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HeLa-cell adherence patterns and actin aggregation of enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin-producing *E. coli* (STEC) strains carrying different *eae* and *tir* alleles

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Summary. A collection of 69 *eae*-positive strains expressing 29 different intimin types and eight *tir* alleles was characterized with respect to their adherence patterns to HeLa cells, ability to promote actin accumulation in vitro, the presence of *bfp*A alleles in positive strains, and bundle-forming pilus (BFP) expression. All of the nine typical enteropathogenic *Escherichia coli* (tEPEC) studied harbored the enteropathogenic *E. coli* adherence factor (EAF) plasmid, as shown by PCR and/or EAF probe results. In addition, they were positive for *bfp*A, as shown by PCR, and BFP expression, as confirmed by immunofluorescence (IFL) and/or immunoblotting (IBL) assays. Localized adherence (LA) was exclusively displayed by those nine tEPEC, while localized-adherence-like (LAL) was the most frequent pattern among atypical EPEC (aEPEC) and Shiga-toxin-producing *E. coli* (STEC). All LA and LAL strains were able to cause attaching and effacing (AE) lesions, as established by means of the FAS test. There was a significant association between the presence of *tir* allele α1 and *bfp*A-positive strains, and consequently, with the LA pattern. However, intimin type or *bfp*A was not associated with the adherence pattern displayed in HeLa cells. Among the eight *bfp*A alleles detected, a new type (β10; accession number FN391178) was identified in a strain of serotype O157:H45, and a truncated variant (β3.2-t; accession number FN 391181) in four strains belonging to different pathotypes. [**Int Microbiol** 2009; 12(4):243-251]

Keywords: enteropathogenic *E. coli* · Shiga-toxin-producing *E. coli* · HeLa-cell adherence · intimin

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Introduction

Attaching and effacing *Escherichia coli* (AEEC) are capable of attaching to intestinal epithelial cells of human and animals, leading to diarrhea. Intimin, which is encoded by the chromosomal gene *eae* as part of a pathogenicity island termed the locus for enterocyte effacement (LEE) [33], is responsible for the intimate adherence of these bacteria to the

enterocyte membranes, which, in turn, triggers the attaching and effacing (AE) lesion of the brush-border microvilli [14]. Analysis of the variable C-terminal-encoding sequence of *eae* defined at least 29 distinct intimin types (α1, α2, β1, β2, β3, γ1, θ1, κ, δ, ε1, ε2, ε3, ε4, ε5, ζ1, ζ3, η1, η2, ι1-A, ι1-B, ι1-C, ι2, λ, μ, ν, ο, π, ρ, σ) [6,7,8,21] associated with tissue tropism [43]. Another factor encoded by the LEE is the intimin receptor (Tir), which is translocated to the enterocyte cell surfaces and shows allelic diversity [21].

AE strains represent a variety of enteric pathotypes of both humans and animals, including enteropathogenic *E. coli* (EPEC), some Shiga-toxin-producing *E. coli* (STEC), *Escherichia albertii*, and *Citrobacter rodentium* [1,5,7, 29,48,52]. EPEC strains are defined as intimin-containing diarrheagenic *E. coli* able to form AE lesions on intestinal cells and lacking Shiga-toxin genes [32]. Most human classic EPEC strains belong to a series of O antigenic groups known as EPEC serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 [50]. EPEC are further classified as typical (tEPEC) when they carry an EPEC adherence factor (EAF) plasmid that encodes localized adherence (LA) to the surfaces of HeLa and HEp-2 cells. Adherence is associated with the formation of compact bacterial microcolonies after 3 h of incubation [46] and is mediated by the bundle-forming pilus (BFP). BFP is a type IV fimbria whose expression depends on a 14-gene operon, with *bfp*A encoding the major structural subunit. Sequence comparisons of *bfp*A alleles have provided compelling evidence for diversifying selection at the molecular level. The polymorphism of *bfp*A has important implications for the divergence of BFP structure and function and for its interaction with the host immune system. In addition, the gene can be used as an epidemiological marker [9,10,36]. Atypical EPEC (aEPEC) strains, by contrast, produce the AE lesion but do not express BFP and lack Shiga-toxin genes [29]. In general, they form loose bacterial microcolonies that are detected only after a more prolonged incubation period (6-h) and display a localized-adherence-like (LAL) pattern [44]. Finally, STEC strains produce Shiga toxins (Stx1 and Stx2), are negative for BFP, and do not have LA to cultured mammalian cells [41]; they are zoonotic food-borne pathogens, with ruminants being a significant reservoir [5,38].

