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Atypical enteropathogenic *Escherichia coli* as aetiologic agents of sporadic and outbreak-associated diarrhoea in Brazil

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Enteropathogenic *Escherichia coli* (EPEC) are important agents of diarrhoea in industrialized as well as developing countries, such as Brazil. The hallmark of EPEC pathogenesis is the establishment of attaching and effacing lesions in enterocytes, in which pedestal-like structures are formed underneath adherent bacteria. EPEC are divided into two subgroups, typical (tEPEC) and atypical (aEPEC), based on the presence of the EPEC adherence factor plasmid in tEPEC and its absence in aEPEC. This study was designed to characterize 82 aEPEC isolates obtained from stool samples of diarrhoeic patients during 2012 and 2013 in Brazil. The majority of the aEPEC were assigned to the phylo-group B1 (48.8 %), and intimin subtypes θ (20.7 %), β1 (9.7 %) and λ (9.7 %) were the most prevalent among the isolates. The *nleB* and *nleE* genes were concomitantly detected in 32.9 % of the isolates, demonstrating the occurrence of the pathogenicity island O122 among them. The O157-plasmid genes (*ehxA* and/or *espP*) were detected in 7.3 % of the isolates, suggesting that some aEPEC could be derived from Shiga-toxin-producing *E. coli* that lost the *stx* genes while trafficking in the host. PFGE of 14 aEPEC of serotypes O2 : H16, O33 : H34, O39 : H9, O108 : H$^-$ and ONT : H19 isolated from five distinct outbreaks showed serotype-specific PFGE clusters, indicating a high degree of similarity among the isolates from the same event, thus highlighting these serotypes as potential aetiologic agents of diarrhoeal outbreaks in Brazil.

INTRODUCTION

The main feature of enteropathogenic *Escherichia coli* (EPEC) infections is the formation of a histopathological lesion in infected enterocytes, termed attaching and effacing, which is characterized by intimate EPEC adherence, destruction of brush border microvilli and formation of a pedestal-like structure that is rich in F-actin and other cytoskeletal elements (Kaper *et al.*, 2004). The proteins involved in attaching and effacing lesion formation are encoded by genes located in a pathogenicity island known as locus of enterocyte effacement (LEE) region (McDaniel *et al.*, 1995). The LEE region harbours the *eae* and *tir* genes, which encode the adhesive protein called intimin and its translocated receptor (Tir), respectively (Jerse *et al.*, 1990; Kenny *et al.*, 1997). Variations found in the C-terminal sequence of the *eae* gene define many different intimin subtypes and can be used as an additional tool for EPEC typing (Vieira *et al.*, 2001; Abe *et al.*, 2009; Mora *et al.*, 2009).

EPEC are divided into typical (tEPEC) and atypical (aEPEC), based on the presence of a large virulence plasmid designated EPEC adherence factor (pEAF) in tEPEC and its absence in aEPEC (Trabulsi *et al.*, 2002; Hernandes *et al.*, 2009). The pEAF harbours a set of genes that encode bundle-forming pili, responsible for developing compact bacterial micrcolonies on the surface of infected cells (Donnenberg *et al.*, 1992).

Epidemiological studies have indicated aEPEC as one of the most prevalent diarrhoeagenic *E. coli* pathotypes among diarrhoeic children and adults in different geographic areas

Abbreviations: aEPEC, atypical enteropathogenic *Escherichia coli*; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; STEC, Shiga-toxin-producing *E. coli*; tEPEC, typical enteropathogenic *E. coli*.

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(Gomes *et al.*, 2004; Cohen *et al.*, 2005; Foster *et al.*, 2015; Dias *et al.*, 2016). However, the association of these bacteria with diarrhoeal disease is still questionable due to similar identification rates found in both diarrhoeic and asymptomatic subjects in some studies (Hernandes *et al.*, 2009). In addition, aEPEC are recognized as a very heterogeneous group of isolates (Vieira *et al.*, 2001; Gomes *et al.*, 2004), in which only some isolates are truly enteropathogenic. Therefore, it is believed that outbreak-associated isolates are potentially the most pathogenic.

The occurrence of diarrhoeal outbreaks due to aEPEC infections have been reported in several parts of the world, such as Finland, Japan and China (Viljanen *et al.*, 1990; Yatsuyanagi *et al.*, 2003; Hao *et al.*, 2012), while, in Brazil, outbreaks caused by these pathogens have not been recorded, so far. In addition, aEPEC were implicated as the cause of persistent diarrhoea in studies conducted in Norway and Australia (Afset *et al.*, 2004; Nguyen *et al.*, 2006).

