

Adherence and virulence genes of *Escherichia coli* from children diarrhoea in the Brazilian Amazon

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Abstract

The bacterial pathogen most commonly associated with endemic forms of childhood diarrhoea is *Escherichia coli*. Studies of epidemiological characteristics of HEp-2 cell-adherent *E. coli* in diarrhoeal disease are required, particularly in developing countries. The aim of this study was evaluate the presence and significance of adherent *Escherichia coli* from diarrhoeal disease in children. The prevalence of LA, AA, and DA adherence patterns were determined in HEp-2 cells, the presence of virulence genes and the presence of the O serogroups in samples obtained from 470 children with acute diarrhoea and 407 controls in Porto Velho, Rondônia, Brazil. *E. coli* isolates were identified by PCR specific for groups of adherent *E. coli*. Out of 1,156 isolates obtained, 128 (11.0%) were positive for *eae* genes corresponding to EPEC, however only 38 (29.6%) of these amplified *bfpA* gene. EAEC were isolated from 164 (14.1%) samples; of those 41(25%), 32 (19%) and 16 (9.7%) amplified *egg*, *aggA* or *aafA* genes, respectively and *aggA* was significantly associated with diarrhoea ($P = 0.00006$). DAEC identified by their adhesion pattern and there were few isolates. In conclusion, EAEC was the main cause of diarrhoea in children, especially when the *aggA* gene was present, followed by EPEC and with a negligible presence of DAEC.

Key words: Enteroadherent *Escherichia coli*, diarrhoea, children.

Introduction

Diarrhoea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries (Nakhjavani *et al.*, 2013). The bacterial pathogen most commonly associated with endemic forms of childhood diarrhoea is *Escherichia coli*, which can be identified in about 50% of cases as presented by Chandra *et al.* (2012). At least six categories of diarrhoeagenic *E. coli* strains are recognized on the basis of distinct epidemiological and clinical features, specific virulence determinants, and an association with certain serotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Torres *et al.*, 2005). EPEC, EAEC, and DAEC isolates are

characterized by their distinct patterns of adherence to cultured epithelial cells *in vitro*. EPEC strains are responsible for a large number of cases of infantile diarrhoea in several developing countries (Abba *et al.*, 2009; Chandra *et al.*, 2012; Contreras *et al.*, 2012; Nakhjavani *et al.*, 2013). These strains belong to specific serotypes within different *E. coli* serogroups (O groups) and produce a characteristic adherence pattern in tissue culture cells called localized adherence (LA) (Humphries and Armstrong, 2010).

In the LA pattern, bacteria bind to localized areas of the cell surface, forming compact microcolonies (bacterial clusters) that can be visualized after 3 h of contact between the bacteria and the cells (Humphries and Armstrong, 2010). This phenomenon is associated with the presence of the EPEC plasmid adherence factor (EAF) (Bardiau *et al.*, 2010). The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E) characterized by

microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment (Humphries and Armstrong, 2010; Torres *et al.*, 2005). Atypical EPEC strains that do not carry the EAF plasmid can exhibit different adherence patterns including localized adherence like (LAL) pattern, and have been isolated from acute infantile diarrhoea in Brazil (Arenas-Hernández *et al.*, 2012; Bardiau *et al.*, 2010; Ochoa and Contreras, 2011).

EAEC strains are defined by their characteristic aggregative adherence (AA) to HEp-2 cells in bacterial culture (Weintraub, 2007). EAEC produce at least three fimbrial adhesins encoded on a 60- to 65-MDa virulence plasmid required for expression of the AA pattern: *aggA* (AAF/I), *aafA* (AAF/II), and *agg-3* (AAF/III) (Huang *et al.*, 2006).

