes might also be capable of causing diarrhoea and underscores the importance of defining EPEC by virulence properties rather than by serotypes.

The clinical significance of invasion in the pathogenesis of EPEC infection is not clear. EPEC strains do not cause invasive diarrhoea. However, it has been noted that EPEC can enter the blood stream, disseminate late in the infection (24,25), and cause sepsis (26). It is likely that a similar process might also be operative with regard to non-EPEC infection.

ACKNOWLEDGEMENTS

This research was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), which is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors include the aid agencies of the Governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, the Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, the United Kingdom and the United States; international organisations including the United Nations Development Programme, the United Nations Population Fund, the United Nations Children's Fund and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank Manzurul Haque for secretarial assistance.

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