This study was designed to investigate phenotypic and molecular features of 82 aEPEC isolates obtained, during 2012 and 2013, from stool samples of diarrhoeic patients and to investigate the putative involvement of these pathogens as aetiologic agents of diarrhoeal outbreaks in Brazil.

METHODS

Atypical enteropathogenic *E. coli* isolates. The 82 aEPEC isolates (68 from sporadic cases and 14 from five diarrhoeal outbreaks) used in this study were obtained from stool samples of diarrhoeic patients at the Instituto Adolfo Lutz (a public health laboratory and the Brazilian reference center for pathogenic *E. coli*), during 2012 (35 isolates) and 2013 (47 isolates). The classification of *E. coli* isolates as aEPEC was based on the detection of the presence of the *eae* gene, located in the LEE region, and the absence of *bfpA* and *stx* genes (Hernandes *et al.*, 2009), using primers and PCR conditions as previously described by Peresi *et al.* (2016).

Serotyping. Identification of the O : H serotypes of the 82 aEPEC was performed by standard agglutination tests with absorbed antisera to somatic (O1 to O183) and flagellar (H1 to H56) antigens produced at the Instituto Adolfo Lutz, São Paulo, Brazil.

Pulsed-field gel electrophoresis (PFGE). The 14 outbreak-associated aEPEC isolates were subjected to chromosomal DNA restriction with 50 U XbaI enzyme (New England Biolabs) according to the PulseNet protocol for Shiga-toxin-producing *E. coli* (STEC) non-O157. PFGE was performed in a CHEF-DR III apparatus (BioRad Laboratories) using *Salmonella* Braenderup H9812 (Hunter *et al.*, 2005) as molecular size marker. The restriction profiles were analysed in Bionumerics v7.5 (Applied Maths), and a dendrogram was generated by the unweighted pair group method with arithmetic mean applying the Dice coefficient with optimization and tolerance set at 1.5 %.

Phylo-group determination. The *E. coli* phylo-groups (A, B1, B2, C, D, E and F) and *Escherichia* clades of the 82 aEPEC isolates studied were determined by the quadruplex PCR method as previously described by Clermont *et al.* (2013).

Intimin subtyping. The intimin gene (*eae*) was subtyped into $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, δ, γ, θ, $\varepsilon 1$, $\varepsilon 2$, ζ, $\eta 1$, $\eta 2$, $\iota 1$, $\iota 2$, λ, μ, ν, ξ and o, employing primers and PCR assay conditions previously described (Blanco *et al.*, 2006). aEPEC isolates where PCR products were detected with more than one pair of primer or not detected with any of the primers tested were classified as having a non-typed intimin (NT). The positive controls for the 19 distinct intimin subtypes tested were EPEC or STEC from a previous study (Mora *et al.*, 2009), and the negative control was *E. coli* K12 C600.

Investigation of the virulence-factor-encoding genes. The occurrence of several genes encoding virulence factors, such as adhesins (*afaBC*, *ecpA*, *efa1/lifA*, *iha*, *paa* and *saa*), toxins (*astA*, *cdt*, *cnf*, *ehxA*, *espP* and *subAB*) and non-LEE effector proteins (*cif*, *esp*T, *ibe*, *nleA*, *nleB*, *nleC*, *nleD*, *nleE* and *nleF*), was determined by DNA amplification. PCR was performed using Green master mix (Promega) with 0.34 μM of each of the primers. All primer sequences and PCR assay conditions used for the detection of virulence genes are described in the references cited in Table 1.

RESULTS AND DISCUSSION

The 82 diarrhoeic patients included in the present study were from three different Brazilian states: São Paulo (63.4 %), Minas Gerais (18.3 %) and Santa Catarina (18.3 %); they were residents of the state's capital and countryside cities as well. The majority of the patients were children (62.2 %) up to 9 years old, while the adults were 19–59 years old (36.6 %), except for one 87-year-old elderly patient (1.2 %). Regarding the patients' gender, 51.2 % of them were female, and 48.8 % were male. Other bacterial enteropathogens, such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Aeromonas* spp. and *Plesiomonas* spp., were not detected in the stool samples analysed in this study (data not shown).