The aim of our study was to characterize the adherence pattern to HeLa cells of *eae*-positive strains carrying 29 different intimin types and eight *tir* alleles. The late goal was to determine whether intimin (or Tir receptor) expression correlated with the adherence pattern displayed. In addition, we used fluorescent actin-staining (FAS) to monitor actin accumulation in these strains in vitro, and analyzed both the type of *bfp*A harbored by positive EPEC strains and BFP expression.

Materials and methods

Strain collection. The 69 strains analyzed in this study expressed 29 intimin types (α1, α2, β1, β2, β3, γ1, θ1, κ, δ, ε1, ε2, ε3, ε4, ε5, ζ,1, ζ,3, η1, η2, ι1-A, ι1-B, ι1-C, ι2, λ, μ, ν, ο, π, ρ, σ), and carried eight *tir* alleles (α1, β1, β3, γ1, θ, ν, ο, ρ). All strains were of clinical origin [4,5,7,8,21] and had been isolated from humans (53), cattle (8), poultry (2), pigs (2), goats (2), sheep (1), or a monkey (1).

Serotyping. O and H antigens were identified as described by Guinée et al. [25], employing all available O (O1–O185) and H (H1–H56) antisera. Antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove non-specific agglutinins. The O antisera were obtained from the *E. coli* Reference Laboratory (University of Santiago de Compostela, Lugo, Spain), and the H antisera from the Statens Serum Institute (Copenhagen, Denmark).

PCR assays and sequencing. Virulence genes were detected by PCR using specific primers: stx_1 and stx_2 [4], *eae* [4], *bfpA* [26], and EAF plasmid [19]. In addition, the presence of the high-molecular weight virulence plasmid of enteroaggregative *E. coli* (EAEC) was studied in strains showing an aggregative adherence pattern (AA), by detection of the *aatA* sequence [42,47]. The amplification procedures were described elsewhere [7,8]. The intimin genes (*eae* α1, α2, β1, β2, β3, γ1, θ1, κ, δ, ε1, ε2, ε3, ε4, ε5, ζ1, ζ3, η1, η2, ι1-A, ι1-B, ι1-C, ι2, λ, μ, ν, ο, π, ρ, σ) and the Tir genes (*tir* α1, β1, β3, γ1, θ, ν, ο, ρ) were typed by PCR and sequencing as previously described [7,8,21]. The amplification products were purified with the Spinclean PCR purification kit (Mbiotech) and their sequences determined using the dideoxynucleotide triphosphate chain-termination method of Sanger, with the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3730xl Genetic Analyzer (Applied Biosystems). The *bfp*A alleles were analyzed by PCR and sequencing, and the results were compared with the available allelic sequences [9,10,31,36]. New allelic sequences were deposited in GenBank. Finally, as proposed by Hyma et al. [30], multiplex PCR was used to detect conserved sequences that distinguish members of the *E. albertii* lineage, all of which are positive carriers of the *eae* gene, from *E. coli* in order to avoid controversial results of biochemical and antigenic phenotypes.

Adherence properties in HeLa cells. Adherence assays were done at 37°C for 3 and 6 h as described by Cravioto et al. [15]. HeLa cells were cultivated for 48 h (60–70% confluence) in minimal essential medium (MEM) supplemented with 2% fetal bovine serum and 2% D-mannose and then infected with 1:50 dilutions of bacteria grown overnight in LB broth. After 3 h of incubation at 37°C, the preparations were washed either six times (3-h assay) with phosphate-buffered saline (PBS), or three times and then incubated in fresh medium for an additional 3 h (6-h assay) followed by six washes with PBS. All preparations (3- and 6-h assays) were fixed with methanol, stained with May-Grünwald-Giemsa, and examined by light microscopy. The adherence patterns were classified as: localized adherence (LA), when the bacteria adhered to the cell surface as tight clusters; localized-like-adherence (LAL), when the bacteria adhered to the cell surface, forming loose clusters; aggregative adherence (AA), when the bacteria adhered to the cell surface and to the cover slip in a stacked-brick pattern; and diffuse adherence (DA), when the bacteria adhered diffusely to the cell surface [40,44,46]. *E. coli* strains E2348/69, C1845, and 0431-4 were used as controls for the localized, diffuse, and aggregative adherence patterns, respectively. *E. coli* HB101 was used as the negative control. Each strain was tested at least twice, and three different examiners read the slides.