DAEC strains are characterized by their dispersing adherence (DA) pattern on cultured epithelial cells (Servin, 2005a). Two adhesins capable of mediating the diffuse-adherence phenotype have been characterized for DAEC strains. F1845 is a fimbrial adhesin that mediates the adherence of the DAEC strain C1845 to epithelial cells (Servin, 2005a, b). F1845 fimbriae are encoded by five genes, designated *daaABCDE*. The significance of F1845 in virulence remains unclear because DAEC strains of faecal origin rarely express F1845 adhesin (Campos *et al.*, 1999; Torres *et al.*, 2005). The adhesin involved in diffuse adherence (AIDA-I) is a plasmid-encoded protein of the clinical DAEC strain 2787. The AIDA-I precursor protein is encoded by the *aida* gene and its mature form mediates diffuse adherence to HeLa cells (Benz and Schmidt, 1992a, b).

Studies evaluating the epidemiological characteristics of HEp-2 cell-adherent *E. coli* in diarrhoeal disease are required, particularly in developing countries. For this reason, and in order to determine the significance of the EAEC, EPEC, and DAEC strains as possible pathogenic microorganisms causing infantile diarrhoea, we deter-

mined the prevalence of the LA, AA, and DA patterns, the presence of virulence genes and the presence of O serogroups in samples obtained from children with acute diarrhoea and controls in Porto Velho, Rondônia, Brazil.

Material and Methods

Search for pathogens in the samples

Faecal samples from 470 diarrheic children (ages 0 to 72 months) and 407 children without diarrhoea (controls) were collected. The samples were collected at the Infantile Hospital Cosme and Damião in Porto Velho, Rondonia, Brazil, from March 2000 to March 2002.

Faecal samples were collected after natural excretion or through stimulation with a glycerin suppository. Samples were divided into two fractions: one fraction was used for parasitological examination for helminths eggs and protozoa cysts. The second aliquot was processed by routine microbiological and biochemical studies to identify *E. coli*. Five lactose-fermenting colonies and up to three lactose-negative colonies from each child were selected from McConkey plates to be tested by conventional and PCR procedures. Reference strains used as positive controls in the PCR tests included the *E. coli* strains O44H18, EDL933, 6085, O157:H7, H19 and C600PEB1 provided by Pasteur Institute (Paris, France). The non-pathogenic *E. coli* strain HB101 was used as a negative control and to monitor for PCR contamination.

PCR analysis

All strains were evaluated by PCR for identification of virulence genes from a distinct category of *E. coli*: *eaeA* and *bfpA* for EPEC; *astA* (encoding the toxin EAST1), *eagg*, *aafA* and *aggA* for EAEC; and *daaE* for DAEC (Pass *et al.*, 2000), using primer sequences presented in Table 1. PCR amplifications were performed as follows: 5.0 µL of bacterial extract was added to a reaction mixture (Life Technologies, Carlsbad, CA, USA) with a final volume of

Table 1 - Target genes and primers used to identify adherent *Escherichia coli* obtained from children diarrhoea in the Brazilian Amazon.

Target	Gene	Primer	EP	Reference
EPEC	<i>eaeA</i>	fp : 5'- TGAGCGGCTGGCATGAGTCATAC-3'	241	Pass <i>et al.</i> (2000)
		bp : 5'- TCGATCCCCATCGTACCAGAGG-3'		
EPEC	<i>bfp</i>	fp : 5'- AATGGTGCTTGCCTTGTCTGC-3'	324	Gunzburg <i>et al.</i> (1995)
		bp : 5'- GCCGCTTTATCCAACCTGGTA-3'		
EAEC	<i>eagg</i>	fp : 5'- AGACTCTGGCGAAAGACTGTATC-3'	194	Pass <i>et al.</i> (2000)
		bp : 5'- ATGGCTGTCTGTAATAGATGAGAAC-3'		
EAEC	<i>aggA</i>	fb : 5'- GCTAACGCTGCGTTAGAAAGACC-3'	352	Piva <i>et al.</i> (2003)
		bp : 5'- GGAGTATCATTCTATATTCGCC-3'		
EAEC	<i>aafA</i>	fp : 5' - GACAACCGCAACGCTGCGCTG-3'	307	Piva <i>et al.</i> (2003)
		p : 5' - GATAGCCGGTGAATTGAGCC-3'		

Note - EP: expected product; fp, forward primer; bp, backward primer.