Before molecular tools became available for EPEC identification, serological determination of certain O and H antigens was the predominant method used for this purpose (Robins-Browne, 1987; Nataro & Kaper, 1998). In this study, regarding the typing of the somatic (O) antigen, which is the basis for the division of *E. coli* into serogroups, 12 isolates were O-non-typeable, 6 were autoagglutinable (rough) and the remaining 64 aEPEC isolates were distributed in 36 distinct serogroups (Tables 2 and 3). Only 13 isolates (15.8 %) belonged to the classical EPEC serogroups (Table 2), where the serogroups detected were as follows: O26 (three isolates), O86 (one isolate), O111 (one isolate), O119 (one isolate), O125 (one isolate), O126 (two isolates), O127 (three isolates) and O142 (one isolate).

Among the 58 O : H serotypes identified, O33 : H34 (8.5 %) and O2 : H16 (4.9 %) were the most prevalent (Table 2). In a previous study performed in Brazil, among 59 aEPEC isolates, 35 serotypes were identified, and the serotype O51 : H40 was one of the most frequent (Vieira *et al.*, 2001). The occurrence of this serotype among aEPEC was also observed in other Brazilian studies (Moreira *et al.*, 2008; Abe *et al.*, 2009), but in our investigation, it was not detected. These data demonstrate how the prevalence of distinct aEPEC serotypes can vary between different studies, probably due to the period in which the aEPEC were isolated.

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Table 1. Primers used for amplification of virulence-factor-encoding genes in the aEPEC isolates studied

Target gene	Primer (5′→3′)	Amplicon size (bp)	Reference
Adhesins			
afaBC	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750	Le Bouguenec *et al.* (1992)
ecpA	GCAACAGCCAAAAAAGACACC CCAGGTCGCGTCGAACT	477	Hernandes *et al.* (2011)
efa1/lifA	AAGGTGTTACAGAGATTA TGAGGCGGCAGGATAGTT	268	Nicholls *et al.* (2000)
iha	CAGGTCGGGGTTACCAAGT CAAATGGCTCTCTTCCGTCAATGC	925	Szalo *et al.* (2002)
paa	GGATCCATGAGGAACATAA CTCGAGAGTGCCTTTCCTGG	605	Batisson *et al.* (2003)
saa	CGTGATGAACAGGCTATTGC ATGGACATGCCTGTGGCAAC	119	Paton & Paton (2002)
Toxins			
astA	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	111	Yamamoto & Echeverria (1996)
cdt	GAAARTAAATGGAAYAYAMATGTCCG AATCWCCWRSAATCATCCAGTTA	466	Tennant *et al.* (2009)
cnf	TTATATAGTCGTCAAGATGGA CACTAAGCTTTACAATATTGA	633	Tóth *et al.* (2003)
ehxA	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	Paton & Paton (1998)
espP	GTCCATGCAGGGACATGCCA TCACATCAGCACCGTTCTCTAT	547	Boisen *et al.* (2009)
subAB	GTACGGACTAACAGGGAACTG GCAAAAGCCTTCGTGTAGTC	1844	Paton *et al.* (2004)
Non-LEE effectors			
cif	AACAGATGGCAACAGACTGG AGTCAATGCTTTATGCGTCAT	383	Afset *et al.* (2006)
espT	AATCTCATTCTCTTATC TCATGTGATGAGTGGATG	263	Arbeloa *et al.* (2009)
ibe	TTCTTAAGTGCGGAGATGA TTAGTCAATATCTGTGGGTT	425	Buss *et al.* (2009)
nleA/espI	ATGAACATTCAACCGACCATACAATCTG TTAGACTCTTGTTTCTTGGATTATATCA	1326	Afset *et al.* (2006)
nleB	GGTGTGCTGGTAGATGGA CAGGGTATGATTCTTGTTTATG	175	Afset *et al.* (2006)
nleC	TATGCGGATTACGTTTTGGA TACGGCATCACGAAATGTCT	192	Afset *et al.* (2006)
nleD	TGCTCCATGTTTTCCACAAT CTGTTGGAATCCCAGACTCA	245	Afset *et al.* (2006)
nleE	CTAATACTCAGGGCGTGTCC ACCGTCTGGCTTTCTCGTTA	192	Afset *et al.* (2006)
nleF	TTTGTAAGTGGGCTGATGGA CTGAAGAGTGATGCGGACAT	192	Afset *et al.* (2006)