Enteropathogenic *Escherichia coli* (EPEC) adherence (EAF) probe. The EAF sequence was detected by colony blot hybridization assays under stringent conditions [45]. DNA probe fragments were

amplified from strain E2348/69 using primers described elsewhere [19] and then labeled with [P^{32}] dCTP using DNA Labeling beads (Amersham).

Fluorescent actin-staining (FAS). The ability of a strain to cause the AE lesion was determined by means of the FAS test, as described by Knutton et al. [34]. At the end of the above-described adherence assay, the preparations were fixed in 3% formalin, washed three times with PBS, and treated with 0.1% Triton X-100 (5 min). They were then washed three times with PBS, incubated for 30 min with 5 µg fluorescein isothiocyanate-labeled phaloidin (Sigma)/ml, washed another three times (10-min incubation period each), and examined by fluorescence microscopy. *E. coli* HB101 and *E. coli* E2348/69 were used as the negative and positive control, respectively.

Immunofluorescence (IFL) assays for BFP detection. Strains bearing the BFP structural gene *bfpA* were examined for BFP expression according to the method described by Knutton et al. [35], using a ploypolyclonal anti-BFP antibody at a dilution of 1:100 [23] and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (GAR-FITC-Sigma) diluted 1:50 in PBS. Preparations were visualized and photographed using a fluorescence microscope (Axioskop, Zeiss, Germany) connected to a digital camera (DFC 300FX-Leica, Germany).

Immunoblotting (IBL) analysis. A 1-ml aliquot of a Dulbecco's modified eagle medium (DMEM) overnight culture of each strain was centrifuged at 14,000× *g* for 20 min; the supernatants were discarded and the pellet resuspended in 20 µl of sample buffer, boiled at 100°C for 5 min, and applied to a 15% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Hybond-ECL-Amersham) at 100 V for 18 h at 4°C. The membrane was blocked with 5% skim milk in PBS for 2 h and reacted with polyclonal anti-BFP antibody (diluted 1:1000) for 18 h at 4°C. The membrane was then washed and incubated for 2 h with horseradish-peroxidase-conjugated anti-rabbit antibody (1:5000) and exposed with quimioluminescence substrate and ECL-exposed X-ray films (Pierce).

Statistical analysis. Prevalence rates were compared between groups by Fisher's exact test, with $P < 0.05$ considered significant.

Results

Typical EPEC (tEPEC). This study included nine tEPEC (Table 1) carrying eight types of intimin (α1, β1, β2, θ1, δ, η1, η2, μ) and three *tir* alleles (α1, β1, θ). Furthermore, all harbored the EAF plasmid, as confirmed by PCR and/or EAF probe results; were positive for *bfp*A by PCR; and positive for BFP expression by immunofluorescence (IFL) and/or immunoblotting (IBL) assays. The IFL assay was less sensitive (five out of nine tEPEC) than the IBL assay (eight out of nine) in the detection of BFP expression. Also, PCR (seven out of nine) was less sensitive than the EAF probe (nine out of nine) in the detection of EAF plasmid.

As expected, all nine tEPEC strains showed the characteristic LA pattern of adherence to HeLa cells (strain FV 10129 showed a mixed pattern LA/AA); in fact, LA was exclusively displayed by tEPEC strains. In addition, all nine strains were able to cause AE lesions, as determined by means of the FAS test.

