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Autotransporter Protein-Encoding Genes of Diarrheagenic *Escherichia coli* Are Found in both Typical and Atypical Enteropathogenic *E. coli* Strains

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Autotransporter (AT) protein-encoding genes of diarrheagenic *Escherichia coli* (DEC) pathotypes (*cah*, *eatA*, *ehaABCDJ*, *espC*, *espI*, *espP*, *pet*, *pic*, *sat*, and *tibA*) were detected in typical and atypical enteropathogenic *E. coli* (EPEC) in frequencies between 0.8% and 39.3%. Although these ATs have been described in particular DEC pathotypes, their presence in EPEC indicates that they should not be considered specific virulence markers.

Enteropathogenic *Escherichia coli* (EPEC) was the first pathotype of diarrheagenic *E. coli* (DEC) described (1). In 1995, EPEC strains were classified into typical (tEPEC) and atypical (aEPEC) groups on the basis of the presence and absence of the EPEC adherence factor plasmid (pEAF), respectively (2). Later, additional differential characteristics of the members of these groups, such as serotypes, virulence characteristics, adherence patterns, and reservoir, were reported (3, 4). However, the two groups have in common the central mechanism of EPEC pathogenesis: an intestinal histopathological lesion called attaching and effacing, whose genetic determinants are located on the locus of enterocyte effacement (LEE) (5).

In the past, tEPEC strains were more common in developing countries, where they have been found to be strongly associated with acute diarrhea in children <1 year of age, while aEPEC strains were more frequently isolated from children of industrialized countries (4). Recent epidemiological studies have demonstrated that aEPEC is more prevalent than tEPEC in both developing and industrialized countries, where strains have been found in association with endemic diarrhea in children and diarrhea outbreaks (3, 6).

In general, tEPEC strains are more homogeneous than aEPEC strains in their virulence traits. tEPEC strains mainly produce the virulence factors encoded by LEE and pEAF, while aEPEC strains frequently express non-LEE-encoded effectors and carry genes encoding virulence factors of other DEC pathotypes, in addition to the LEE-encoded factors (3, 4).

Indeed, phylogenetic studies have indicated that aEPEC strains have a genomic background with characteristics that allow the acquisition, retention, and expression of genes encoding virulence factors of other DEC pathotypes (7, 8).

In the past decade, several autotransporter (AT) proteins have been described. Members of this family of proteins have been identified in *E. coli* and other Gram-negative bacteria and are often associated with virulence functions such as adherence, aggregation, invasion, biofilm formation, and toxicity (9–11). The ATs are secreted by the type V secretion system, the most widespread secretion pathway for the transportation of molecules across the outer membrane of Gram-negative bacteria, including the AT pathway (also known as AT-1 or type Va), the two-partner secretion pathway (also known as type Vb), and the Oca system, also known as AT-2, type Vc, or trimeric AT adhesion (10). AT proteins possess an overall unifying structure comprising three functional domains: the amino-terminal leader sequence, which initiates transport of the precursor across the inner membrane; the passenger domain, which confers the function of the secreted protein; and a carboxy-terminal (β) domain, which forms a β-barrel pore to allow secretion of the passenger protein through the outer membrane (12).

Thus, the aim of this study was to evaluate the presence of genes encoding AT virulence proteins produced by DEC pathotypes among tEPEC and aEPEC strains.

The 117 EPEC strains selected for this study were characterized in previous studies (4, 13–15). The 72 aEPEC strains of several serotypes were isolated during an epidemiological study of the etiology of acute diarrhea in the city of Salvador (State of Bahia, Brazil), between 2003 and 2004 (14). The 45 tEPEC strains belonging to the 12 classic EPEC O serogroups (4) were isolated from sporadic cases of acute diarrhea in Brazil and other countries, during different time periods (4, 15).