Molecular typing methods, such as multilocus sequence typing and whole-genome sequencing, are constantly increasing our knowledge of the phylogenetic structure of *E. coli* isolates. Nowadays, eight different phylo-groups are recognized, and distribution of the *E. coli* isolates within these groups appears to be related to their origin (Clermont *et al.*, 2015). The triplex PCR used for classification of *E. coli* isolates into four main phylo-groups (A, B1, B2 and D) (Clermont *et al.*, 2000) has been substituted by a quadruplex PCR, which allows the assignment of *E. coli* isolates to the seven phylo-groups and *Escherichia* clades currently recognized (Clermont *et al.*, 2013). Using quadruplex PCR, we found that the aEPEC isolates studied belonged mainly to the phylo-group B1 (48.8 %), B2 (24.4 %) or A (24.4 %), as previously observed in other studies using triplex PCR (Afset *et al.*, 2008; Bando *et al.*, 2009).

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Table 2. Phylo-groups, intimin subtypes and serotypes found among 82 aEPEC isolates studied

Phylo-group	Intimin subtype	No. of isolates	Serotypes (no. of isolates)
A (*n*=20)	β1	4	O2 : H16 (4)
	δ	2	O88 : H$^-$ (1), O88:HNT (1)
	ε1	1	O157 : H16 (1)
	λ	1	OR : H$^-$ (1)
	θ	7	O117 : H40 (1), O117 : H$^-$ (1), O127 : H40 (2), ONT : H40 (2), OR : H40 (1)
	NT	5	O76 : H$^-$ (1), O86 : H$^-$ (1), O127 : H21 (1), ONT : H2 (1), ONT : HR (1)
B1 (*n*=40)	β1	4	O26 : H11 (3), O119 : H21 (1)
	ε1	1	O109:H23 (1)
	ε2	5	O88 : H25 (1), O123 : H19 (3), O126 : H19 (1)
	ι1	3	O39 : H9 (2), O98 : H$^-$ (1)
	θ	9	O66 : H21 (1), O108 : H21 (1), O111 : H8 (1), O146 : H21 (1), O170 : H49 (1), ONT : H8 (1), ONT : H21 (1), ONT : H$^-$ (1), OR : H$^-$ (1)
	ζ	1	O106 : H$^-$ (1)
	NT	17	O35 : H19 (1), O71 : H$^-$ (1), O85 : H4 (2), O91 : H23 (1), O103 : H4 (1), O108 : H$^-$ (2), O126 : H19 (1), O160 : H19 (2), ONT : H4 (1), ONT : H19 (2), ONT : H21 (1), OR : H19 (1), OR : H21 (1)
B2 (*n*=20)	α1	1	O177 : H$^-$ (1)
	α2	2	O63 : H6 (2)
	δ	1	O71 : H49 (1)
	ι1	1	O145 : H34 (1)
	λ	7	O33 : H34 (7)
	ζ	2	O156 : H1 (1), OR : H1 (1)
	NT	6	O37 : H$^-$ (1), O63 : H$^-$ (1), O121 : H$^-$ (1), O125 : H5 (1), O142 : H34 (1), ONT : H6 (1)
E (*n*=2)	γ	1	O145 : H$^-$ (1)
	θ	1	O153 : H31 (1)

NT, non-typed intimin.

Unlike previously noted (Afset *et al.*, 2008; Bando *et al.*, 2009), none of the 82 aEPEC studied were classified in the phylo-group D. For comparative purposes, all aEPEC were also tested by the triplex PCR method, and among the differences observed, the most notable was that the seven isolates assigned to the phylo-group D by triplex PCR were reclassified to the phylo-group B1 (six isolates) or E (one isolate), thus explaining the differences found between studies (data not shown).

Among the 82 aEPEC studied, 11 distinct intimin subtypes were detected, although in 34.1 % of the isolates, the intimin allele was non-typeable with the primers used (Table 2). The most prevalent intimin subtype was θ, detected in 17 isolates (20.7 %), followed by β1 (9.7 %), λ (9.7 %) and ε2 (6.0 %). The high prevalence of β1 and θ intimin subtypes among aEPEC isolates was previously reported in other studies (Abe *et al.*, 2009; Contreras *et al.*, 2010; Xu *et al.*, 2016). However, such prevalence can vary according to the geographic region and time at which the aEPEC isolates were isolated (Hernandes *et al.*, 2009). Interestingly, aEPEC harbouring intimin subtype θ was more common among isolates obtained in 2013 than in isolates obtained in 2012 (data not shown).

