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MINIREVIEW

An overview of atypical enteropathogenic *Escherichia coli*

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Abstract

The enteropathogenic *Escherichia coli* (EPEC) pathotype is currently divided into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC). The property that distinguishes these two groups is the presence of the EPEC adherence factor plasmid, which is only found in tEPEC. aEPEC strains are emerging enteropathogens that have been detected worldwide. Herein, we review the serotypes, virulence properties, genetic relationships, epidemiology, reservoir and diagnosis of aEPEC, including those strains not belonging to the classical EPEC serogroups (nonclassical EPEC serogroups). The large variety of serotypes and genetic virulence properties of aEPEC strains from nonclassical EPEC serogroups makes it difficult to determine which strains are truly pathogenic.

Introduction

Escherichia coli is one of the best known and best characterized bacterial species. *Escherichia coli* strains associated with the human host are classified as commensals, enteric pathogens [diarrheagenic *E. coli* (DEC)] or extraintestinal pathogens according to their set of virulence genes and clinical properties (Russo & Johnson, 2000; Kaper *et al.*, 2004). The latter pathogens comprise various pathotypes, which can cause human and/or animal infections.

DEC strains have been classified into six different pathotypes: enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli*, enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) (reviewed by Kaper *et al.*, 2004). The various *E. coli* pathotypes tend to be clonal groups that share O (somatic) and H (flagellar) antigens, which define their serogroups (O antigen only) or serotypes (O and H antigens) (reviewed by Kaper *et al.*, 2004). The World Health Organization recognized that EPEC comprises strains of 12 O serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (WHO, 1987), also known as the classical EPEC serogroups.

In 1995, EPEC was divided into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC). The basic difference between the two groups is the presence of the EPEC adherence factor plasmid (pEAF) in tEPEC and its absence in aEPEC (Kaper, 1996). Trabulsi *et al.* (2002) described many other differential characteristics between tEPEC and aEPEC. By the time that the latter review was published, most studies encompassed aEPEC strains mainly belonging to the classical EPEC serogroups. However, various *E. coli* strains that fit the concept of aEPEC, but that belong to nonclassical EPEC serogroups, have been identified in the last several years. Thus, we can define aEPEC as *E. coli* strains that may or may not belong to the classical EPEC serogroups that produce the characteristic histopathologic lesion known as attaching and effacing on intestinal cells, but do not express the bundle-forming pilus (BFP) and lack Shiga-toxin genes.

Although the epidemiological association of aEPEC with diarrhea is still controversial, its high prevalence worldwide

and the involvement of some strains with diarrheal outbreaks support the concept that some aEPEC strains are diarrheagenic. The aim of this review is to gather information regarding serotypes, virulence properties, genetic relationships, epidemiology, reservoir and diagnosis of aEPEC strains, including those not belonging to the classical EPEC serogroups.

Serotypes

A vast serotype diversity of aEPEC strains, particularly when considering nonclassical EPEC serogroups, has been reported by several authors worldwide (Supporting Information, Table S1). Although most of these studies (62.5%) include strains isolated from preschool children both with and without diarrhea, some of them include strains from diarrheic adults. Regarding serogroups, 113 different types have been reported in a total of 746 strains, including the 12 classical EPEC serogroups. Approximately 81% of the aEPEC strains reported in Table S1 do not belong to the classical EPEC serogroups, and 26.6% of them are O nontypeable. The most frequent serogroup is O51 (4.7%), followed by O145 (4.3%), O26, O55 and O111 (2.5% each), and O119 (2.4%). The O-typeable strains reported so far belong to $>$ 200 different serotypes, including many nonmotile and H nontypeable strains.

Pathogenesis

Attaching and effacing (A/E) lesion

tEPEC and aEPEC have in common the ability to form A/E lesion, which is the main pathogenic mechanism in both groups. This lesion results from intimate bacterial adherence, local microvillus effacement and accumulation of polymerized actin and other elements of the cytoskeleton underneath adherent bacteria forming pedestal-like structures (Rothbaum *et al.*, 1982; Moon *et al.*, 1983). A/E lesion production has also been detected in enterohemorrhagic *E. coli* (EHEC), a subgroup of STEC strongly associated with severe human illnesses (reviewed by Nataro & Kaper, 1998).