PCR was used to detect the presence of 17 genes encoding autotransporter proteins: *aida-I*, *cah*, *eatA*, *ehaABCDJ*, *epeA*, *espC*, *espI*, *espP*, *pet*, *pic*, *sab*, *sat*, and *tibA*. The specific primers, cycle conditions, sizes of amplified fragments, and controls are described in Table 1. Amplification was performed in a total volume of 50 μl containing 40 pmol of each primer; dATP, dTTP, dCTP, and dGTP (0.1 mM each); 1.5 U *Taq* DNA polymerase (Invitrogen); 5.0 μl 10× PCR buffer (Invitrogen); $MgCl_2$ (2 mM); and 2.0 μl of DNA template, obtained from a colony from culture on Luria-Bertani agar boiled in 500 μl of water for 10 min. Statistical analyses were performed using Fisher's exact and χ^2 tests.

The frequencies of AT genes in EPEC and their distribution among the tEPEC and aEPEC groups are shown in Table 2. With the exception of *aida-I*, *epeA*, and *sab*, all of the genes investigated

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TABLE 1 Primers used for PCR, size of amplified products, annealing temperatures, origin, and description of target genes[a]

Gene	Description of target (DEC pathotype)	Known function (reference)	Primer sequence (5′–3′)	Size of PCR product (bp)	Annealing temp (°C)	Primer source or reference
aida-I	Adhesin involved in diffuse adherence (DAEC)	Adherence and biofilm formation (25)	GTTCTCTCTGATGGTTATGC AACATTGACCATACCGCCG	342	60	This study (GenBank accession no. GU810159.1)
cah	Calcium-binding antigen 43 homologue (EHEC)	Biofilm formation and autoaggregation (26)	CGTATCGCTGTGCCCGATAAC CCGTATACGAGTTGTCAGAATCA	707	58	27
eatA	ETEC autotransporter A (ETEC)	Serine protease (28)	CAGGAGTGGGAACATTAAGTCA CGTACGCCTTTGATTTCAGGAT	743	60	27
ehaA	EHEC autotransporter A (EHEC)	Biofilm formation (11)	CACAGATGACAGAAGGGAC GTTTACCCCACTCGTCAG	326	59	This study (GenBank accession no. AE005174.2)
ehaB	EHEC autotransporter B (EHEC)	Biofilm formation (11)	CAG GGTTATGAGTGGGAAG CCACTTGCTGCCGTTGTT	423	59	This study (GenBank accession no. AE005174.2)
ehaC	EHEC autotransporter C (EHEC)	Unknown (11)	TAATGACGGCAAAGGTGGT CATTCATCAGGGAGTTGCT	599	59	This study (GenBank accession no. AE005174.2)
ehaD	EHEC autotransporter D (EHEC)	Biofilm formation (11)	GGCAGTTGACACGATTATTA CTGTCGCTTTGCCATTATC	821	59	This study (GenBank accession no. AE005174.2)
ehaJ	EHEC autotransporter J (EHEC/EPEC)	Biofilm formation (9)	ACGGGCTGCTGAGTATTTT GTAGTTTGCCACATCACCG	455	60	This study (GenBank accession no. NC_011601.1)
epeA	EHEC plasmid-encoded autotransporter (EHEC)	Protease and mucinolytic activity (29)	GGGAGAGTTCAGGCATTTA CAGCGTTACCTTACTTGAG	783	57	This study (GenBank accession no. AY258503.2)
espC	*E. coli* secreted protein C (EPEC)	Enterotoxic activity and cleavage of spectrin, pepsin, and factor V (30)	TAGTGCAGTGCAGAAAGCAGTT AGTTTTCCTGTTGCTGTATGCC	301	55	27
espI	*E. coli* secreted protease (EHEC)	Degradation of plasma proteins (19)	ATGGACAGAGTGGAGACAG GCCACCTTTATTCTCACCA	560	52	19
espP	Extracellular serine protease (EHEC)	Cleavage of spectrin, pepsin, and factor V (31)	GTCCATGCAGGGACATGCCA TCACATCAGCACCGTTCTCTAT	547	55	27
pet	Plasmid-encoded toxin (EAEC)	Enterotoxic and cytopathic toxin effects and cleavage of spectrin (32)	GGCACAGAATAAAGGGGTGTTT CCTCTTGTTTCCACGACATAC	302	58	27
pic	Protein involved in colonization (EAEC)	Mucinase activity and cleavage of factor V (33)	GGGTATTGTCCGTTCCGAT ACAACGATACCGTCTCCCG	1,176	60	33
sab	STEC autotransporter (EHEC)	Biofilm formation (34)	GGTGGATACAGCAGGTAATG TATCTCACCACCTGCTATCG	163	59	34
sat	Secreted autotransporter toxin (DAEC)	Cytopathic toxin effects and cleavage of spectrin and factor V (35)	TCAGAAGCTCAGCGAATCATTG CCATTATCACCAGTAAAACGCACC	930	59	17
tibA	Tib A (ETEC)	Biofilm formation, adherence, and autoaggregation (36)	ATGGTTGGCAGTGACGGTA GGTTGTTGACGGACGGAAA	480	58	This study (GenBank accession no. FN649414.1)