The allelic variability found in *eae* and other critical virulence LEE genes among different aEPEC isolates reflects the differences found in the LEE region (Müller *et al.*, 2009). Recently, 185 aEPEC from the Global Enteric Multicenter Study were sequenced and compared, showing the existence of 30 distinct LEE types among these isolates (Ingle *et al.*, 2016). In this study, we observed that some aEPEC isolates harbouring the same intimin subtype were assigned to different phylo-groups (Table 2), reinforcing the hypothesis that distinct *E. coli* lineages have independently acquired the LEE region at different times during the evolutionary history of this pathogen (Hazen *et al.*, 2013).

The prevalence of all virulence-factor-encoding genes investigated and combinations found in each aEPEC serotype as well are described in Table 3. Since aEPEC lack bundle-forming pilus production, some other fimbrial and non-fimbrial adhesins have been reported, which appears to support the initial step of bacteria–cell interaction (Hernandes *et al.*, 2011). Regarding the adhesin-encoding genes investigated, the most prevalent was *ecpA* (65.8 %), followed by *paa* (28.0 %). A previous study reported that *ecpA* was highly prevalent among aEPEC isolates and that *E. coli* common pilus production was detected in aEPEC adhered to cultured epithelial cells (Hernandes *et al.*, 2011). The

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Table 3. Virulence features detected in the 82 aEPEC isolates studied

Serogroup	H type	Virulence genes encoding for†:			No. of isolates
		Adhesin	Toxin	Non-LEE effector	
O2	H16	*ecpA*, *efa1*	*cdt*	*nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*	4
O26*	H11	*efa1*, *paa*	*ehxA*, *espP*	*cif*, *ibe*, *nleB*, *nleC*, *nleE*	1
	H11	*ecpA*, *efa1*, *iha*, *paa*	*ehxA*, *espP*	*cif*, *ibe*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*	1
	H11	*ecpA*, *efa1*, *iha*, *paa*	*ehxA*, *espP*	*cif*, *ibe*, *nleB*, *nleE*, *nleF*	1
O33	H34	–	–	–	6
	H34	–	–	*nleF*	1
O35	H19	*ecpA*	–	*ibe*, *nleA*, *nleD*, *nleF*	1
O37	H$^-$	*paa*	*cdt*	*cif*, *nleC*, *nleD*, *nleF*	1
O39	H9	*ecpA*	–	*espT*, *ibe*, *nleA*, *nleF*	2
O63	H6	*paa*	–	*cif*, *nleC*, *nleF*	1
	H6	*ecpA*, *paa*	–	*cif*, *nleF*	1
	H$^-$	–	–	*nleC*, *nleD*	1
O66	H21	*ecpA*	–	*cif*, *nleB*, *nleD*, *nleE*, *nleF*	1
O71	H49	–	*astA*	*nleD*	1
	H$^-$	*ecpA*, *paa*	–	*nleA*, *nleC*, *nleD*, *nleF*	1
O76	H$^-$	*ecpA*	*ehxA*, *espP*, *astA*	*ibe*, *nleC*, *nleD*	1
O85	H4	*ecpA*, *paa*	–	*cif*, *ibe*	1
	H4	*ecpA*, *paa*	*espP*	*cif*, *ibe*, *nleF*	1
O86*	H$^-$	*ecpA*	–	*ibe*, *nleC*	1
O88	H25	–	–	*nleC*, *nleF*	1
	HNT	*ecpA*	–	*cif*, *nleA*, *nleC*, *nleF*	1
	H$^-$	*ecpA*	–	*cif*, *nleC*, *nleF*	1
O91	H23	–	–	–	1
O98	H$^-$	*paa*	–	*nleC*, *nleD*, *nleF*	1
O103	H4	*ecpA*, *paa*	–	*cif*, *ibe*, *nleC*, *nleD*, *nleF*	1
O106	H$^-$	*iha*	–	*espT*, *ibe*, *nleC*, *nleD*, *nleF*	1
O108	H21	*ecpA*, *paa*	–	*cif*, *nleB*, *nleC*, *nleE*, *nleF*	1
	H$^-$	*ecpA*	–	–	2
O109	H23	*ecpA*	–	*ibe*	1
O111*	H8	*efa1*, *paa*	–	*cif*, *nleA*, *nleB*, *nleE*, *nleF*	1
O117	H40	*ecpA*	–	*cif*, *nelB*, *nleC*, *nleE*, *nleF*	1
	H$^-$	*ecpA*	–	*cif*, *nelB*, *nleC*, *nleE*, *nleF*	1
O119*	H21	*ecpA*, *efa1*	–	*cif*, *nleB*, *nleE*, *nleF*	1
O121	H$^-$	–	–	*cif*, *nleF*	1
O123	H19	–	–	*ibe*, *nleC*, *nleF*	2
	H19	*ecpA*	–	*ibe*, *nleF*	1
O125*	H5	–	–	*cif*	1
O126*	H19	*ecpA*	–	*ibe*, *nleC*, *nleF*	2
O127*	H21	*ecpA*	–	*ibe*	1
	H40	*ecpA*	–	*cif*, *nleB*, *nleC*, *nleE*	1
	H40	*ecpA*	–	*cif*, *nleB*, *nleC*, *nleE*, *nleF*	1
O142*	H34	–	–	*nleA*, *nleC*	1
O145	H34	*paa*	–	*nleD*, *nleF*	1
	H$^-$	*ecpA*, *efa1*, *iha*, *paa*	*ehxA*, *espP*	*cif*, *ibe*, *nleB*, *nleC*, *nleE*, *nleF*	1
O146	H21	*ecpA*	–	*cif*, *espT*, *nleB*, *nleC*, *nleE*, *nleF*	1
O153	H31	*ecpA*, *paa*	–	*cif*, *espT*, *nleB*, *nleE*, *nleF*	1
O156	H1	*ecpA*	*cdt*	–	1
O157	H16	*ecpA*	–	*ibe*, *nleC*, *nleD*, *nleF*	1
O160	H19	*ecpA*	–	*nleC*, *nleF*	1
	H19	*ecpA*	–	*ibe*, *nleC*, *nleF*	1

