Attaching-Effacing Lesions and Intracellular Penetration in HeLa Cells and Human Duodenal Mucosa by Two "Escherichia coli" Strains Not Belonging to the Classical Enteropathogenic "E. coli" Serogroups

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In the present study, we compared two strains of serotypes O88:H25 and O145:H45 with an enteropathogenic "Escherichia coli" (EPEC) adherence factor-positive (EAF⁺) strain of the classic enteropathogenic "E. coli" serotype O111ab:H2 for their association with HeLa cells and with biopsies of human duodenal mucosa. Both strains not belonging to the classic EPEC serotype showed virulence properties similar to those of the serotype O111ab:H2 strain, i.e., the production of attaching-effacing lesions and intracellular penetration in both systems. These virulence properties associated with the relatively high frequency at which the two serotypes had been detected in infant diarrhea in Säo Paulo, Brazil (T. A. T. Gomes, M. A. M. Vieira, I. K. Wachsmuth, P. A. Blake, and L. R. Trabulsi, J. Infect. Dis. 160:131-135, 1989) allowed us to suggest that strains of serotypes O88:H25 and O145:H45 should be included in the EAF⁺ EPEC category.

Enteropathogenic "Escherichia coli" (EPEC) serogroups have been identified as important agents of infant diarrhea in many developing countries (2, 3, 5, 6, 10, 11, 13, 14). More recently, only the following specific serotypes (classic serotypes) within these serogroups were recognized as belonging to the EPEC category: O26:H-, O26:H11, O55:H-, O55:H6, O55:H7, O86:H34, O111ab:H-, O111ab:H2, O111ab:H21, O114:H2, O119:H6, O125ac:H21, O126:H-, O126:H27, O127:H-, O127:H6, O128ab:H2, O128ab:H7, O142:H6, and O158:H23 (5).

In Säo Paulo, Brazil, a study conducted to examine the interrelationships of EPEC serotypes and serogroups, EPEC adherence factor (EAF) genes, and strains not belonging to the classic EPEC serogroups were detected, and localized adherence (LA) production by these strains in HeLa cells was confirmed. Although as a group these serotypes were not associated with diarrhea, at least three of them (O88:H25, O145:H45, and O153:H7) were individually found at frequencies that were higher than the frequency of EAF⁺ strains of some classic EPEC serotypes. However, other virulence mechanisms presently attributed to EAF⁺ EPEC serotypes, such as attaching-effacing (A/E) lesions (8) and intracellular penetration, should be searched for in these non-EPEC serotypes to better understand their role in infant diarrhea.

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FIG. 1. Electron micrographs of HeLa cells after incubation for 7 h at 37°C with O111ab:H2 (A), O88:H25 (B), and O145:H45 (C). Bacteria enclosed in membrane-bound vacuoles were seen in most cells (initial ratio, 4 × 10⁵ bacterial cells per HeLa cell) (arrows). Magnifications: A, ×8,000; B, ×6,000; C, ×3,125.

In the present study, we compared two E. coli EAF+ strains of serotypes O88:H25 and O145:H45 with an EAF+ strain of the classic EPEC serotype O111ab:H2 for their association with HeLa cells and with biopsies of human duodenal mucosa. We found that both strains not belonging to the classic EPEC serogroups had virulence properties similar to those of the serotype O111ab:H2 strain, i.e., the production of A/E lesions and intracellular penetration in both systems.

The three LA-positive EAF+ E. coli strains studied were isolated from infant diarrheic feces in which no other recognized enteropathogens had been identified (5) and were stored at −70°C. The eae probe (7) was used to detect DNA sequences associated with A/E lesions. All three strains reacted with this probe.

For the HeLa cell and duodenal mucosa assays, bacterial strains were grown overnight in 3 ml of tryptic soy broth (TSB). E. coli K-12 HB101 was used as a negative control. Invasion of HeLa cells was detected by the method of Miliotis et al. (12). After incubation for 7 h, infected HeLa cells were washed in phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 30 min, gently scraped, and washed in 9% saccharose (Sigma) in 0.05 M cacodylate buffer (pH 7.2). Cell pellets were placed in 1% agarose (Sigma), and small blocks were cut and prepared for transmission electron microscopy (TEM).

