Adherence Factors in Atypical Enteropathogenic *Escherichia coli* Strains Expressing the Localized Adherence-Like Pattern in HEp-2 Cells[▽]

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Although atypical enteropathogenic *Escherichia coli* (aEPEC) strains are frequently implicated in childhood diarrhea in developing countries, not much is known about their adherence properties. The phenotypic and genotypic characterization of 29 aEPEC strains expressing the localized adherence-like pattern points toward the involvement of *E. coli* common pilus (ECP), intimins, and other known *E. coli* adhesins in this pattern.

Atypical enteropathogenic *Escherichia coli* (aEPEC) strains are increasingly recognized as an emerging pathotype responsible for childhood diarrhea in many countries (2–4, 19, 23). Atypical EPEC strains together with typical EPEC (tEPEC) strains constitute two distinct groups of organisms that have in common the locus of enterocyte effacement (LEE), a pathogenicity island responsible for the development of attaching-effacing (A/E) lesions. This island encodes the type III secretion system with multiple secreted proteins and a bacterial adhesin called “intimin” encoded by the *eae* gene (12, 16–18). Unlike tEPEC, aEPEC strains do not possess the EPEC adherence factor (EAF) virulence plasmid that contains the bundle-forming pili (BFP) responsible for a localized adherence (LA) pattern on cultured epithelial cells (5, 8, 28), but aEPEC strains display different adherence patterns. The typical EPEC strain exhibits only the LA pattern, while aEPEC strains display LA-like (LAL), diffuse adherence (DA), or aggregative adherence (AA) patterns (1, 10, 26, 32, 33). In addition, aEPEC strains belong to serotypes other than those of tEPEC strains (1, 10, 32, 33).

Despite the clear differences in adherence patterns, only the factors mediating the LA pattern have been extensively studied in tEPEC, and little is known about the factors mediating adherence of aEPEC strains. In a previous study, we identified in an aEPEC strain belonging to the O26 serogroup an adhesin gene designated as *lda*, for “locus for diffuse adherence,” which encodes a nonfimbrial structure conferring the DA phenotype (27).

Recently, in a collection of 126 aEPEC strains isolated from Brazilian children, we found many putative *E. coli* adhesin-encoding genes besides *lda* (25), such as *efal* (enterohemorrhagic *E. coli* [EHEC] factor adherence 1) (13, 20), *tox*B (a plasmidial locus found in EHEC O157:H7 implicated in adhesion) (31), *lpfA* (long polar fimbriae) (9), *iha* (IrgA-homolo-

gous adhesion) (30), and *paa* (porcine A/E-associated gene) (6). In the present study, we characterize these strains regarding intimin types, HEp-2 adherence patterns, and ability to promote actin accumulation in vitro.

The *eae* gene was subtyped according to the restriction fragment length polymorphism assay described by Ramachandran et al. (21). This method permits detection of the intimin types α , β , γ , ϵ , ζ , θ , ι , κ , λ , ν , ξ , $\omicron$, ρ , and σ . As can be seen in Table 1, intimins α (22 strains), β (26 strains), and γ (20 strains) were the most frequently found types. Forty-three (34%) strains had a nontypeable intimin. Intimins ξ , ι , κ , λ , and ν were not found among the entire collection of 126 aEPEC strains. In agreement with previous data (10), a close relationship between classic serotypes and intimin types was seen in this study: intimin α was found mainly among O142:H2 strains, and intimin β was detected in O26:H11, O119:H2, and O128:H2 strains, whereas intimin γ was seen in O55 strains.

The HEp-2 adherence patterns of aEPEC strains were determined according to the method described by Scaletsky et al. (28). Infected monolayers were examined after a 3-h incubation period, and when the adherence pattern was weak or negative, a new preparation was made and examined after a 6-h incubation period. Twenty-nine aEPEC strains (23%) showed the LAL pattern, characterized by the presence of loose bacterial clusters in the 3-h assay and compact clusters, identical to LA of tEPEC, in the 6-h assay (Fig. 1). Other less frequently found patterns included aggregative adherence (AA) (three strains) and diffuse adherence (DA) (two strains), with both patterns detected only in the 6-h assay. Eleven strains (8.7%) promoted cell detaching. Thirty-two strains (25.4%) did not adhere to HEp-2 cells in the 3-h assay, but in the 6 h assay showed an indeterminate adherence pattern. Finally, 49 strains (38.9%) were nonadherent after 6 h. Comparable results were reported by others who found LAL to be the most frequent adherence pattern among aEPEC strains, whereas AA and DA patterns were found in lower frequencies (1, 10, 32).