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Table 3. cont.

Serogroup	H type	Virulence genes encoding for†:			No. of isolates
		Adhesin	Toxin	Non-LEE effector	
O170	H49	*ecpA*, *paa*	–	*cif*, *nleB*, *nleD*, *nleE*, *nleF*	1
O177	H$^-$	*ecpA*, *iha*	*cdt*	*cif*, *ibe*, *nleC*, *nleF*	1
ONT	H2	*efa1*	–	*ibe*, *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*	1
	H4	*ecpA*, *paa*	–	*ibe*, *nleD*	1
	H6	*ecpA*, *paa*	–	*cif*, *nleC*	1
	H8	*efa1*, *paa*	–	*cif*, *nleA*, *nleB*, *nleE*, *nleF*	1
	H19	*ecpA*	–	*ibe*, *nleC*, *nleF*	2
	H21	–	–	*ibe*	1
	H21	*ecpA*, *paa*	*astA*	*cif*, *ibe*, *nleB*, *nleC*, *nleD*, *nleE*	1
	H40	–	–	*nleB*, *nleE*	1
	H40	*ecpA*	–	*cif*, *nleB*, *nleC*, *nleE*	1
	HR	*ecpA*	–	*ibe*, *nleA*	1
	H$^-$	*ecpA*, *paa*	–	*ibe*, *nleB*, *nleD*, *nleE*, *nleF*	1
OR	H1	–	*cdt*	–	1
	H19	*ecpA*	–	*nleF*	1
	H21	*ecpA*	–	*nleC*	1
	H40	*ecpA*	–	*cif*, *nleB*, *nleC*, *nleE*, *nleF*	1
	H$^-$	*ecpA*, *paa*	–	*ibe*, *nleB*, *nleD*, *nleE*, *nleF*	1
	H$^-$	*ecpA*, *iha*	*astA*	*ibe*	1

ONT, O-non-typeable; OR, rough isolates; H$^-$, non-motile isolates.
*Classical EPEC serogroups.
†Virulence-factor-encoding genes (% of positive aEPEC) investigated: *afaBC* (0.0 %), *ecpA* (65.8 %), *efa1/lifa* (14.6 %), *iha* (7.3 %), *paa* (28.0 %), *saa* (0.0 %), *astA* (4.9 %), *cdt* (9.7 %), *cnf* (0.0 %), *ehxA* (6.0 %), *espP* (7.3 %), *subAB* (0.0 %), *cif* (37.8 %), *espT* (6.0 %), *ibe* (40.2 %), *nleA* (17.0 %), *nleB* (32.9 %), *nleC* (51.2 %), *nleD* (28.0 %), *nleE* (32.9 %) and *nleF* (62.2 %).

