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Characterization of Atypical Enteropathogenic *Escherichia coli* Strains That Express Typical Localized Adherence in HeLa Cells in the Absence of the Bundle-Forming Pilus[▽]

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The characterization of nine atypical enteropathogenic *Escherichia coli* strains expressing localized adherence in HeLa cells in the absence of the bundle-forming pilus revealed a diversity of serotypes, plasmids, and virulence genes. Although the strains lacked known *E. coli* adhesin genes, the identification of new adhesins could contribute to the characterization of similar enteropathogenic *E. coli* isolates.

The term "enteropathogenic *Escherichia coli*" (EPEC) was first used by Neter et al. (16) to distinguish certain *E. coli* serogroups (somatic O groups) that were associated with diarrhea from those found in patients with extraintestinal infections or in healthy individuals.

The main EPEC virulence mechanism is the ability to promote attaching-effacing (A/E) lesions, a property that is also observed in strains of enterohemorrhagic *E. coli* (EHEC), another diarrheagenic *E. coli* pathotype that produces Shiga toxins (Stx). In A/E lesions, intimate bacterial adherence to epithelial cells is observed along with localized destruction of microvilli and the formation of actin-rich pedestal-like structures on the apical cell membrane (15). The various proteins involved in the establishment of A/E lesions are encoded in a pathogenicity island named the *l*ocus of *e*nterocyte *e*ffacement (LEE) (14). They comprise regulatory proteins, the adhesive outer membrane protein intimin, its translocated receptor (*t*ranslocated *i*ntimin *r*eceptor [Tir]), and other effector proteins that are injected into the affected eukaryotic cell by a type III secretion system (5, 6).

EPEC strains also carry the large *E*PEC *a*dherence *f*actor (EAF) plasmid (pEAF), which encodes the *b*undle-*f*orming *p*ilus (BFP), a type IV fimbrial adhesin, and Per (*p*lasmid-*e*ncoded *r*egulator), a complex regulator of virulence genes (12).

Since many epidemiological studies in distinct geographic regions have detected LEE-positive *E. coli* strains devoid of pEAF and the Stx-encoding genes, in 1995 the EPEC pathotype was further subgrouped into typical EPEC (tEPEC) strains, which carry pEAF, and atypical EPEC (aEPEC) strains, which are devoid of this plasmid (12, 21). Although many aEPEC strains belong to the traditional EPEC serogroups, it has been shown that various aEPEC strains belong to non-EPEC serogroups (21).

For many decades, tEPEC was the main bacterial enteropathogen in infants in developing countries. However, recent studies in Brazil have shown a decrease in their isolation rates and an apparent increase in the frequency of aEPEC (9, 17, 18, 21), but the role of the latter group in diarrhea has not been established. Since aEPEC strains show a very diverse repertoire of virulence genes, studies of subgroups of strains sharing specific properties might help in the identification of novel virulence factors and/or combinations of known virulence genes that could confirm their role as enteropathogens (10, 22).

tEPEC strains produce localized adherence (LA) to HeLa/HEp-2 cells within 3 h of incubation, with compact bacterial microcolonies being formed by means of BFP. In contrast, aEPEC strains, which lack BFP expression, generally present the LA-like (LAL) pattern of adherence, in which loose bacterial microcolonies are produced (21). However, while characterizing a collection of aEPEC strains of non-EPEC serogroups with regard to various phenotypic and genotypic traits, Vieira et al. (22) identified nine strains that produced compact LA much like that produced by tEPEC, despite the fact that this phenotype was detected only in prolonged assays (6 h). Thus, in the present study we sought to analyze the LA-producing aEPEC strains lacking BFP expression by comparing published and new data with regard to various virulence traits.