The ability of adherent strains to cause A/E lesions was evaluated by the fluorescent-actin staining (FAS) test (15). All but two adherent aEPEC strains were able to cause the A/E lesions, indicating the functionality of the LEE region (16).

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TABLE 1. Characteristics of 126 atypical enteropathogenic *Escherichia coli* strains^a

Serotype (no. of strains)	Intimin type	No. of strains with HEp-2 adhesion pattern:						FAS test result (no. of strains)	Adhesin gene(s) ^b
		AA	DA	LAL	IA	NA	DE		
O4:H4 (2)	μ		2					+	<i>iha</i>
O15:HND (2)	NT					2		NT	<i>lpfA iha</i>
O26:NM (3)	NT			2	1			+	<i>toxB</i>
O26:NM (3)	NT					3		NT	
O26:H11 (1)	β			1				+	<i>efa1 toxB lpfA iha paa</i>
O26:HND (3)	β			3				+	<i>efa1 toxB lpfA iha paa lda afa</i>
O33:H6 (2)	γ				1	1		NT	<i>lpfA</i>
O35:H19 (2)	ε				1	1		NT	<i>lpfA</i>
O37:NM (1)	NT					1		NT	<i>efa1 toxB</i>
O49:HND (1)	NT					1		NT	<i>toxB</i>
O55:NM (3)	γ				3			+	<i>efa1</i>
O55:HND (2)	γ			2				+	<i>efa1 lpfA iha</i>
O61:HND (1)	NT					1		NT	<i>iha</i>
O63:HNM (1)	NT					1		NT	<i>toxB</i>
O79:HND (1)	NT					1		NT	
O85:H40 (1)	NT			1				+	<i>lpfA iha</i>
O96:NM (1)	NT					1		NT	
O98:HND (1)	NT				1			+	<i>toxB lpfA paa</i>
O101:NM (1)	λ					1		NT	
O103:NM (2)	α, β				2			+	<i>lpfA iha</i>
O105:H7 (1)	γ			1				+	<i>efa1 lpfA</i>
O108:H31 (2)	γ						2	NT	<i>efa1 lpfA paa</i>
O109:H54 (1)	NT					1		NT	<i>efa1</i>
O111:NM (1)	α			1				+	<i>lpfA iha paa</i>
O111:NM (2)	NT			2				+	<i>lpfA</i>
O111:NM (1)	NT					1		NT	
O114:NM (1)	NT					1		NT	
O117:HND (1)	NT					1		NT	
O119:H2 (8)	β			1	4	3		+	<i>efa1</i>
O119:HND (2)	β			2				+	<i>efa1 lpfA afa</i>
O125:HND (1)	α					1		NT	
O126:NM (1)	NT			1				+	
O127:NM (3)	α			1		2		+	<i>afa</i>
O127:H40 (2)	γ			2				+	<i>lpfA</i>
O128:HNT (2)	β			1	1			+	
O132:HND (1)	NT					1		NT	
O141:NM (1)	NT					1		NT	
O142:H2 (3)	α		3					+	<i>efa1 paa</i>
O142:HNT (1)	α					1		NT	<i>paa</i>
O142:HNT (6)	α				3		3	+	
O153:H11 (1)	θ				1			+	<i>efa1 lpfA iha paa</i>
O153:H11 (1)	θ				1			+	<i>efa1 lpfA</i>
O156:H16 (1)	α					1		NT	
O157:NM (1)	δ			1				+	<i>iha lda</i>
O157:NM (1)	δ				1			+	<i>iha</i>
O157:NM (1)	δ				1			+	
O167:H6 (1)	NT				1			+	<i>iha</i>
O169:H6 (1)	NT					1		NT	
O175:HND (1)	NT					1		NT	
ONT:H18 (2)	v			2				+	<i>efa1 toxB lpfA iha paa lda</i>
ONT:HND (4)				2	2			+	<i>efa1 toxB lpfA iha paa</i>
ONT:HND (2)					2			+	<i>efa1 iha paa afa</i>
ONT:HND (1)					1			+	<i>efa1 paa afa</i>
ONT:HND (2)						2		NT	<i>paa</i>
ONT:HND (31)		3			5	17	6	+	
Total		3	2	29	32	49	11		