The ability to produce A/E lesions can be indirectly examined *in vitro* by the fluorescent actin staining (FAS) assay (Knutton *et al.*, 1989). However, although a FAS-positive assay confirms the potential of a strain to promote A/E lesions, a negative FAS result should be regarded with caution as positivity may depend on the cell lineage used. Moreira *et al.* (2008) have shown that intestinal T84 cells were more reliable than HeLa and intestinal Caco-2 cells for detecting aEPEC strains of serotype O51:H40 that cause A/E lesions in the rabbit ileal loop model. Furthermore, Bai *et al.* (2008) have shown that although aEPEC strains of serotype O125:H6 trigger inefficient actin polymerization *in vitro*, these strains can form A/E lesions in human intestinal biopsies. These authors consequently proposed that strains unable to polymerize actin on *in vitro* cultured cells (FAS-negative strains) should be confirmed as nonpathogenic by alternative methods, such as infection of cultured human intestinal explants (*in vitro* organ culture).

Locus of enterocyte effacement (LEE) region

The genes necessary for the establishment of A/E lesions are located on the pathogenicity island (PAI) designated as the LEE (McDaniel *et al.*, 1995). LEE encodes the structural components of a type III secretion system (T3SS), regulators, translocators, chaperones and effector molecules that alter diverse cell signaling processes (reviewed by Garmendia *et al.*, 2005a). LEE also encodes the outer membrane adhesive protein intimin and its translocated receptor Tir (translocated intimin receptor) (Jerse *et al.*, 1990; Kenny *et al.*, 1997).

The LEE PAI of the clinical aEPEC isolates 3431-4/86 (O8:HNM), 0181-6/86 (O119:H9), B6 (O26) and 9812 (O128:H2) were sequenced and compared (Gärtner & Schmidt, 2004; Bielaszewska *et al.*, 2007a). These LEE islands exhibited genetic organization analogous to that of the prototype tEPEC strain E2348/69 and other pathogenic *E. coli* strains (Bielaszewska *et al.*, 2007a). Although the genes constituting the T3SS apparatus are highly conserved, the LEE-encoded effector proteins and the flanking regions of the LEE exhibit major differences.

LEE is inserted at different sites in the *E. coli* chromosome, downstream of the genes encoding the tRNA for selenocysteine (*selC*) or phenylalanine (*pheU* or *pheV*) (McDaniel *et al.*, 1995; Sperandio *et al.*, 1998; Jores *et al.*, 2001). The variable location of the LEE within different EPEC strains suggests that different strains may have acquired the LEE from different progenitors at various times (reviewed by Donnenberg & Whittam, 2001), or that LEE can be mobilized and reintegrated within individual *E. coli* strains (Tauschek *et al.*, 2002). Furthermore, it has been shown that LEE insertion sites vary according to the clonal origin of the *E. coli* strains (Wieler *et al.*, 1997). In most aEPEC strains, the LEE is inserted in *selC* or *pheU* (Sperandio *et al.*, 1998; Vieira *et al.*, 2001; Dulguer *et al.*, 2003). However, the LEE of O51:H40 aEPEC strains is located in *pheV*, as indicated by PCR analysis (Moreira *et al.*, 2008).

Intimin

Intimin, which is encoded by the *E. coli* attaching and effacing (*eae*) gene, is required for intimate adhesion to epithelial cells and cytoskeletal reorganization (Jerse *et al.*, 1990). The variable 280-amino acid C-terminal sequence of intimin (Int_{280}) defines many different intimin subtypes (Frankel *et al.*, 1994; Adu-Bobie *et al.*, 1998; Lacher *et al.*, 2006). Correlation between the expression of some intimin

subtypes and tissue tropism has been demonstrated *in vitro* (Phillips & Frankel, 2000). Although the host tissue distribution of EPEC strains is probably multifactorial, characterization of different intimin subtypes could provide information regarding tissue tropism (reviewed by Torres *et al.*, 2005).

Intimin subtypes α, β, γ, ζ, δ and ε appear to be the most frequent among aEPEC strains of different serotypes worldwide, while other intimin subtypes are less frequent (Gomes *et al.*, 2004; Blanco *et al.*, 2006a, b; Jenkins *et al.*, 2006; Abe *et al.*, 2007). aEPEC strains of a single serotype isolated in different geographic areas were shown to carry the same intimin subtype, for example, aEPEC serotype O51:H40 isolated in Brazil, Uruguay and Spain possessing intimin subtype θ (Blanco *et al.*, 2006a, b; Moreira *et al.*, 2008). Conversely, certain aEPEC serotypes carry different intimin subtypes, for example, O80:H26 carries either intimin subtype β or ε (Blanco *et al.*, 2006a; Jenkins *et al.*, 2006). The occurrence of aEPEC strains of a single serotype carrying different intimin subtypes confirms the diversity of aEPEC strains and reinforces the proposal that the LEE may have been acquired at different periods of time by different strains (Donnenberg & Whittam, 2001).