The origin and properties of the nine strains studied are presented in Table 1. They were isolated from five children (16 to 41 months old) with diarrhea and four without diarrhea in the city of São Paulo, São Paulo State, Brazil (22). Figure 1 presents the LA patterns (at 6 h) (LA_6) of HeLa cells of four representative aEPEC strains compared to the LA pattern (at 3 h) of tEPEC strain E2348/69 and the LAL pattern of aEPEC strain 1711-4. The LA pattern (at 6 h) was observed in HeLa cells for all strains studied. The nine strains belonged to at least eight distinct serotypes, demonstrating that they comprised distinct clones (22). A lack of the *bfp* operon in the nine strains was presumed previously because of the absence of the *bfpA*

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TABLE 1. Origins and properties of nine atypical EPEC strains of non-EPEC serogroups expressing typical localized adherence in HeLa cells in the absence of the bundle-forming pilus

Strain	Presence of diarrhea	Patient age (mo)	Serotype[a]	Size(s) (MDa) of plasmid band(s)[b]	Intimin type[c]	Virulence genotype[d]
0471-1	Yes	24	ONT:H19	60.0, 42.7	NT	*eae irp2*
0621-6	Yes	16	O41:H$^-$	103.2, 80.0	NT	*ler eae paa*
1331-2	Yes	35	O70:H2	68.0	NT	*ler eae irp2*
1551-2	Yes	23	ONT:H$^-$		NT	*eae*
2041-1	Yes	41	ONT:H2	39.0	NT	*ler eae*
1112-6	No	24	R:H11,21	73.0	NT	*ler eae irp2 astA pet hly*
2932-2	No	34	O153:H7	63.4	β	*ler eae*
3522-6	No	23	ONT:H11	70.0, 24.5	NT	*eae irp2 astA pet hly*
4632-3	No	16	ONT:H$^-$	50.0	β	*ler eae irp2 astA lpfA*

[a] NT, nontypeable with antisera for O1 to O173 and H1 to H56; H$^-$, nonmotile; R, rough strains.
[b] Includes only high-molecular-mass (>15-MDa) bands.
[c] NT, nontypeable with the sequences tested (α, β, γ, δ, ε, ι, and ζ).
[d] The *E. coli* virulence sequences tested were as follows: previously tested sequences included *bfpA*, *perA*, EAF, *E-hly*, enteroaggregative *E. coli*, *aggR*, *aggC*, *aafC*, *aspU*, *shf*, *irp2*, *pet*, *pic*, *astA*, *pap*, *afa*, *sfa*, *daaC*, *cdt*, *cnf*, and *hly* (10, 22); and sequences tested in this study were *saa*, *paa*, *toxB*, *iha*, *lda*, *lpfA*, and *ler*.

gene (encoding the BFP pilin) (22); this observation was confirmed in this study by immunogold experiments (not shown).

Vieira et al. (22) previously demonstrated that these nine strains were potentially able to promote A/E lesions in HeLa and Caco-2 intestinal cells, since they were positive in a fluorescent actin staining test (13). In this study, we confirmed the ability to promote A/E lesions in HeLa cells for all strains by transmission electron microscopy. Figure 2 shows pedestal formation underneath intimately adherent bacteria (characteristic of A/E lesions) of one representative strain (1551-2).

The occurrence of large plasmids compatible with pEAF was analyzed in agarose gels after extraction by the alkaline lysis method (4). Although eight of the nine strains presented one or two high-molecular-mass plasmid bands (between 27 and 110 MDa), one strain (1551-2) presented no plasmids (Fig. 3; Table 1). Moreover, only two strains (0471-1 and 2932-2) carried plasmid bands of 60 to 65 MDa, which is compatible with the size of pEAF. These results suggest that the compact LA

