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Escherichia coli O125ac:H6 Encompasses Atypical Enteropathogenic *E. coli* Strains That Display the Aggregative Adherence Pattern▽§

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O125 is an enteropathogenic *Escherichia coli* (EPEC) serogroup, which includes the O125ac:H6 serotype, defined as atypical EPEC. Strains of this serotype displayed the aggregative adherence (AA) pattern with HEp-2, Caco-2, T84, and HT-29 cells, possessed all the LEE region genes, and expressed intimin, Tir, and EspABD, although the attaching-effacing lesion was not detected in vitro. These results confirm that *E. coli* O125ac:H6 is atypical EPEC that displays the AA pattern and indicate the necessity of testing for EPEC genes combined with the determination of the adherence pattern for atypical EPEC identification.

Infectious diarrheal diseases are still a major public health concern, affecting mainly children in developing countries (19). *Escherichia coli* as the etiological agent of such infections is referred to as diarrheagenic *E. coli* and classified into six pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli*, and diffusely adherent *E. coli* (17).

Among these pathotypes, EPEC has been responsible for a vast number of cases of acute infantile diarrhea in Brazil and several other countries (1, 11, 13, 21, 27). The diagnosis of EPEC is commonly based on the serological determination of the lipopolysaccharide (O) antigen. However, the 12 EPEC O serogroups are composed of serotypes that may include different pathogens (32).

The EPEC serogroup O125 has been isolated from cases of diarrhea throughout the world (9, 11, 13, 30, 31). This serogroup mainly encompasses serotypes associated with the EAEC pathotype, i.e., *E. coli* strains displaying the aggregative adherence (AA) pattern with HeLa cells and that are reactive with the EAEC diagnostic probe (5). However, among the diversity of serotypes, O125ac:H6 shows an exclusive profile, including strains described as displaying a nondefined adherence pattern with HeLa cells and harboring the EPEC adhesin intimin-encoding gene (*eae*) (5). Further characterization of this serotype has demonstrated that these strains display the AA pattern with HEp-2 cells in the 6-h assay and lack EAEC virulence markers, including the EAEC probe (8). Since these strains carry the *eae* gene but not the EPEC adherence factor plasmid, they are classified as atypical EPEC (5, 8, 32). Therefore, the main objective of this study was to investigate the adherence patterns with different epithelial cell lines and the atypical EPEC attributes of strains belonging to this serotype.

For this purpose, six O125ac:H6 strains isolated in Brazil (strains EC292/84 and 1794/80), Germany (strains CB1924 and CB5304), and Australia (strains CB3114 and CB3338) were selected. The strains isolated from cases of diarrhea in Brazil were described previously (5). Strains CB1924 and CB5304