occurrence of *paa* in aEPEC has already been noted in other studies, and a case–control analysis has demonstrated an association of this gene with diarrhoeal disease (Afset *et al.*, 2006; Scaletsky *et al.*, 2009, Gomes *et al.*, 2011).

aEPEC of serotypes O26 : H11 (three isolates), O76 : H$^-$ (one isolate) and O145 : H$^-$ (one isolate) harboured the *ehxA* and *espP* genes, while the aEPEC of serotype O85 : H4 (one isolate) harboured only *espP* (Table 3). These are genes located in the O157 plasmid (pO157) and were first described in STEC of serotype O157 : H7 (Makino *et al.*, 1998). The occurrence of pO157 genes in aEPEC may suggest that these isolates probably comprise STEC that lost *stx* genes while trafficking in the host (Bielaszewska *et al.*, 2008), especially considering aEPEC belonging to well-recognized STEC serotypes, such as O26 : H11 and O145 : H$^-$, where the occurrence of the *ehxA* and *espP* genes, located on virulence plasmids, was already described by analysing sequence data (Fratamico *et al.*, 2011; Yan *et al.*, 2012). Moreover, the intimin subtypes found in aEPEC of serotypes O26 : H11 (intimin β1) and O145 : H$^-$ (intimin γ) are consistent with those found in STEC of the same serotype, thus reinforcing the hypothesis discussed earlier (Oswald *et al.*, 2000). This finding deserves special attention to prevent life-threatening complications associated with STEC infections, such as haemolytic uremic syndrome.

Several genes encoding non-LEE type 3 secretion system-dependent effector proteins involved in bacterial invasion (*espT*), modulation of epithelial cell cycle (*cif*), inhibition of protein export from the endoplasmic reticulum (*nleA/espI*), inhibition of pro-inflammatory signalling (*nleB*, *nleC*, *nleD* and *nleE*), inhibition of apoptosis (*nleF*) and enhancement of pedestal formation (*ibe*) have been investigated (Wong *et al.*, 2011; Vossenkämper *et al.*, 2011). Among these genes, the most prevalent were *nleF* (62.2 %), *nleC* (51.2 %) and *ibe* (40.2 %), while *espT* (6.0 %) was the least prevalent, as previously reported in other studies aimed at characterizing aEPEC (Afset *et al.*, 2006, Salvador *et al.*, 2014, Arbeloa *et al.*, 2009).

The genes from the pathogenicity island O122 (PAI-O122) *nleB* and *nleE* were concomitantly detected in 32.9 % of the isolates, while *efa1/lifA* was observed in only 14.6 % (12 isolates). It is important to clarify that all 12 aEPEC isolates that possessed the *efa1/lifA* gene also harboured the other genes from PAI-O122 investigated (*nleB* and *nleE*). Some studies have demonstrated the association of these genes with diarrhoeal disease, while in other studies, such association was not observed (Afset *et al.*, 2006; Scaletsky *et al.*, 2009; Salvador *et al.*, 2014). Interestingly, one study found that a complete PAI-O122 (*efa1*, *pagC*, *sen*, *nleB* and *nleE*) was significantly more common in aEPEC isolated from

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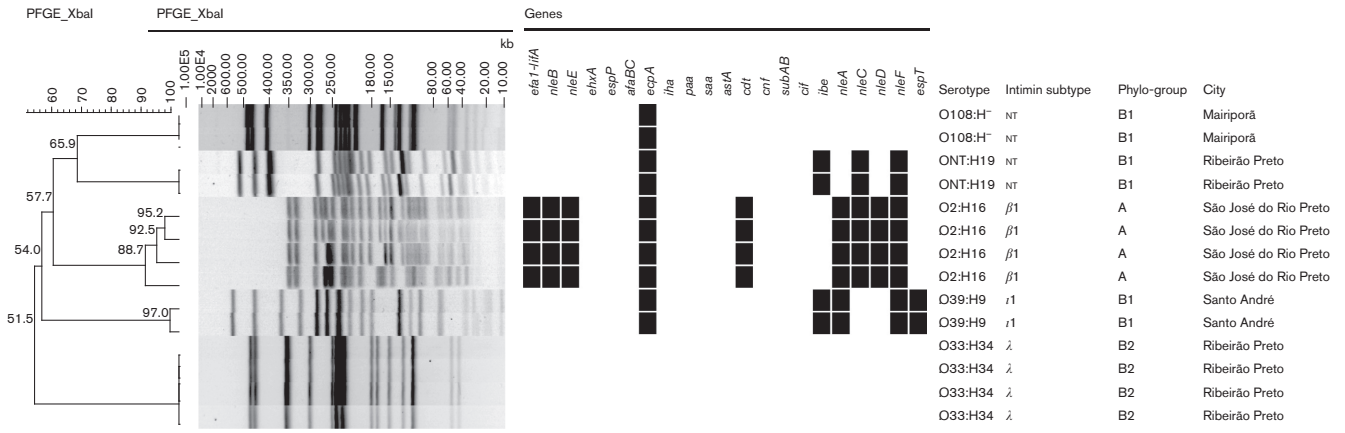