Typical EPEC-like (tEPEC-like). In this study, and for comparative purposes, nine *bfp*A-positive *E. coli* strains negative for BFP expression were referred to as typical EPEC-like (tEPEC-like). Two additional *E. albertii bfp*A-positive strains were included in this comparison. Table 2 shows the adherence patterns of the 11 strains, which carried eight types of intimin (α1, β2, κ, ε2, ε4, ι1-B, ι1-C, μ) and two *tir* alleles (α1, β1).

The *bfp*A alleles carried by the 20 *bfp*A-positive strains were identified and characterized. Comparative analysis with the

Table 1. Adherence patterns to HeLa cells of tEPEC strains included in the study

Strain	Serotype	Origin	*eae*	*tir*	*bfp*A PCR	*bfp*A IFL	*bfp*A BLOT	EAF PCR	EAF probe	Adherence 3 h (FAS)
FV 10087	O55:H6	H	α1	α1	α1	+	–	+	+	LA (+)
FV 10089	O157:H45	B	α1	α1	β10	–	+	–	+	LA (+)
FV 10095	O111:H2	H	β1	β1	α2	+	+	+	+	LA (+)
FV 10097	O119:H6	H	β2	α1	α3	+	+	+	+	LA (+)
FV 10111	O127:H40	H	θ1	θ	β5	–	+	+	+	LA (+)
FV 10114	O86:H34	H	δ	α1	α1	+	+	+	+	LA (+)
FV 10127	O125:HNM	H	η1	α1	α1	+	+	+	+	LA (+)
FV 10129	ONT:H45	B	η2	α1	β7.1	–	+	–	+	LA/AA (+)
FV 10137	O55:HNM	H	μ	α1	β5	–	+	+	+	LA (+)

Abbreviations: IFL, immunofluorescence assay; BLOT, immunoblotting assay; HNM, non-motile; ONT, O antigen non-typable; H, human; B, bovine; LA, localized adherence; AA, aggregative adherence.

Table 2. Adherence patterns to HeLa cells of tEPEC-like and two *Escherichia albertii* strains included in the study

Strain	Serotype	Origin	*eae*	*tir*	*bfp*A PCR	*bfp*A IFL	*bfp*A BLOT	EAF PCR	EAF probe	Adherence 3 h (FAS)	Adherence 6 h (FAS)	Pathogenic group
FV 12049	O157:H45	H	α1	α1	β7.1	–	–	–	+		NC (–)	tEPEC-like
FV 10138	O55:H51	H	μ	α1	β5	–	–	+	+	NC (+)		tEPEC-like
FV 10139	O55:HNM	H	μ	α1	β5	–	–	+	+	NC (+)		tEPEC-like
FV 10099	O167:H6	M	β2	α1	α4	–	–	–	–		NC (–)	tEPEC-like
FV 10112	O86:H34	H	κ	α1	α1	–	–	–	–		LAL (+)	tEPEC-like
FV 10131	O153:H8	H	ι1-B	α1	β7.1	–	–	–	–		LAL (+)	tEPEC-like
FV 10132	O119:H8	B	ι1-C	α1	β7.1	–	–	–	–		LAL (+)	tEPEC-like
FV 10113	O118:H5	H	κ	α1	β3.2-t	–	–	–	–		LAL (+)	tEPEC-like
FV 12050	O88:H25	H	ε2	α1	β3.2-t	–	–	–	–		NC (–)	tEPEC-like
FV 10123	O109:HNM	H	ε4	β1	β3.2-t	–	–	–	–		LAL (+)	*E. albertii*
FV 9968	O109:HNM	H	ε4	β1	β3.2-t	–	–	–	–		LAL (+)	*E. albertii*

Abbreviations: IFL, immunofluorescence assay; BLOT, immunoblotting assay; HNM, non-motile; H, human; M, monkey; B, bovine; LAL, localized-adherence-like; NC, non-characteristic adherence pattern.

sequences available in GenBank [16–18,22] revealed the existence of a new allele (β10; accession no. FN 391178) in a strain of serotype O157:H45, and a truncated variant (β3.2-t; accession no. FN 391181) in two tEPEC-like strains and two strains of *E. albertii*. Among the 20 strains, eight alleles were detected: α1 (four strains), α2 (one strain), α3 (one strain), α4 (one strain), β5 (four strains), β7.1 (four strains), and β3.2-t (four strains), β10 (one strain) (Table 1). As expected, all four strains carrying a truncated *bfp*A gene lacked BFP expression.