Cultured human intestinal mucosa assays were performed as described by Knutton et al. (8) with some modifications. Duodenal mucosa biopsies were taken from adult patients undergoing endoscopy after informed consent had been obtained. Biopsies were taken immediately to the laboratory after being placed in ice-cold NCTC 135 medium (Sigma) with 200 µg of gentamycin per ml and washed in medium without antibiotic. Two biopsy samples from different patients were placed on sterile filters (AP15; Millipore) in plastic petri dishes (35 by 10 mm; Corning). After the addition of 4.8 ml of the organ culture medium described by Embaye et al. (4) and containing 1% D-mannose (Sigma), the dishes were maintained for 15 min at 37°C in an incubator with 95% O2-5% CO2. Two hundred microliters of bacterial culture was added to the biopsy samples, and the mixtures were incubated for 2 h at 37°C with gentle agitation in an incubator with 95% O2-5% CO2. Biopsy samples were then washed six times in NCTC 135 medium and incubated with 5 ml of organ culture medium without antibiotic for an additional 6 h and with antibiotic for an additional 10 or 16 h under the conditions described above. After these incubations, the biopsy samples were washed and prepared for

![FIG. 2. SEM views of surfaces of human duodenal biopsies infected with O111ab:H2 (A), O88:H25 (B), and O145:H45 (C) for 8 h at 37°C. Bacteria can be seen (arrows) in contact with each other and attached to the epithelial surfaces. The surrounding epithelial surfaces appear unaltered. Magnifications: A, ×6,540; B, ×6,250; C, ×7,500.](http://iai.asm.org/)

![FIG. 3. TEM views of human duodenal biopsies infected with O111ab:H2 (A), O88:H25 (B), and O145:H45 (C) for 8 h at 37°C. Bacilli can be seen closely adherent to enterocyte membranes (arrowheads). The characteristic effacement of microvilli (mv), membrane cupping, and pedestal formation at the sites of attachment can be seen (stars). Magnifications: A, ×12,000; B, ×12,500; C, ×12,115.](http://iai.asm.org/)
TEM. For scanning electron microscopy (SEM), medium without antibiotic was always used.

The HeLa cell invasion assays performed with the three strains showed a few bacterial cells undergoing internalization and others enclosed in membrane-bound vacuoles (Fig. 1). *E. coli* K-12 HB101 was not associated with HeLa cells.

SEM of duodenal mucosa infected by O88:H25, O111ab:H2, and O145:H45 demonstrated that the mucosal surface was colonized by bacteria during 8 h of incubation (Fig. 2). For the same period, TEM demonstrated that the three *E. coli* strains caused characteristic A/E lesions, i.e., brush border effacement, cup and pedestal formation with bacterial and enterocyte membranes being in close proximity, and elongated microvilli located at sites of bacterial attachment (Fig. 3). After 12 h of incubation, a few bacterial cells of the three serotypes were observed to be undergoing internalization (Fig. 4). After 18 h of incubation, intracellular penetration by the three serotypes studied and bacteria free in the cytoplasm of enterocytes could be observed (Fig. 5). *E. coli* K-12 HB101 was not found in duodenal biopsy samples.

In a case control study, Echeverria et al. (3) reported the occurrence of EAF+ *E. coli* strains in 3.3% of infants with diarrhea and 1.9% of control infants. Although these strains did not show a statistical association with diarrhea, they were able to produce A/E lesions, as identified in the fluorescent-actin staining assay (9). More recently, Albert et al. (1) demonstrated that three EAF+ non-EPEC strains of serotypes O2:H2, O2:H25, and O15:H2 and isolated from infant diarrhea caused A/E lesions in HeLa cells, as detected by use of the fluorescent-actin staining assay and rabbit ileal segments.

In this study, besides causing A/E lesions, the non-EPEC serotype O88:H25 and O145:H45 strains were able to invade HeLa cells and human duodenal mucosa.

In a previous study conducted in our laboratory, strains of serotype O88:H25 were isolated from six patients with...
diarrhea but not from controls, this difference being statistically significant (5). Moreover, strains of serotype O145:H45, although not statistically associated with diarrhea (three patients and one control), occurred at frequencies similar to or higher than those observed for other recognized EAF+ EPEC serotypes, such as O55:H−, O86:H34, O127:H6, and O142:H6.

The relatively high frequencies at which serotypes O88:H25 and O145:H45 occurred in infant diarrhea in São Paulo, Brazil (5), and the demonstration of specific, EPEC-related virulence factors in a strain of each of these two serotypes suggest that when similar behavior is observed for other strains of these serotypes, these strains should be included in the EAF+ EPEC category.

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