Tir

Tir is inserted in the plasma membrane of eukaryotic cells, exposing its middle segment at the cell surface. The amino and carboxy portions of Tir remain exposed in the cytosol, interacting with host proteins and other bacterial proteins (reviewed by Frankel & Phillips, 2008).

Ooka *et al.* (2007) have recently found two human aEPEC strains (serotypes O104:H12 and ONT:H19) that harbor new variant Tir proteins, which exhibit $< 65\%$ sequence identity to Tir molecules reported previously. While the Tir intimin-binding domains of these strains are highly conserved, the cytoplasmic terminal regions are highly divergent. As the functional analysis of these Tir variants is lacking, more information is required to better understand the implications of these findings in aEPEC pathogenesis.

Non-LEE-encoded putative T3SS effectors

Various effector proteins encoded outside the LEE, which are secreted by T3SS, have been described in EHEC O157, *Citrobacter rodentium* and tEPEC (Deng *et al.*, 2004; Tobe *et al.*, 2006; Iguchi *et al.*, 2009). The contribution of these various putative effectors (e.g. NleA/EspI, NleB, NleC, NleD, NleE, NleF, NleG and NleH) to virulence is still under investigation (Gruenheid *et al.*, 2004; Mundy *et al.*, 2004; Marchès *et al.*, 2005; Kelly *et al.*, 2006; Li *et al.*, 2006; reviewed by Dean & Kenny, 2009). Another non-LEE-encoded effector is the cycle-inhibiting factor (Cif), which is encoded by a lambdoid prophage in several tEPEC and EHEC strains and causes an irreversible cytopathic effect in host cells (Marchès *et al.*, 2003; Taieb *et al.*, 2006). EspG2, which is encoded on the EspC PAI, triggers the disruption of the microtubule network of the host cells (Shaw *et al.*, 2005).

Studies on the frequency of these non-LEE effectors among aEPEC strains are scarce. In a case control study in Norwegian children, Afset *et al.* (2006) found that in aEPEC strains, the *nleB* and *nleE* genes were statistically associated with diarrhea. In addition, in a study conducted in Brazil, the Nle-encoding genes were more frequent in tEPEC than in aEPEC strains, with *espG2* and *nleC* being the most, and *nleD* the least frequent in both groups. The *espG2* and *nleC* genes occurred in 70% and 58% of 63 aEPEC strains, respectively (T.A.T. Gomes *et al.*, unpublished data).

In contrast to the LEE of tEPEC, the LEE of EHEC is not sufficient for A/E lesion formation (Elliott *et al.*, 1999). EHEC injects into the host cell its own adaptor protein, Tir cytoskeleton coupling protein, which connects EHEC Tir to N-WASP and promotes actin accumulation via Arp2/3 complex activation (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). To date, two variants of the *tccP* gene have been described: *tccP* (carried on prophage CP-933U/Sp14) and *tccP2* (carried on prophage Sp4/CP-933 M). Studies concerning the characterization of aEPEC have shown the presence of *tccP* and *tccP2*, and have demonstrated that aEPEC strains can trigger actin polymerization by Tir-Nck (which depends on the phosphorylation of Y474) and/or Tir-TccP pathways (Garmendia *et al.*, 2005b; Ooka *et al.*, 2007; Frankel & Phillips, 2008).

EPEC interaction with host cells

Characteristically, after 3 h of incubation, tEPEC strains produce localized adherence (LA) to HeLa/HEp-2 cell surfaces, forming compact microcolonies (Fig. 1a) (Scaletsky *et al.*, 1984). LA is mediated by the BFP, a type IV pilus encoded by pEAF, which interconnects bacteria within microcolonies, promoting their stabilization (Girón *et al.*, 1991).

A variant LA pattern has been described in aEPEC strains, which is characterized by the presence of loose clusters of bacteria in few cells, and was named LA like (LAL) (Fig. 1b) (Rodrigues *et al.*, 1996). This pattern is defined only in assays using prolonged incubation periods (6-h assays) and is the most frequent pattern among aEPEC strains (Pelayo *et al.*, 1999; Scaletsky *et al.*, 1999; Vieira *et al.*, 2001; Dulguer *et al.*, 2003; Gomes *et al.*, 2004; Abe *et al.*, 2007). LAL probably corresponds to the poor LA pattern as described previously by Knutton *et al.* (1991). Notably, many variations of the LA pattern can be found, in which most show a tendency toward the formation of loose clusters (Vieira *et al.*, 2001; Hernandes *et al.*, 2006).