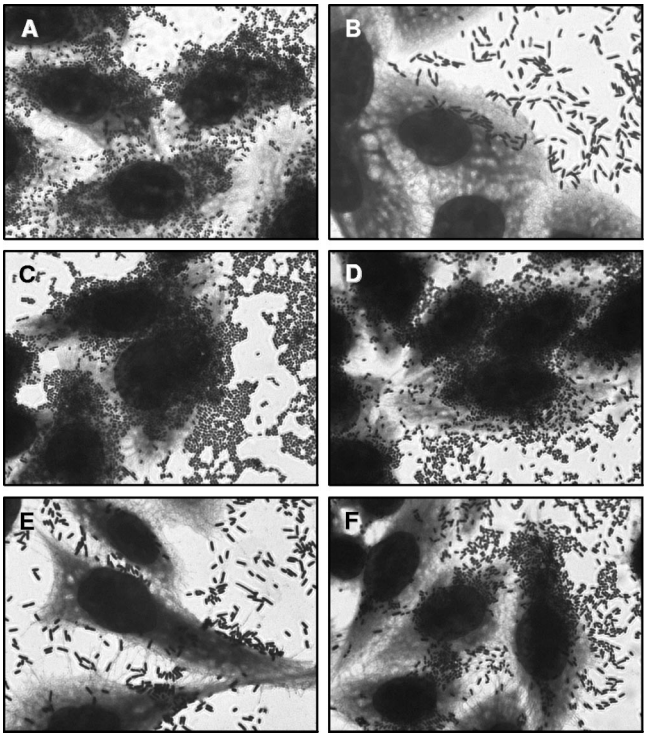

FIG. 1. AA pattern with HEp-2 cells (6-h assay) of six atypical EPEC strains belonging to the O125ac:H6 serotype. Strains EC292/84 (A), 1794/80 (B), CB1924 (C), CB3114 (D), CB3338 (E), and CB5304 (F) are shown. Cells were subjected to Giemsa and May-Grünwald staining. Original magnification, ×1,000.

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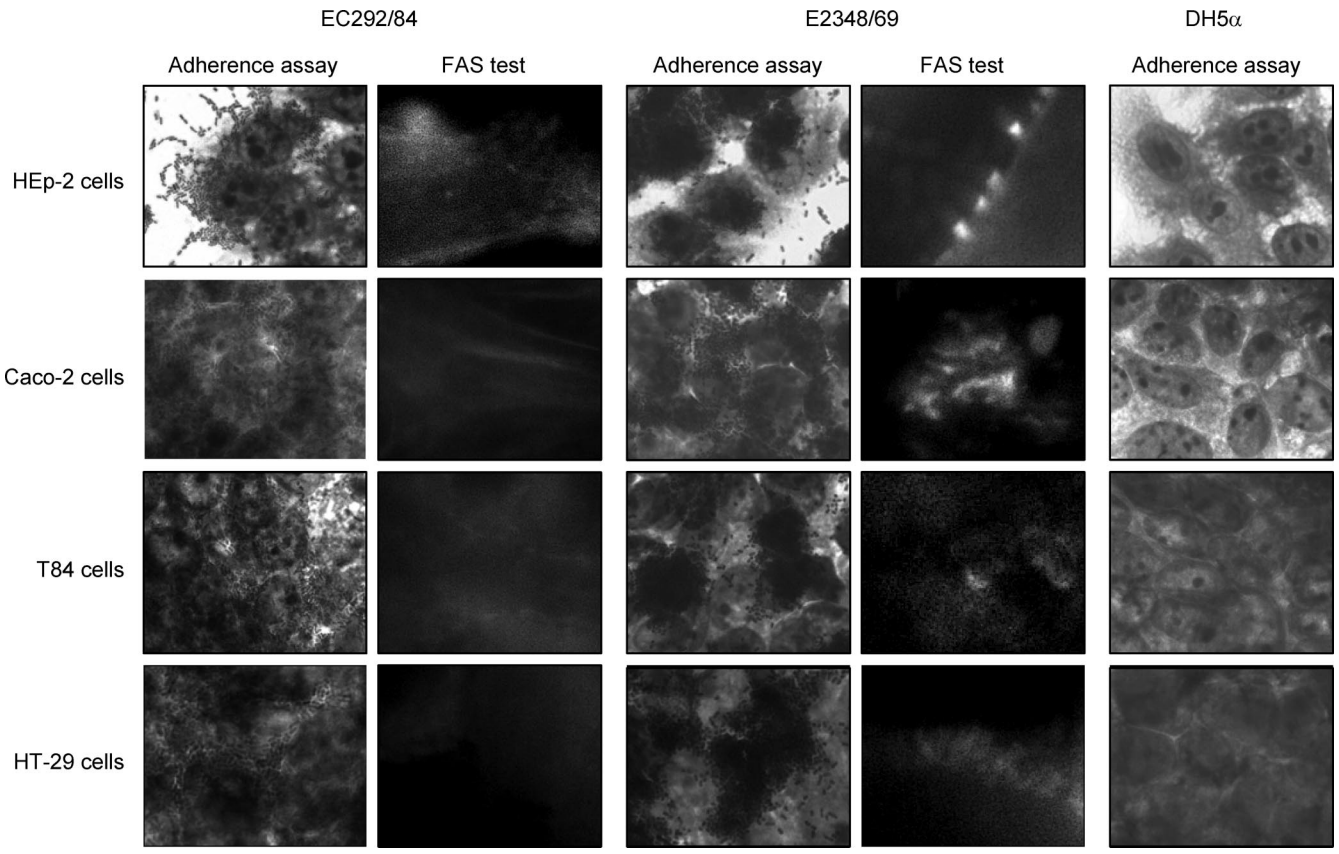