[a] EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EHEC, enterohemorrhagic *E. coli*; EAEC, enteroaggregative *E. coli*; DAEC, diffusely adherent *E. coli*. Positive controls: *cah*, *ehaABCD*, and *espP*, EHEC EDL933 (26); *eatA* and *tibA*, ETEC H10407 (28); *pet* and *pic*, EAEC 042 (32); *ehaJ* and *espC*, EPEC E2348/69 (30); *epeA* and *sab*, EHEC EH41 (29); *aida-I*, DAEC 2787 (25); *sat*, DAEC FBC114 (37); *espI*, STEC O113:H21 (38).

TABLE 2 Distribution of autotransporter virulence genes among typical and atypical EPEC strains

Gene	No. (%) of positive strains		
	tEPEC ($n = 45$)	aEPEC ($n = 72$)	Total ($n = 117$)
aida-I	0 (0.0)	0 (0.0)	0 (0.0)
cah	0 (0.0)	3 (4.2)	3 (2.6)
eatA	4 (8.8)	3 (4.2)	7 (6.0)
ehaA	13 (28.9)	33 (45.8)	46 (39.3)
ehaB	22 (48.9)	24 (33.3)	46 (39.3)
ehaC	7 (15.5)	32 (44.4)[a]	39 (33.3)
ehaD	12 (26.7)	31 (43.1)	43 (36.7)
ehaJ	13 (28.9)[a]	3 (4.2)	16 (13.7)
epeA	0 (0.0)	0 (0.0)	0 (0.0)
espC	22 (48.8)	21 (29.2)	43 (36.7)
espI	0 (0.0)	5 (6.9)	5 (4.3)
espP	0 (0.0)	3 (4.2)	3 (2.6)
pet	8 (17.8)[a]	4 (5.6)	12 (10.3)
pic	0 (0.0)	1 (1.4)	1 (0.8)
sab	0 (0.0)	0 (0.0)	0 (0.0)
sat	2 (4.4)	0 (0.0)	2 (1.7)
tibA	1 (2.2)	0 (0.0)	1 (0.8)

[a] $P < 0.05$ by Fisher's exact test (tEPEC versus aEPEC).