Fig. 1. Dendrogram showing the relationships among aEPEC isolates of serotypes O2:H16, O33:H34, O39 : H9, O108 : H$^-$ and ONT : H19, obtained from five diarrhoeal outbreaks, with additional information regarding the phylo-group, intimin subtype and occurrence of several virulence-factor-encoding genes. The numbers (65.9, 57.7, etc.) represent the degree of similarity (%) among the isolates.

diarrhoeic patients than from asymptomatic subjects (Vieira *et al.*, 2010), probably indicating that the proteins encoded by this set of genes can cooperatively enhance the virulence of these isolates.

Of importance, the aEPEC of serotypes O2 : H16 (four isolates), O33 : H34 (four isolates), O39 : H9 (two isolates), O108 : H$^-$ (two isolates) and ONT : H19 (two isolates) were isolated from diarrhoeic patients involved in five distinct outbreaks, investigated by the São Paulo State Epidemiological Surveillance Center. The five outbreaks investigated in the present study occurred in four different cities in São Paulo State, including Ribeirão Preto, São José do Rio Preto, Santo André and Mairiporã, as illustrated in Fig. 1. The presence of aEPEC isolates belonging to the same serotype in two or more diarrhoeic patients, involved in the same outbreak, indicated these pathogens as possible aetiologic agents of these events.

To evaluate this hypothesis, the outbreak-associated aEPEC isolates were subjected to PFGE, and the analyses confirmed the suspicion of outbreak isolates. The dendrogram generated showed the formation of serotype-specific PFGE clusters (Fig. 1), which indicated that aEPEC isolates from each outbreak were highly related, since a single restriction pattern, with 100 % similarity, was observed among isolates belonging to each of the serotypes: O108 : H$^-$, ONT : H19 and O33 : H34. Different but highly related restriction patterns were observed between the aEPEC of serotypes O39 : H9 (97.0 % similarity) and O2 : H16 (88.7 % similarity), where these differences were probably explained by the occurrence of acquisitions and/or deletions of mobile genetic elements, such as prophages and large plasmids, as well as recombination events that could have occurred within the genomic DNA.

None of the virulence markers investigated could be used to discriminate between outbreak-associated aEPEC from the other isolates studied. Interestingly, the outbreak-associated aEPEC of serotype O33 : H34 was devoid of all virulence-encoding genes investigated (Fig. 1), suggesting the existence of novel virulence mechanisms to cause damage in the host and produce disease. All phenotypic and genotypic features of the outbreak-associated aEPEC are illustrated in Fig. 1.

To the best of our knowledge, this is the first study to identify aEPEC of serotypes O2 : H16, O33: H34, O39 : H9, O108 : H$^-$ and ONT : H19 as potential aetiologic agents of diarrhoeal outbreaks in Brazil, although the serotypes O2 : H16 and ONT : H19 were previously isolated among Brazilian children with diarrhoea (Nunes *et al.*, 2003; Abe *et al.*, 2009). In the present study, the serotype O33 : H34 was isolated as an aetiologic agent of an outbreak (four isolates), as well as from three sporadic cases of diarrhoea during 2012 (one isolate) and 2013 (two isolates). Altogether, these lines of evidence indicate that some of the outbreak-associated aEPEC serotypes identified in this study are actually circulating and causing diarrhoea in the Brazilian setting.

In conclusion, our data demonstrated that the aEPEC studied were heterogeneous with respect to the serotypes and virulence traits investigated, hampering the identification of a common marker among the outbreak-associated aEPEC isolates. Since the identification of truly pathogenic aEPEC is still an open question, further studies with aEPEC outbreak-associated isolates are necessary to find phenotypic and/or molecular fingerprints that could potentially distinguish between pathogenic and non-pathogenic aEPEC in routine diagnosis.

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