FIG. 2. Adherence and FAS assays (6-h assays). HEp-2, Caco-2, T84, and HT-29 polarized cells were infected with the representative O125ac:H6 atypical EPEC strain EC292/84. EPEC E2348/69 and *E. coli* DH5α were used as positive and negative control strains, respectively. Cells were subjected to Giemsa and May-Grünwald staining (adherence assays) or labeled with fluorescein isothiocyanate-phalloidin (FAS assays). Original magnification, ×1,000.

were isolated from cases of infantile diarrhea in Germany and belong to Lothar Beutin's laboratory collection. Strains CB3114 and CB3338 were isolated from a case of infantile diarrhea and from a healthy baby in Australia, respectively, and were kindly donated by Karl Bettelheim (University of Melbourne, Melbourne, Australia).

Initially, the pattern of adherence to HEp-2 cells was determined in 3- and 6-h assays, following the protocol described by Cravioto et al. (4). Figure 1 shows that all six strains displayed the AA pattern, since they adhered to the cells and to the coverslip surface in a stacked-brick pattern (24) after 6 h of incubation. However, bacteria were predominantly found adhered to the coverslip surface, which has been considered a variation of the AA pattern (14). It is interesting to note that the adherence to HeLa cells was much less intense than that displayed on HEp-2 cells (data not shown), which can explain the early description of this serotype as demonstrating a noncharacteristic adherence pattern with HeLa cells (5).

Expression of AA in the adherence assay is the main characteristic that defines the EAEC pathotype (17). However, strains belonging to the O125ac:H6 serotype have been described as harboring the *eae* gene (5, 8). For this reason, the capacity to induce the histopathological lesion on epithelial cells, known as the attaching-effacing (AE) lesion (23), was investigated by means of the fluorescent-actin staining (FAS) assay, which detects actin accumulation under the adherent bacteria (18). All six strains were unable to cause the AE lesion in HEp-2 cells after 3 or 6 h of bacterium-cell interaction. Figure 2 shows the FAS-negative reaction of a representative strain (EC292/84) after a 6-h period.

Due to the fact that a difference in AA pattern expression between HEp-2 and HeLa cells was observed with our strains, we also investigated the adherence pattern and the capacity of these strains to cause AE lesions with some intestinal cell lines cultivated in vitro. For this purpose, strains EC292/84 and 1794/80 were selected for the 3- and 6-h adherence assays employing Caco-2, T84, and HT29 polarized cell lines cultivated in vitro (25, 26). As shown in Fig. 2, the representative strain EC292/84 demonstrated a similar AA pattern, as displayed by both strains on HEp-2 cells, with these three other cell lines, as well as the inability to cause the AE lesion, indicated by the negative FAS test.

Recently Bai et al. (2) demonstrated that strains belonging to the O125:H6 serotype lack the ability to utilize either the Nck or TccP/TccP2 pathway to activate the N-WASP protein and are therefore unable to activate actin polymerization in vitro, the basis of the AE lesion. However, such a phenotype could be observed by employing human intestinal biopsies, demonstrating that strains of this serotype colonize the intestinal mucosa via Nck- and TccP-independent mechanisms. Our