The *tir* type was found to correlate with the presence of *bfp*A, in that 16 (80%) of the tEPEC (seven out of nine) and tEPEC-like (nine out of nine) strains carried the *tir* allele α1, as opposed to 11 (22.4%; ten aEPEC and one STEC) of the 49 *bfp*A-negative strains ($P = 0.001$). This result suggested a correlation between *tir* α1 and LA, as seven (78%) out of those nine tEPEC LA-positive strains carried this type of *tir*, compared with 20 out of 60 (33.3%) LA-negative strains ($P = 0.015$). By contrast, there was no correlation between intimin type and adherence pattern.

Atypical enteropathogenic *Escherichia coli* (aEPEC). Table 3 shows the adherence pattern of 32 aEPEC. Additionally, eight *E. albertii* strains were included in this table. All 40 strains tested negative for genes encoding BFP, EAF plasmid, and Shiga toxins. They carried 20 different intimin types (α2, β1, β2, β3, γ1, ε1, ε2, ε3, ε5, ζ1, ζ3, η2, ι1-A, ι2, λ, ν, ο, π, ρ, σ) and seven different Tir alleles (α1, β1, β3, γ1, ν, ο, ρ). Atypical EPEC strains displayed heterogeneous patterns of adherence to HeLa cells, although 15 of 32 (46.8%) adhered to the cells with the LAL pattern in the 6-h assay. Of the other 17 aEPEC strains, the following patterns were seen: non-characteristic (NC), seven strains; aggregative adherence (AA), seven strains; and no adherence, three strains. Likewise, the eight *E. albertii* strains displayed different patterns: LAL (three strains), NC (three strains), localized adherence at 6 h (LA6, one strain), and a mixed pattern of diffuse adherence (DA) and LAL (one strain).

Seven strains displaying AA (intimins α2, ε1, ε2, ζ1, ο, π) adhered to cells at 3 h (six strains), while only one such strain adhered at 6 h. The presence of the *aatA* sequence of enteroaggregative *E. coli* (EAEC) was negative by PCR in all strains displaying this pattern. The *E. albertii* strain showing the LA6 pattern [seen in only one other strain: FV10103 STEC (Table 4)] belonged to serotype O128:HNM and carried *eae* β3, *tir* β3. A preliminary test established the invasiveness of this strain. All strains of LAL pattern, whether aEPEC or not, were positive for the FAS test. Only ten out of the 32 aEPEC strains (five strains with the AA pattern and five with NC) tested negative for FAS.

Table 3. Adherence patterns to HeLa cells of the aEPEC and eight *Escherichia albertii* strains included in the study