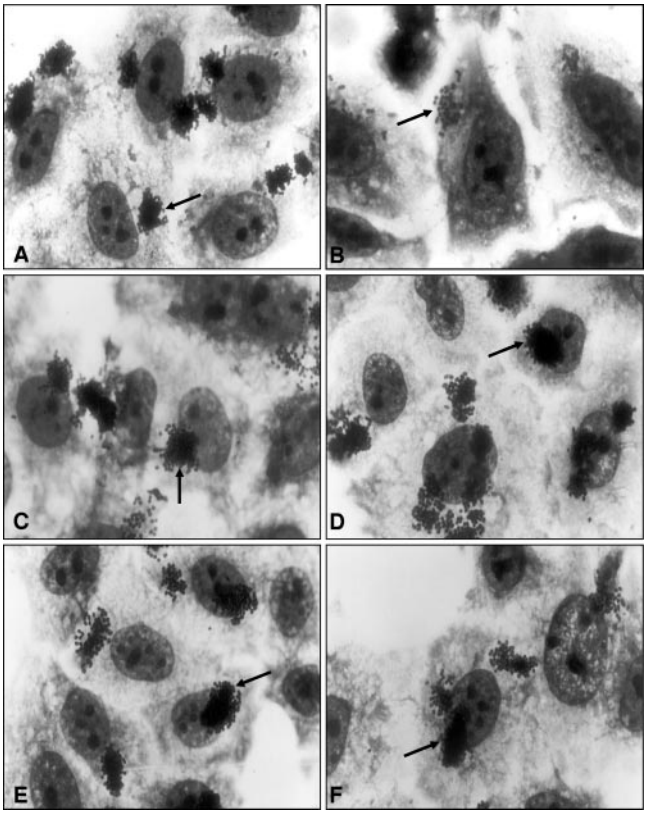

FIG. 1. Localized adherence patterns in HeLa cells of representative atypical EPEC strains that lack the bundle-forming pilus. (A) LA pattern (compact microcolonies, 3 h) of typical EPEC strain E2348/69; (B) LAL pattern (loose microcolonies, 6 h) of atypical EPEC strain 1711-4; (C to F) LA_6 (compact microcolonies, 6 h) of four representative atypical EPEC strains, namely, strain 0621-6 (C), strain 1331-2 (D), strain 1551-2 (E), and strain 1112-6 (F). Original magnification, ×1,000.

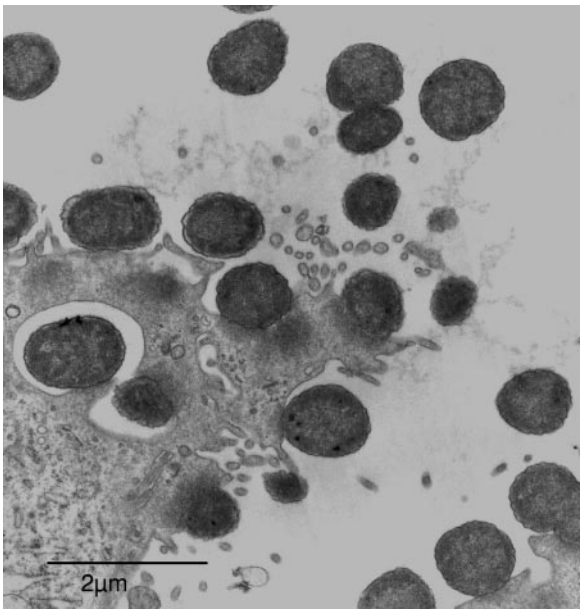

FIG. 2. Transmission electron micrograph of HeLa cells infected with a representative atypical EPEC strain of a non-EPEC serogroup, i.e., strain 1551-2, that expresses typical localized adherence (LA_6) in the absence of the bundle-forming pilus. Note the pedestal-like structures that characterize the A/E lesions underneath intimate adherent bacteria, which were observed for all nine strains studied.