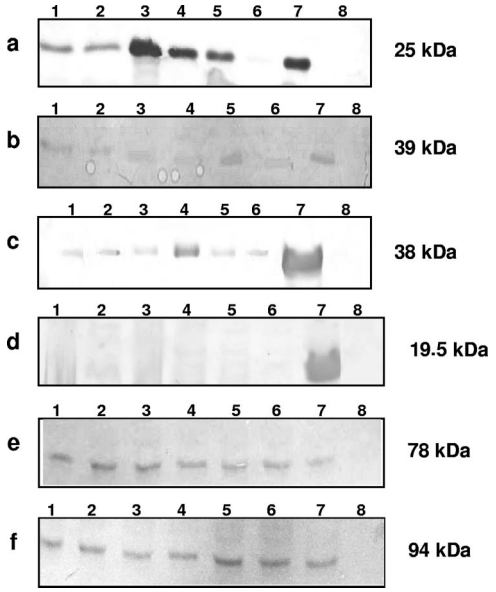

FIG. 3. Western immunoblot analyses of EspA (a), EspD (b), EspB (c), bundle-forming pilus (d), Tir (e), and intimin (f) in O125ac:H6 atypical EPEC strains. Secreted (a to c) or whole-cell (d to f) proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, and the transferred nitrocellulose membranes were immunodetected with the corresponding polyclonal antiserum. Lanes: 1, EC292/84; 2, 1794/80; 3, CB1924; 4, CB5304; 5, CB3114; 6, CB3338; 7, positive controls (E2348/69 [a to d and f] or EDL933 [e]); 8, negative controls (UMD872 [a], UMD870 [b], UMD864 [c], JPN15 [d], or *E. coli* DH5α [e and f]). The apparent molecular masses are indicated on the right.

data acquired by employing three different polarized cell lines of intestinal origin support the idea that in vitro assays of the capacity to cause AE lesions has limitations (2). In fact, strains EC292/84 and 1794/80 were negative for the *tccP* and *tccP2* genes, as determined by PCR (see the supplemental material), and tyrosine phosphorylation of the intimin translocated receptor (16) was not observed in these strains, which is in agreement with previous reports (2, 28). However, these previous works have not distinguished the O subgroups (ab and ac) of the O125:H6 strains.

All genes necessary to mediate the expression of the AE lesion are located in a pathogenicity island known as the locus of enterocyte effacement (LEE) (22). The presence of 31 genes of LEE was investigated by PCR for strains EC292/84 and 1794/80 (see the supplemental material). All genes assayed were detected in both strains, demonstrating the presence of the LEE genes. The expression of the main proteins involved in the establishment of the AE lesion was also examined, employing immunoblots of secreted or whole-cell proteins and specific polyclonal antisera (10, 20, 29, 33). As presented in Fig. 3, the expression of intimin, Tir, and EspABD was observed in all six strains, as was the lack of bundle-forming pilus expression, supporting the classification of O125ac:H6 as atypical EPEC. These data indicate that the main LEE-encoded proteins are expressed in vitro but there are differences in signal transduction between cultured epithelial cells and intestinal mucosa, since the AE lesion is expressed only in human biopsies (2).

Finally, the presence of three additional EAEC virulence factors was investigated for all the strains, since the lack of the *aatA*, *aggA*, *aafA*, *aggR*, *aap*, *shf*, *pet*, *pic*, *irp2*, and *astA* genes has been previously reported (8). The AAF/III usher (*agg3C*) and pilin (*agg3A*) subunits (3), the EAEC major subunit of type IV pili (*pilS*) (6), and the *aaiA* gene of a pathogenicity island inserted at *pheU* site of the chromosome of EAEC 042 (7) were sought by PCR (see the supplemental material), and none of them was detected. Therefore, our strains are devoid of the main plasmid and chromosomal virulence markers of EAEC described so far (15).

We conclude that the strains of serotype O125ac:H6 are atypical EPEC strains which express the AA pattern in different cultured epithelial cells. The fact that they are devoid of all EAEC virulence markers contributes to this classification. Atypical EPEC strains displaying aggregative, diffuse, or localized adherence have been described, indicating the heterogeneity of this subgroup of EPEC (12, 33). The characterization of the adhesin mediating the AA pattern of this serotype is currently under investigation in our laboratories.

In summary, our data clearly demonstrate the importance of complementing the serological diagnosis of EPEC by employing adherence assays and genetic detection of virulence markers. Moreover, O-antigen subgrouping is also indicated.

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