Strain	Serotype	Origin	*eae*	*tir*	Adherence 3 h (FAS)	Adherence 6 h (FAS)	Pathogenic group
FV 10090	O125:H6	H	α2	γ1	AA (–)		aEPEC
FV 10091	O63:H33	H	α2	γ1		NC (–)	aEPEC
FV 10092	O132:H34	H	α2	γ1		LAL (+)	aEPEC
FV 10096	O177:H11	B	β1	β1		LAL (+)	aEPEC
FV 10098	O113:H6	H	β2	α1		NC (–)	aEPEC
FV 10101	O128:HNM	H	β3	β3		DA/LAL (+)	*E. albertii*
FV 10102	O128:HNM	H	β3	β3		LA6 (+)	*E. albertii*
FV 10106	O145:H28	H	γ1	γ1		NC (+)	aEPEC
FV 10116	O26:HNM	H	ε1	β1		LAL(+)	aEPEC
FV 10117	O157:H16	H	ε1	β1	AA (–)		aEPEC
FV 5713	O1:H45	H	ε1	β1		LAL (+)	aEPEC
FV 10118	O6:H19	H	ε2	α1	AA (–)		aEPEC
FV 10119	O103:H19	H	ε2	α1		NC (–)	aEPEC
FV 10120	O123:H19	H	ε2	α1		NC (–)	aEPEC
FV 10121	O181:HNM	H	ε3	β1		LAL (+)	*E. albertii*
FV 10143	O80:H2	P	ε5	β1		LAL (+)	aEPEC
FV 10144	O157:HNM	P	ε5	β1		LAL (+)	aEPEC
FV 10125	O156:HNM	H	ζ1	α1	AA (–)		aEPEC
FV 10126	O85:H31	H	ζ3	α1		LAL (+)	aEPEC
FV 10128	ONT:H45	H	η2	α1		LAL (+)	aEPEC
FV 10130	O145:H4	H	ι1-A	α1		LAL (+)	aEPEC
FV 10133	ONT:H45	H	ι2	ν		LAL (+)	aEPEC
FV 10134	O34:HNM	H	λ	γ1		NA	aEPEC
FV 10135	O33:HNT	H	λ	γ1		NA	aEPEC
FV 10136	O101:H33	H	λ	γ1		LAL (+)	aEPEC
FV 10140	O10:HNM	H	ν	ν	NC (+)		*E. albertii*
FV 10141	ONT:HNM	H	ν	ν	NC (+)		*E. albertii*
FV 10145	O129:HNM	H	ο	ο		AA (+)	aEPEC
FV 10146	O84:HNM	H	ο	ο	NC (+)		*E. albertii*
FV 12052	O84:HNM	H	ο	ο		LAL (+)	aEPEC
FV 12053	O105:H4	H	ο	ο		NC (–)	aEPEC
FV 9969	ONT:H4	H	ο	ο	AA (–)		aEPEC
FV 12054	ONT:HNM	H	ο	ο		NA	aEPEC
FV 10147	O14:H5	H	π	α1	NC (+)		aEPEC
FV 10148	ONT	H	π	α1	AA (+)		aEPEC
FV 10149	O149:HNM	C	ρ	ρ		LAL (+)	aEPEC
FV 10150	O149:HNM	C	ρ	ρ		LAL (+)	aEPEC
FV 10151	O180:HNM	B	ρ	ρ		LAL (+)	aEPEC
FV 10152	O86:HNM	A	σ	β1		LAL (+)	*E. albertii*
FV 10153	O86:HNM	A	σ	β1		LAL (+)	*E. albertii*

Abbreviations: ONT, O antigen non-typable; HNM, non-motile; H, human; B, bovine; P, porcine; C, caprine; A, avian; AA, aggregative adherence; LAL, localized-adherence-like; DA, diffuse adherence; LA6, localized adherence in 6-h assay; NC, non-characteristic adherence pattern; NA, non-adherent.

Table 4. Adherence patterns to HeLa cells of the STEC included in the study

Strain	Serotype	Origin	*eae*	*tir*	*stx*	Adherence 6 h (FAS)
FV 10094	O26:H11	H	β1	β1	*stx1*	LAL (+)
FV 10103	O157:H7[a]	H	γ1	γ1	*stx1 stx2*	LA6 (+)
FV 10104	O157:H7[a]	H	γ1	γ1	*stx1 stx2*	LAL (+)
FV 10108	O157:HNM[b]	H	γ1	γ1	*stx2*	NC (+)
FV 10109	O111:HNM	B	θ1	θ	*stx1*	LAL (+)
FV 10110	O111:H8	H	θ1	θ	*stx1 stx2*	LAL(+)
FV 10115	O103:H2	B	ε1	β1	*stx1*	LAL (+)
FV 10142	O80:HNM	B	ε5	β1	*stx1*	NA
FV 10124	O156:HNM	O	ζ1	α1	*stx1*	AA (–)

Abbreviations: HNM, non-motile; H, human; B, bovine; O, ovine; LAL, localized-adherence-like; LA6, localized adherence in 6-h assay; NC, non-characteristic adherence pattern; AA, aggregative adherence; NA, non-adherent.
[a]Non-sorbitol-fermenting strain.
[b]Sorbitol-fermenting strain.