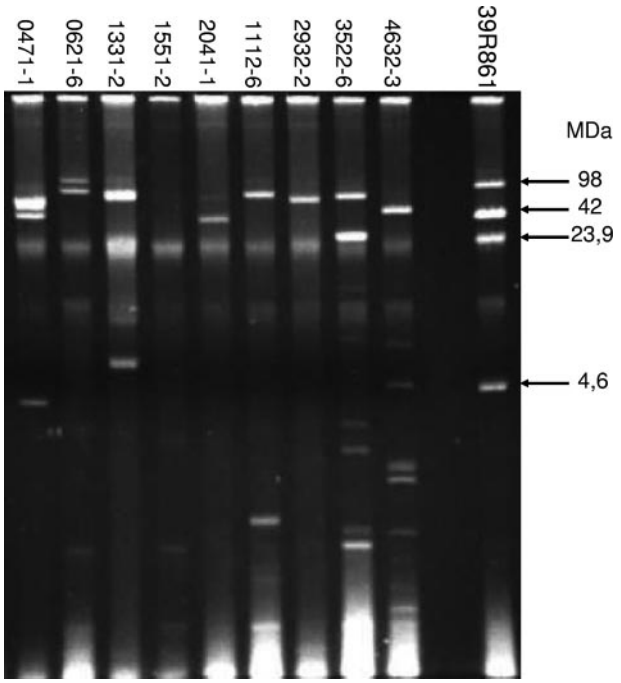

FIG. 3. Plasmid contents of nine atypical EPEC strains of non-EPEC serogroups expressing typical localized adherence (LA_6) in HeLa cells in the absence of the bundle-forming pilus. Lanes: 1, strain 0471-1; 2, strain 0621-6; 3, strain 1331-2; 4, strain 1551-2; 5, strain 2041-1; 6, strain 1112-6; 7, strain 2932-2; 8, strain 3522-6; 9, strain 4632-3; 39R861, *E. coli* strain carrying plasmids of known molecular sizes.

sites in these aEPEC strains probably result from the expression of one or more chromosomal adhesin genes.

The intimin subtypes of the strains were previously analyzed by PCR for 5 (α, β, γ, δ, and ε) (22) of 10 known intimin subtypes (1, 23). In the present study, this analysis was extended to two other intimin subtypes (ι and ζ) (23); it was observed that two strains carried intimin β and that the seven remaining strains carried a nontypeable intimin (Table 1).

The involvement of various adhesive structures which were recently described for *E. coli*, i.e., Saa (Shiga toxin-producing *E. coli* autoagglutinating adhesin), Paa (porcine A/E-associated gene), ToxB (a plasmidial locus found in EHEC O157:H7 implicated in adhesion), Iha (IrgA-homologous adhesin), Lpf (long polar fimbria), and Lda (locus for diffuse adherence), in the adherence properties of the strains studied was sought by PCR, as previously reported (2, 3, 8, 11, 19, 20). The presence of *ler* (LEE-encoded regulator) was evaluated as reported by Deng et al. (7). The data regarding the virulence gene sequences found were combined with previously reported data (10, 22) and are presented in Table 1. As observed with serotyping, the strains also differed regarding their combinations of virulence genes. Except for *paa* and *lpfA*, which were present in one strain each (0621-6 and 4632-3, respectively), the remaining adhesin-encoding genes were not found. Interestingly, three strains lacked the regulatory gene *ler*, or the sequences of this gene in these strains differed in the primer annealing regions.

In summary, we report the characterization of a collection of nine aEPEC strains of non-EPEC serogroups that share the ability to adhere to HeLa cells, forming compact microcolonies very similar to those formed by tEPEC. This phenotype is interesting because most aEPEC strains form the LAL pattern of adherence in this cell lineage, regardless of the serogroups to which they belong (21). Despite their common phenotype, we demonstrated that these strains are diverse with regard to their serotypes, plasmid contents, and profiles of virulence genes. Furthermore, the nature of the structure that mediates bacterial aggregation within the microcolonies is not known. Our data suggest that it is unrelated to recently described adhesin genes reported for other *E. coli* pathotypes.

Notwithstanding the large genotypic and phenotypic diversity found, it is still possible to presume that the strains studied bear a common new adhesive structure involved in the formation of compact microcolonies, which could be used as a marker for the characterization of such aEPEC isolates. Therefore, future studies should focus on the structure involved in the formation of compact microcolonies in aEPEC strains in the absence of BFP. Strain 1551-2 is a natural candidate for such a study, since it neither produces plasmid bands nor carries additional known virulence genes that could contribute to this phenomenon.

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