TABLE 3 Distribution of DEC autotransporter virulence genes among the typical EPEC groups

Gene	No. (%) of positive strains	
	EPEC 1[a] ($n = 26$)	EPEC 2[b] ($n = 19$)
eatA	0 (0.0)	4 (21.0)[c]
ehaA	1 (3.8)	12 (63.2)[c]
ehaB	18 (69.2)[c]	4 (21.0)
ehaC	5 (19.2)	2 (10.5)
ehaD	6 (23.1)	6 (31.6)
ehaJ	12 (46.1)[c]	1 (5.3)
espC	18 (69.2)[c]	4 (21.0)
pet	8 (30.8)[c]	0 (0.0)
sat	0 (0.0)	2 (10.5)
tibA	0 (0.0)	1 (5.3)

[a] Serotypes: O55:H6, O86:H34, O119:H6, O127:H6, O142:H6, and O142:H34.
[b] Serotypes: O111:H2, O114:H2, O119:H2, O126:H2, and O128:H2.
[c] $P < 0.05$ by Fisher's exact test (EPEC 1 versus EPEC 2).

were detected. The five most frequent genes were *ehaABCD* and *espC*, found in both groups of EPEC. The other investigated genes were detected at low frequencies: *ehaJ*, *eatA*, and *pet* were detected in both groups; *cah*, *espI*, *espP*, and *pic* were detected only in the aEPEC group; and *sat* and *tibA* were detected only in the tEPEC group. On the basis of their comparative frequencies in the tEPEC and aEPEC groups, *ehaJ* and *pet* were statistically associated with the typical EPEC group and *ehaC* with the atypical EPEC group (Table 2).

A few previous studies have investigated the presence of AT genes in EPEC, but none of them searched for all sequences as in the present work or used a bigger strain collection. *ehaDJ*, *espC*, *espP*, *espI*, *pet*, and *pic* have been previously detected at frequencies similar to those detected in our collection (9, 16–19). On the other hand, Easton et al. (9) found higher frequencies of *ehaABC* than in our study when they examined the prevalence of these genes (100% for *ehaAB* and 86% for *ehaC*) in a collection of 21 EPEC strains isolated in Australia.

We further evaluated the distribution of the AT genes in tEPEC, classifying the strains into two groups: EPEC 1, possessing flagellar antigens H6 or H34, and EPEC 2, possessing flagellar antigen H2 (4, 20). The majority of the AT genes detected were found in strains belonging to group 1. Genes *ehaB*, *ehaJ*, *espC*, and *pet* were statistically associated with EPEC 1 and genes *eatA* and *ehaA* with EPEC 2 (Table 3).

Thus, we conclude that the majority of the AT virulence genes studied here, originally described as corresponding to specific pathotypes of DEC, are also present in EPEC. However, analysis of our data should take into consideration that we have not evaluated a very large number of strains.

The detection of these genes, particularly in the aEPEC subgroup, is in accordance with a study showing that aEPEC strains belonging to particular phylogenetic clusters have genetic similarity to enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), or enteroaggregative *E. coli* (EAEC) (8).

The AT proteins encoded by the genes found in our study have several distinct functions, such as enterotoxicity, cytotoxicity, adherence, invasion, biofilm formation, and protease and mucinase activities (9–11). The role of these proteins in EPEC pathogenesis is unclear. Additional factors involved in adherence (such as *cah* and *tibA*) may compensate for the lack of bundle-forming pili (BFP) in early stages of intestinal colonization in aEPEC (21, 22). Other studies have shown the presence of adherence factors, other than EspA, in aEPEC (21, 23, 24), but none of them are ATs. Also, genes encoding proteins involved in biofilm formation (such as *cah*, *ehaABCDJ*, *sab*, and *tibA*) might be important in the persistence of both EPEC groups in the intestine.

It is worthwhile to mention the presence of *espC*, *espP*, and *pet*, cytotoxin-encoding genes of EPEC, EHEC, and EAEC, respectively, and *pic*, encoding a mucinase of EAEC involved in colonization (10). Expression of Pet and Pic was detected in all aEPEC strains bearing their respective encoding genes in this study (data not shown). The role of these toxins in aEPEC pathogenesis is currently under study by our group. In summary, our data show that several AT protein-encoding genes are present in EPEC. Consequently, they should not be considered markers for any specific DEC pathotype.

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