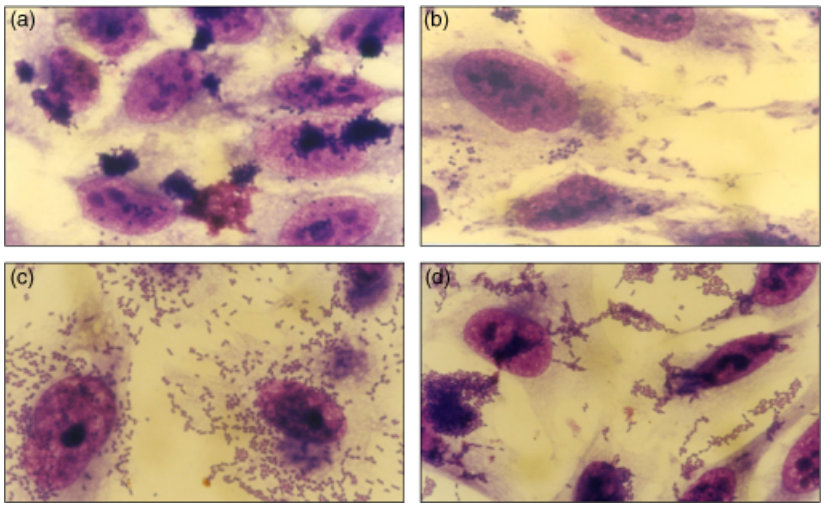

Fig. 1. Distinct adherence patterns exhibited by aEPEC strains in HeLa cells (a) LA, (b) LAL, (c) DA and (d) AA.

Furthermore, some *E. coli* strains, fitting the concept of aEPEC, can express adherence patterns distinct from LAL, as diffuse adherence (DA) (Fig. 1c) or aggregative adherence (AA) (Fig. 1d) (Vieira *et al.*, 2001; Dulguer *et al.*, 2003; Nunes *et al.*, 2003; Robins-Browne *et al.*, 2004; Abe *et al.*, 2007). In some DA-producing aEPEC strains, this pattern results from the expression of *daaC* and *afa*, which are sequences associated with the Dr family of adhesins (Beinke *et al.*, 1998; Vieira *et al.*, 2001; Keller *et al.*, 2002). Scaletsky *et al.* (2005) cloned a 15-kb genomic region of an aEPEC O26:H11 strain designated the locus for DA (*lda*), which encodes a nonfimbrial structure conferring DA phenotype, and whose expression is induced by bile salts (Torres *et al.*, 2007).

aEPEC strains of serotype O125ac:H6 express AA in HEp-2 cells and lack EAEC-virulence-associated markers (Elias *et al.*, 2002; Barros *et al.*, 2008). An outer membrane protein has been identified as a putative aggregative adhesin in these strains (W.P. Elias *et al.*, unpublished data).

One very large protein called lymphocyte inhibitory factor (LifA) was originally characterized in EPEC strains as the toxin that inhibits lymphocyte activation (Klapproth *et al.*, 2000). An adhesin encoded by the O island 122 (OI-122) PAI termed EHEC factor adhesin 1 (Efa1) was identified in EHEC strains of serotype O111:HNM, showing a similarity of 99.9% with LifA (Nicholls *et al.*, 2000). Although Efa1/LifA has been implicated in the attachment of aEPEC strains to host cells (Badea *et al.*, 2003), its association with diarrheal diseases is controversial (Robins-Browne *et al.*, 2004; Afset *et al.*, 2006). In a recent study in Brazil, the *efa1/lifA* gene was found to be more frequent among tEPEC (60%) than among aEPEC (28%) strains (M.A.M. Vieira *et al.*, unpublished data).

Although we have recently identified many other putative *E. coli* adhesin-encoding genes (*paa*, $lpfA_{O113}$, *iha*, *toxB* and *lda*G) in a collection of aEPEC strains (T.A.T. Gomes *et al.*, unpublished data), the role of such adhesins remains to be established.