^a AA, aggregative adherence; DA, diffuse adherence; LAL, localized-like adherence; IA, indeterminate adherence; NA, nonadherence; DE, detachment; NT, nontypeable; v, variable.

^b DNA sequences previously tested included *bfpA*, *efa1*, *lpfA*_{O113}, *paa*, *toxB*, *iha*, *saa*, *spfA*, *lda*, and *afa* (25).

The strains presenting LAL resulted in areas of discrete and intense fluorescence in the FAS test after 6 h of incubation (data not shown). The strains presenting AA or DA were also able to accumulate actin in the adherence site. The ability to

aggregate actin in HEp-2 cells by LAL, AA, or DA has also been reported by others (1, 23).

The origins and properties of the 29 aEPEC strains expressing LAL are presented in Table 2. Most of the strains were

FIG. 1. Localized adherence-like (LAL) patterns in HEP-2 cells of representative classic aEPEC strains. (A and B) LAL patterns of strain HDV133-1 in the 3-h assay and 6-h assay, respectively. (C to E) LAL patterns of strains AMB6-3-3 (C), HSP23-5 (D), and SC241-1 (E) in the 6-h assay. (F) Localized adherence (LA) pattern of tEPEC strain E2348/69 in the 3-h assay.

isolated from patients with diarrhea, belonged to the classical EPEC serotypes, and, as previously reported, carried at least one known *E. coli* adhesin gene (25). As can be seen in Table 2, common genetic profiles, represented in boldface, could be observed among strains belonging to the same serotype. These common genetic profiles led us to evaluate the presence of common plasmids in the 29 aEPEC strains. One or two high-molecular-mass plasmid bands (between 23.9 and 98 MDa)

were found after DNA extraction by the alkaline lysis method (7) in all strains (Fig. 2), but no common plasmid profile could be detected. Interestingly, a plasmid band of 60 to 65 MDa was observed in almost all 29 strains, although none of them had the EAF plasmid (data not shown).

The involvement of the recently described *E. coli* common pilus (ECP) in the adherence properties of the 29 aEPEC strains displaying the LAL pattern was sought by PCR (22). All

TABLE 2. Origins and properties of 29 atypical EPEC strains presenting a localized adherence pattern to HEp-2 cells^a