Shiga-toxin-producing *Escherichia coli* (STEC). The nine STEC strains included in this study tested positive for Shiga-toxin genes and negative for BFP and EAF (Table 4). Most of them were representative of the classical STEC serotypes (O26:H11, O157:H7/H-, O111:H-; O103:H2, O156:HNM) and carried six *eae* alleles (β1, γ1, θ1, ε1, ε5, ζ1) and four Tir types (α1, β1, γ1, θ). The adhesion response to HeLa cells was not homogeneous. All of the strains, with the exception of FV 10124 (O156:HNM *eae* ζ1, *tir* α1, AA), had a positive FAS test. Note that the three strains of serotype O157:H7/HNM *eae* γ1 *tir* γ1, FV 10103 (non-sorbitol-fermenting strain), FV 10104 (non-sorbitol-fermenting strain), and FV 10108 (sorbitol-fermenting strain), displayed three different adherence patterns (LA6, LAL, and NC, respectively).

Discussion

Differentiation of the *eae*, *tir* and *bfp*A alleles, determination of the BFP expression, and establishment of the adherence pattern together provide an important set of tools for EPEC and STEC typing in diagnostic studies as well as in pathogenesis, epidemiologic, and immunologic investigations [20,37,39].

While a significant correlation between *tir* α 1 and the presence of *bfp*A, and consequently between *tir* α 1 and LA pattern, was detected, no general association between intimin type, *bfp*A type, and adherence pattern in HeLa cells was observed—not even between strains of the same serotype-intimin subtype and adherence pattern.

The LA pattern was exclusively displayed by tEPEC while the LAL pattern was the most frequent among aEPEC (15 of 32 strains) and STEC (five out of eight strains that adhered to HeLa cells) strains, which is in accordance with previous reports [13,17]. In addition, in this study all LA and LAL patterns resulted in actin accumulation in vitro (FAS-positive). Adherence patterns other than LAL were detected among aEPEC (AA) and STEC (AA and LA6). LAL was the most frequent pattern among *E. albertii* (five out of 10 strains). Other patterns included LA6 (one strain), DA/LAL (one strain), and NC (three strains). All ten *E. albertii* strains were FAS-positive. As far as we know, this is the first study to report the adherence patterns of this species, whose behavior does not seem to differ from that of the other *eae*-positive pathotypes analyzed in this work.

The heterogeneous patterns displayed by the strains in this study, including those belonging to the classical EPEC serogroups, were not surprising. In fact, the evolution of EPEC appears to be a dynamic process involving repeated acquisition of the LEE island and transfer of the EAF plasmid. Clonal analysis of the classical EPEC serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142) has shown that they consist of a variety of clones that have acquired distinct combinations of virulence factors, and these combinations correspond to distinct modes of pathogenesis [3,12,16,22,24,44,51]. Lacher et al. [36] reported that at least some aEPEC strains evolved from tEPEC, rather than

tEPEC evolving from aEPEC by acquisition of the plasmid. On the basis of characteristics common to these strains and tEPEC, we propose the name "tEPEC-like" for a group of strains that would be otherwise classified as aEPEC according to the definition of Hernandes et al. [29]. A clear example is that of the three O55 strains (FV 10137, FV 10138, FV 10139; Tables 1 and 2), which carry the same *eae* (μ), *tir* (α1), and *bfp*A (β5) alleles and harbor the EAF plasmid, as confirmed by PCR and/or EAF probe analysis. However, FV 10137 was positive for BFP expression by IBL and thus classified as tEPEC, whereas the other O55 strains were negative for BFP expression. This suggests a genetic modification of the original tEPEC strains.

Comparative sequence analysis of the *bfp*A gene among those 20 positive strains revealed the existence of a new allele of *bfp*A (β10) and a truncated variant (β3.2-t). In this study, most of the previously described *bfp*A alleles [9,10,31,36] were detected and, as expected, they belonged to the same classical serotypes in which they were first described (FV 10087 O55:H6 *eae* α1, *bfp*A α1; FV 10137 and FV 10139 O55:HNM *eae* μ, *bfp*A β5; FV 10112 O86:H34 *eae* κ, *bfp*A α1; FV 10095 O111:H2 *eae* β1, *bfp*A α2; FV 10097 O119:H6 *eae* β2, *bfp*A α3). The new *bpf*A allele (β10) was detected in strain O157:H45 *eae* α1 (FV 10089), while Lacher et al. [36] reported the alleles β6 and β7.1 in strains carrying this serotype and intimin type. We identified the truncated allelic variant (β3.2-t) in four strains of serotypes O118:H5, O88:H25, and O109:HNM, all of which were negative for BFP expression. In two strains of serotypes O128ab:H2 and O119:H2, Bortolini et al. [11] have described a truncated *bfp*A arisen from a transpositional event and resulting in a lack of pilus expression. We detected the allelic variant β3.2-t in two strains of *Escherichia albertii*. As far as we know, this is the first report of the occurrence of the *bfp*A gene (albeit truncated) in this *Escherichia* species.