The invasion of intestinal epithelial cells has been considered a critical virulence characteristic of several important enteric pathogens. Although EPEC is considered an extracellular pathogen, some *in vivo* and *ex vivo* studies have shown the presence of these pathogens inside human enterocytes (Polotsky *et al.*, 1977; Ulshen & Rollo, 1980; Pedroso *et al.*, 1993). Moreover, the invasion ability of EPEC strains has been demonstrated *in vitro* using different cell lines, including HeLa, HEp-2 and Caco-2 cells (Andrade *et al.*, 1989; Donnenberg *et al.*, 1989; Francis *et al.*, 1991). Regarding aEPEC strains, whereas some studies have shown that they invade epithelial cells efficiently (Scaletsky *et al.*, 1996; Rosa *et al.*, 2001; Hernandes *et al.*, 2008), other studies have not (Pelayo *et al.*, 1999; Robins-Browne *et al.*, 2004). The pathogenic role of the invasiveness of aEPEC strains is unknown. As invasive organisms may be protected from destruction by the immune system and some antibiotics that do not penetrate eukaryotic cells, invasion could contribute to the permanence of certain aEPEC strains in the intestine, resulting in the persistent diarrhea reported in recent studies (Afset *et al.*, 2004; Nguyen *et al.*, 2006).

Diversity of virulence properties

In general, aEPEC strains may carry genes encoding virulence factors of other DEC pathotypes more often than tEPEC strains (Trabulsi *et al.*, 2002). Among 59 aEPEC strains of nonclassical EPEC serogroups tested for the presence of 15 virulence DNA sequences of various DEC pathotypes, Vieira *et al.* (2001) observed 17 different combinations of specific virulence sequences (genetic profiles), which reflected the heterogeneity of the group. Extending

the analysis of Vieira *et al.* (2001) to additional putative virulence genes, and including strains from other geographic regions in Brazil, Gomes *et al.* (2004) demonstrated that the aEPEC strains of nonclassical EPEC serogroups are even more diverse. Interesting genetic combinations were observed, for example, extraintestinal pathogenic *E. coli* and EAEC genes, and tEPEC (*bfpA*) and EHEC (*ehly*) genes. In a survey of aEPEC strains isolated in another geographic region of Brazil, genes encoding the DEC autotransporter proteins Pet, Pic, Sat, EspC and EspP were detected. Except for *espC*, detected in 29.2%, the other genes were of low prevalence (*sat*/11.1%, *pet*/5.5%, *espP*/4.2% and *pic*/1.4%) (W.P. Elias *et al.*, unpublished data). These proteins include virulence factors such as toxins, proteases and adhesins (Henderson *et al.*, 2004), but their role in aEPEC pathogenesis remains to be established.

Because genes encoding virulence factors are located on transmissible plasmids, PAIs, transposons or bacteriophages, the emergence of different combinations of virulence gene sequences found in aEPEC strains is not surprising. These sequences could have been transferred horizontally to these strains in the intestine and/or environment. In addition, some of the strains are probably tEPEC that have lost pEAF (or part of it), or EHEC that have lost *stx* phage sequences during infection.

Escherichia coli of serogroup O26 can bear serotypes that cause haemolytic-uremic syndrome (EHEC strains) or aEPEC associated with uncomplicated diarrhea. Bielaszewska *et al.* (2007b) compared the presence of the OI-122, which is present in highly virulent EHEC strains, among aEPEC O26 and EHEC O26 clinical isolates. Most of them contained OI-122 virulence genes (*efa1/lifA, nleB, nleE* and *ent*) and shared a highly conserved PAI, suggesting that aEPEC O26 and EHEC O26 are closely related. In fact, based on serotypes, non-*stx* virulence profiles and multilocus sequence types (MLST), Bielaszewska *et al.* (2008) showed in a study in Germany that most aEPEC strains associated with bloody diarrhea are probably EHEC that had lost Stx-encoding phages (EHEC-LST) during infection. Indeed, recent work in our laboratory has shown the presence of various STEC virulence genes among 117 aEPEC strains, i.e. *paa* (49.6%), $lpfA_{O113}$ (26.5%), *iha* (25.6%), *ehx*, *toxB*, *kat* and *espP* (6.0% each), and *lda*G (2.6%). Interestingly, all *ehx*-positive strains also carried *kat* and *espP*, which are located in the EHEC plasmid (T.A.T. Gomes *et al.*, unpublished data).