Strain	Source	Serotype	Intimin type	FAS test result	<i>E. coli</i> adhesin gene(s) ^b
HDV133-1	Patient	O26:NM	NT	+	<i>toxB ecpA</i>
RN216-5	Control	O26:NM	NT	+	<i>toxB ecpA</i>
HSP7-1	Patient	O26:H11	β	+	<i>toxB ecpA efa1 lpfA iha paa</i>
AMB66-4	Patient	O26:HND	β	+	<i>toxB ecpA efa1 lpfA iha paa lda afa</i>
AMB44-7	Patient	O26:HND	β	+	<i>toxB ecpA efa1 lpfA iha paa lda afa</i>
RN451-1	Patient	O26:HND	β	+	<i>toxB ecpA efa1 lpfA iha paa lda afa</i>
AMB6-3	Patient	O55:HND	γ	+	<i>efa1 lpfA iha ecpA</i>
RP60-3	Patient	O55:HND	γ	+	<i>efa1 lpfA iha ecpA</i>
RN467-5	Patient	O85:H40	NT	+	<i>lpfA iha ecpA</i>
MA540-3	Control	O105:H7	γ	+	<i>lpfA lpfA ecpA</i>
HSP23-5	Patient	O111:NM	NT	+	<i>lpfA ecpA</i>
HSP28-8	Control	O111:NM	α	+	<i>lpfA ecpA iha paa</i>
MA256-1	Control	O111:NM	NT	+	<i>lpfA ecpA</i>
HSP11-1	Patient	O119:H2	β	+	<i>efa1 ecpA lpfA</i>
AMB118-1	Patient	O119:HND	β	+	<i>efa1 ecpA lpfA afa</i>
RP51-1	Patient	O119:HND	β	+	<i>efa1 ecpA</i>
MA343-4	Patient	O126:NM	NT	+	<i>ecpA</i>
MA428-1	Control	O127:NM	α	+	<i>ecpA</i>
HSP35-9	Patient	O127:H40	γ	+	<i>ecpA afa</i>
HSP37-1	Patient	O127:H40	γ	+	<i>ecpA afa</i>
HSP19-7	Patient	O128:NM	β	+	<i>lpfA ecpA</i>
SC241-1	Patient	O142:H2	α	+	<i>efa1 paa ecpA</i>
SC717-7	Patient	O142:H2	α	+	<i>efa1 paa ecpA</i>
RP294-1	Patient	O142:HND	α	+	<i>lpfA efa1 paa ecpA</i>
MA236-4	Control	O157:NM	δ	+	<i>iha lda ecpA</i>
MA225-4	Patient	ONT:H18	NT	+	<i>efa1 toxB lpfA iha paa ecpA lda</i>
RP254-1	Patient	ONT:HND	NT	+	<i>efa1 toxB lpfA iha paa ecpA lda</i>
MA474-2	Control	ONT:NM	NT	+	<i>efa1toxB lpfA iha paa ecpA</i>
RP178-1	Patient	ONT:HND	NT	+	<i>efa1 toxB lpfA iha paa ecpA</i>

^a NT, nontypeable; v, variable.^b DNA sequences previously tested included *bfpA*, *efa1*, *lpfA*_{O113}, *paa*, *toxB*, *iha*, *saa*, *spfA*, *lda*, and *afa* (25); the *ecpA* sequence was tested in this study. Common genetic profiles for strains belonging to the same serotype are in boldface.

strains were positive for *ecpA*, the pilin subunit of ECP. Although this pilus is widespread among *E. coli* strains, including nonpathogenic strains, evidence is accumulating that it may also contribute to epithelial cell adherence of commensal and pathogenic *E. coli* strains, including EHEC (22). In addition, ECP was shown to be an accessory factor contributing to the

multifactorial complex interaction of tEPEC, in association with BFP and other adhesins (24). Unlike the LA pattern of tEPEC which is mediated by multiple factors, including BFP, intimin, ECP, and possibly other adhesins, the mechanism involved in the LAL pattern is still unknown.

It is possible that ECP, in association with other adhesins, is

FIG. 2. Plasmid contents of aEPEC strains expressing the LAL pattern to HEp-2 cells. Lanes: 1, strain HDV133-1; 2, strain RN216-5; 3, strain AMB6-3; 4, strain RP60-3; 5, strain HSP23-5; 6, strain HSP28-8; 7, strain HSP11-1; 8, strain AMB118-1; 9, strain HSP35-9; 10, strain HSP37-1; 11, strain SC241-1; 12, strain SC717-7; 13, strain MA225-4; 14, strain MA245-1; MW, 39R861, an *E. coli* strain carrying plasmids of known molecular sizes.

able to compensate for the absence of BFP and permits bacteria to adhere with a localized pattern to cultured cells in a prolonged assay. All of the 29 aEPEC strains displaying the LAL pattern carried the *ecpA* gene, and all but two carried at least one of the known *E. coli* adhesion genes. In addition, almost all of the 29 aEPEC strains had some type of intimin. Recently, Hernandez et al. (11) showed that the compact microcolony formation of one aEPEC ONT strain was mediated by intimin α (omicon).

In summary, the results obtained in this study suggest that the LAL pattern represents a virulence property of aEPEC strains, particularly of classic aEPEC strains. This pattern has been referred to as “poor LA” by Knutton et al. (14), “LA” by Scotland et al. (29), and “LA6” by Vieira et al. (34). Our data point toward an involvement of ECP, intimins, and other known *E. coli* adhesins in the LAL pattern. However, we cannot rule out the existence of an adhesive structure not yet identified involved with the LAL pattern. Further studies are under way to address these questions.

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