Among the diversity detected in this study, certain serotypes showed common characteristics. For example, the four strains of serogroup O55 carried the same *tir* allele α1, and the three strains *eae* μ (one tEPEC and two tEPEC-like) the same *bfp*A allele β5. However, they displayed different adherence patterns: LA in the two tEPEC of serotypes O55:H6 and O55:HNM, as described by Rodrigues et al. [44], and NC in the tEPEC-like strain.

The profiles of the three strains of serogroup O111 from this study were consistent with reported profiles. Campos et al. [12] described different clones among *E. coli* isolates O111 by electrophoretic types (ETs), ET 12 being the most frequent and corresponding mainly to strains of serotype O111:H2. This serotype carries sequences homologous to EAF and *eae* and has both localized (LA) and intimate (FAS+) adherence. The same characteristics were found in strain FV 10095 O111:H2 *eae* β1 of our study. Clone ET 8, reported by Campos et al. [12], includes STEC strains of serotypes O111:HNM or O111:H8 [49]; similarly, strains FV 10109 (O111:HNM *eae* θ1) and FV 10110 (O111:H8 *eae* θ1) of our study carried *stx* genes and displayed a LAL FAS-positive pattern.

The two strains belonging to serogroup O119 also displayed the expected profiles according to Gonçalves et al. [24], who have reported three virulence patterns among strains of this serogroup. One pattern described by those authors includes only O119:H6 strains, with 90% of them showing LA and 96% being positive for FAS test. Likewise, strain FV 10097 (O119:H6 *eae* β2) of the present study displayed LA and was FAS-positive. A second pattern reported by the same authors consists of strains positive for *eae* and *bfp*A but they do not hybridize with the EAF probe, and most (80%) have the LAL phenotype, as did our tEPEC-like FV 10132 (O119:H8 *eae* ι1-C).

Finally, strains belonging to serotype O125:H6 have been reported to display the AA pattern with HEp-2 cells in the 6-h assay and to lack EAEC virulence markers [2,13,18]. Similarly, the aEPEC strain FV 10090 (O125:H6 *eae* α2) of this study showed the AA pattern and was negative for both the FAS test and PCR detection of the *aat*A sequence associated with EAEC, but, differently, this strain adhered to HeLa cells after 3 h of incubation.

The adherence pattern LA6 was detected in only two strains, consistent with the conclusions of Hernandes et al. [27], who have reported that aEPEC strains presenting LA6 are diverse with regard to serotype, plasmid content, and virulence gene profile. The two LA6-positive strains detected among the 69 included in this study belonged to different pathogenic groups and expressed different intimin and Tir types: *E. albertii* O128:HNM *eae* β3 *tir* β3 (FV 10102) and STEC O157:H7 *eae* γ1 *tir* γ1 *stx*1*stx*2 (FV 10103). Hernandes et al. [28] have also studied the kinetics of this pattern in an aEPEC strain carrying intimin omicron since the key determinants of compact microcolony formation in aEPEC strains are unknown. They have suggested that the LA6 phenotype should be mediated by intimin omicron in that strain. However, none of our six strains carrying *eae* omicron showed LA6; rather, the six strains (one *E. albertii* and five aEPEC) displayed three different adhesion patterns (AA, LAL, NC).

In conclusion, a high heterogeneity was detected among the 69 strains studied, with no general association of intimin type, *bfp*A type, and HeLa-cell adherence pattern. This was also the case for strains of the same serotype-intimin subtype and adherence pattern. However, the *tir* allele α1 was significantly associated with the presence of

*bfp*A, and, consequently, with LA. Further studies are necessary to discover the role of the *tir* allele α1 in the pathogenesis of *bfp*A-positive strains.

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