Studies concerning aEPEC virulence have aimed at identifying diarrhea-associated virulence genes or phenotypic characteristics. Scaletsky *et al.* (1999) showed that aEPEC expressing LAL were significantly associated with diarrhea (Table 1). In a study of infectious intestinal disease in England, the characterization of aEPEC strains by serotyping, intimin subtyping and antimicrobial resistance typing showed no significant differences among aEPEC strains isolated from patients vs. healthy controls (Jenkins *et al.*, 2006). Using DNA microarray analyses to search for diarrhea-linked genes, Afset *et al.* (2006) found that OI-122 genes (*efa1*/*lifA, set*/*ent*, *nleB* and *nleE*) and certain genes located elsewhere (*lpfA*, *paa*, *ehxA* and *ureD*) were associated with diarrhea. In another study, Dulguer *et al.* (2003) demonstrated a strong association between the enteroaggregative *E. coli* heat-stable 1 (East-1) encoding-gene (*astA*) and diarrhea in aEPEC strains isolated in different regions of Brazil. However, whether these virulence-associated genes are expressed in aEPEC is yet to be demonstrated.

Phylogeny

Afset *et al.* (2008) compared the phylogenetic ancestry (groups A, B1, B2 and D) and virulence properties (by DNA microarray analysis) of aEPEC strains to search for an association between ancestry and diarrhea. The strains were serotyped and analyzed by MLST and pulsed-field gel electrophoresis (PFGE). These authors concluded that aEPEC strains are heterogeneous both phylogenetically and by virulence profile.

The investigation of the genetic diversity of aEPEC strains of serotype O51:H40 and their relatedness to tEPEC and EHEC by enterobacterial repetitive intergenic consensus PCR showed a much closer relationship between these strains and EHEC strain EDL933 (68% similarity) than to tEPEC strain E2348/69 (30% similarity) (Moreira *et al.*, 2008).

Epidemiology

Recent epidemiological studies suggest an increasing identification of aEPEC in both developed and developing countries (reviewed by Trabulsi *et al.*, 2002, Table 1). Some of these studies have shown an association of aEPEC with childhood diarrhea (Afset *et al.*, 2004; Cohen *et al.*, 2005; Moreno *et al.*, 2008; Estrada-Garcia *et al.*, 2009), whereas others have not (Vieira *et al.*, 2001; Nunes *et al.*, 2003; Nataro *et al.*, 2006; Spano *et al.*, 2008; D.M. Girão, pers. commun.). The studies in Table 1 show the prevalence of aEPEC worldwide and were performed at different times. Although some studies have shown an association of aEPEC strains with acute childhood diarrhea (Scaletsky *et al.*, 1999; Franzolin *et al.*, 2005; Araujo *et al.*, 2007), recent works have implicated aEPEC as agents of persistent diarrhea (Afset *et al.*, 2004; Nguyen *et al.*, 2006).

Despite the controversial data regarding the epidemiological association of aEPEC strains with diarrhea, the occurrence of aEPEC strains in diarrheic patients of various ages and in patients with AIDS (Gassama-Sow *et al.*, 2004; Gomes *et al.*, 2004) indicate that some aEPEC strains with certain genetic combinations are true enteropathogens.

Table 1. Epidemiological studies on aEPEC

	aEPEC			
Location	Case (%)	Control (%)	Comments*	References
USA (Seattle)	3.8	ND	*eae*$^+$ EAF$^-$	Bokete *et al*. (1997)
Brazil (Rio de Janeiro)	5.1	0.0	*eae*$^+$ EAF$^-$	Rosa *et al*. (1998)
Brazil (São Paulo)†	17.5	2.5	*E. coli* with LAL	Scaletsky *et al*. (1999)
Poland	21.3	8.0	*eae*$^+$ *bfpA*$^-$	Paciorek (2002)
Brazil (São Paulo)	6.0	2.0	*E. coli* with LAL	Scaletsky *et al*. (2002)
Norway	9.8	ND	*eae*$^+$ *bfpA*$^-$	Afset *et al*. (2003)
Brazil (Rio de Janeiro)	7.0	11.0	*eae*$^+$ EAF$^-$	Regua-Mangia *et al*. (2004)
Australia (Melbourne)†	12.8	2.3	*eae*$^+$ *bfpA*$^-$	Robins-Browne *et al*. (2004)
Norway†	14.7	10.0	*eae*$^+$ *bfpA*$^-$	Afset *et al*. (2004)
Mongolia†	1.6	0.0	*eae*$^+$ *bfpA*$^-$	Sarantuya *et al*. (2004)
Vietnam (Hanoi)	6.6	4.4	*eae*$^+$ *bfpA*$^-$	Nguyen *et al*. (2005)
Brazil (Salvador)	10.1	ND	*eae*$^+$ EAF$^-$	Franzolin *et al*. (2005)
Mexico	21.0	ND	*eae*$^+$ *bfpA*$^-$	Estrada-García *et al*. (2005)
USA (Cincinnati)†	6.5	3.9	*eae*$^+$ EAF$^-$	Cohen *et al*. (2005)
Spain (Lugo)	5.2	ND	*eae*$^+$ *bfpA*$^-$	Blanco *et al*. (2006a)
Brazil (Rondonia)†	4.0	0.49	*eae*$^+$ *bfpA*$^-$	Orlandi *et al*. (2006)
USA (Baltimore/New Haven)	4.0	3.4	*eae*$^+$ EAF$^-$ *bfpA*$^-$	Nataro *et al*. (2006)
Iran†	9.3	1.2	*eae*$^+$ *bfpA*$^-$	Alikhani *et al*. (2006)
India	5.5	ND	*eae*$^+$ *bfpA*$^-$	Wani *et al*. (2006)
Brazil (São Paulo)†	5.4	0.7	*eae*$^+$ EAF$^-$	Araujo *et al*. (2007)
Brazil (Salvador)†	10.0	5.9	*eae*$^+$ EAF$^-$	Bueris *et al*. (2007)
Tanzania	0.36	ND	*eae*$^+$ *bfpA*$^-$	Moyo *et al*. (2007)
Mexico (Mexico City)	7.6	1.2	*eae*$^+$ *bfpA*$^-$	Paniagua *et al*. (2007)
Vietnam (Hanoi)	4.5	3.6	*eae*$^+$ EAF$^-$*bfpA*$^-$	Hien *et al*. (2007)
Germany	10.5	ND	*eae*$^+$ *bfpA*$^-$	Kozub-Witkowski *et al*. (2008)
Vietnam (Hanoi)	10.4	6.45	*eae*$^+$ *bfpA*$^-$	Hien *et al*. (2008)
Brazil (Espírito Santo)	5.5	6.9	*eae*$^+$ EAF$^-$	Spano *et al*. (2008)

*Epidemiological studies were selected based on the following criteria: detection of fecal *Escherichia coli* strains presenting *eae* and lacking pEAF genes (EAF sequence and or/*bfpA*), except for two studies detecting *E. coli* strains expressing the LAL phenotype.
†Studies that demonstrate a statistical association of aEPEC strains with diarrhea.
ND, not determined.

Several outbreaks have implicated aEPEC as the causative agent of diarrhea (Viljanen *et al*., 1990; Hedberg *et al*., 1997; Trabulsi *et al*., 2002). In one outbreak involving >100 adults, the implicated pathogen was *E. coli* O39:HNM (Hedberg *et al*., 1997). Another diarrhea outbreak involving >600 people implicated an O111 aEPEC strain (Viljanen *et al*., 1990). A waterborne diarrheal outbreak affecting 41 of 75 students (ages 12–15) was reported in Akita Prefecture, Japan (Yatsuyanagi *et al*., 2003). Among the 41 individuals with clinical symptoms, seven carried aEPEC isolates of serotype ONT:H45. Interestingly, all three outbreak-associated *E. coli* strains (O39:NM, O111 and ONT:H45) also carried *astA*. As *astA* has been detected in commensal *E. coli* strains as well as other DEC pathotypes (Savarino *et al*., 1996), the virulence role of East-1 is questionable.

Another outbreak involving aEPEC was described in infants at a daycare center in Japan. The only diarrheagenic bacterial pathogens isolated from the patients were four aEPEC O55:HNM isolates from four patients, which showed identical PFGE patterns, suggesting that the strains originated from common infectious sources (Yatsuyanagi *et al*., 2002).

Transmission and reservoir

A large number of reports on the isolation of aEPEC from a wide range of diarrheic and healthy animal species have been published. In most of the cases, these aEPEC strains have been identified in the course of characterization of A/E *E. coli* isolates among animals used for food production, such as cattle, sheep, goat, pig and poultry (Aidar *et al*., 2000; Aktan *et al*., 2004; Stephan *et al*., 2004; Blanco *et al*., 2005; Krause *et al*., 2005; Leomil *et al*., 2005; Yuste *et al*., 2006; Ishii *et al*., 2007; Wani *et al*., 2007). Moreover, the detection of aEPEC in domestic animals (dog and cat), horse, deer and marmoset has also been described (Beaudry *et al*., 1996; Goffaux *et al*., 2000; Carvalho *et al*., 2003; Nakazato *et al*., 2004; Krause *et al*., 2005; Ishii *et al*., 2007; A. Cerqueira, pers. commun.).

Although there is no evidence of direct transmission from animals to humans, some aEPEC strains isolated from animals belong to serogroups implicated in human diseases, for example, O26, O103, O119, O128 and O142 (Aidar *et al.*, 2000; Carvalho *et al.*, 2003; Aktan *et al.*, 2004; Nakazato *et al.*, 2004; Blanco *et al.*, 2005; Krause *et al.*, 2005; Leomil *et al.*, 2005; Yuste *et al.*, 2006), suggesting that these animals may represent important reservoirs of aEPEC, which can be transmitted to humans.

Detection and diagnosis

Traditionally, EPEC strains have been identified by the expression of certain serotypes epidemiologically linked to infantile diarrhea. However, as highlighted above, recent studies have shown that aEPEC belong to a large diversity of serotypes, many of which belong to nonclassical EPEC serogroups (Table S1). Furthermore, a substantial percentage of the strains are O and/or H nontypeable and many are nonmotile. In addition, some *E. coli* strains belonging to the classical EPEC serogroups can harbor virulence properties not associated with EPEC but with other DEC pathotypes (Campos *et al.*, 2004). Therefore, phenotypic and genotypic assays based on the presence (or absence) of certain virulence properties should be used for EPEC identification. Table 2 summarizes the most important properties that may distinguish tEPEC and aEPEC strains.

The phenotypic methods to identify tEPEC and distinguish them from aEPEC include the determination of the LA adherence pattern on HeLa/HEp-2 cells and demonstration of BFP expression by immunologic assays (Fig. 1 and Table 2).

The genotypic assays include PCR and multiplex PCR, as well as genetic probes, to detect pEAF (EAF probe), *bfpA* (encoding the major pilin subunit of BFP) and *eae* genes (or other conserved LEE-genes) (Baldini *et al.*, 1983; Jerse *et al.*, 1990; Girón *et al.*, 1993; Aranda *et al.*, 2007; Müller *et al.*, 2007). EPEC strains have been identified by the presence of the LEE region and absence of the *stx* genes (*stx*-negative), which distinguish them from EHEC. tEPEC and aEPEC strains are differentiated mainly by the presence of the EAF sequence and/or the *bfpA* gene in the former group. However, aEPEC strains from serotypes O119:H2 and O128:H2 react with the *bfpA* probe but lack a true pEAF. Instead, these strains have a large plasmid defective in the *bfp* operon and, consequently, do not produce BFP, the reason for their classification as aEPEC (Gonçalves *et al.*, 1997; Bortolini *et al.*, 1999). Conversely, some O142:H6 strains that do not react with the EAF probe but produce BFP, probably have a defective but still functional pEAF and are thus classified as tEPEC (Ghilardi *et al.*, 2003). Based on this information, Trabulsi *et al.* (2002) suggested that the best distinguishing characteristic for tEPEC and aEPEC strains would be production or nonproduction of BFP, respectively. To further confirm the potential pathogenicity of an aEPEC strain, it is necessary to demonstrate its ability to produce A/E lesion on epithelial cells. Nevertheless, the use of such methods is restricted to research laboratories.

It is important to emphasize that in bloody diarrheal cases, isolates characterized as aEPEC could comprise EHEC-LST strains (Bielaszewska *et al.*, 2008). It may be difficult to distinguish between both groups, unless *stx*-positive and *stx*-negative isolates of the same serotype and identical genotypic characteristics are detected in the same patient.

Table 2. Important characteristics of tEPEC and aEPEC

Characteristic	tEPEC	aEPEC
A/E lesion formation	+	+
stx genes	−	−
EAF probe sequence	+	−
bfpA	+	−/+*
BFP expression	+	−
Adherence pattern†	LA	LAL/DA/AA/LA‡/NA

*Incomplete *bfp* operon (Bortolini *et al.*, 1999).

†Adherence pattern in HeLa/HEp-2 cell: LA, localized adherence; LAL, LA like; DA, diffuse adherence; AA, aggregative adherence; NA, nonadherent.

‡The LA phenotype in aEPEC strains is independent of BFP expression.

Conclusions

The heterogeneous nature of strains fitting the concept of aEPEC is probably responsible for their controversial association with human diarrhea. However, the significantly higher prevalence in patients than in healthy controls in certain geographical regions and the occurrence of diarrheal outbreaks strongly confirm that aEPEC strains are truly diarrheagenic pathogens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Serotypes identified among atypical EPEC strains isolated from humans.

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