50 µL containing: 0.1 mM each of dATP, dCTP, dGTP and dTTP PCR buffer (10 mM Tris HCL, pH 8.3; 50 mM KCl; 2 mM MgCl₂); 10 pMol of each PCR primer, and 1U of Taq DNA polymerase. Amplification was performed in a BioRad thermal cyclor. The conditions used for amplification of the target genes are presented in Table 2. After the amplification process the tubes were cooled rapidly at 4 °C. The amplified DNA products were resolved by agarose gel electrophoresis (2%, Life Technologies, Carlsbad, CA, USA) and visualized by UV transillumination after ethidium bromide staining.

HEp-2 adherence test

All *E. coli* isolates were subjected to HEp-2 adherence tests (Cravioto *et al.*, 1991). HEp-2 cells were grown overnight to 50% confluence in Dulbecco's Modified Eagle's medium (Gibco BRL, Gaithersburg-MD, USA) containing penicillin, streptomycin, and 2% foetal bovine serum on eight-well chamber slides. Bacteria were grown for 16 h in Luria broth without shaking. The HEp-2 cells were washed five times with Phosphate Buffered Saline and then the medium was replaced with Dulbecco's Modified Eagle's medium containing 1% mannose. A volume of 10 µL of bacterial suspension was added per well and the slides were incubated at 37 °C in 5% CO₂ for 3 h. The monolayers were washed five times with PBS, then fixed with 70% methanol and Giemsa. The strains were stained with solutions provided in the Panoptic Quick staining kit for one minute. Each strain was tested in duplicate, and appropriate controls were included in the test. Strains that adhered to the monolayers were recorded as adhering in localized, diffuse, or aggregative patterns.

Serotype methods

Enteroadherent *E. coli* isolates grown on nutrient agar were used for the identification of somatic (O) antigens by standard agglutination methods using specific polyvalent and monovalent antisera (Biomerieux, Craonne, Auvergne, France).

Statistical analysis

The prevalence of diarrhoeagenic *E. coli* in patient and control samples was compared by a two-tailed X² test with Yates correction and Fisher's exact test. When analysing the association of two or three pathogens in the same diarrhoeal patient, the same test was used to evaluate the probability of association by random chance as a function of their respective individual frequencies in the population.

Results

A total of 1.156 *E. coli* strains were isolated from faecal specimens from 470 diarrheic children (patients) and 407 children without diarrhoea (controls). The PCR screening results for virulence factors of enteroadherent diarrhoeagenic *E. coli* are presented in Table 3.

Screening for the *eae* sequence, which is specific for both EPEC and EHEC, there were 128 positive isolates comprising 11.7% of the total. Only 38 *eae*-positive patient isolates amplified with *bfpA* primers, indicating that they belonged to typical EPEC. Thus 90 isolates were considered as atypical EPEC, once all isolates were negative for verotoxin genes in previous work (Orlandi *et al.*, 2006). EAEC were isolated from 164 samples; of those 41 (25%), 32 (19%) and 16 (9.7%) amplified for *eagg*, *aggA* and *aafA*, respectively. Of the isolated EAEC, 75 (45.7%) exhibited the AA adhesion pattern in HEp-2 cells, not amplifying for any specific markers for EAEC tested in that State. DAEC were mainly characterized by the pattern of HEp2 cell adhesion. Among the 68 DAEC isolates, only 6 (8.8%) presented the *daaE* gene that encodes biogenesis of the F1845 adhesin. The enteroadherent *E. coli* analysed had higher frequencies in children with acute gastroenteritis (Table 4).

The results show us that all samples belonging to the typical EPEC showed the LA pattern of adhesion after 3 h of incubation; they were distributed in 26 (68.4%) patients and 12 (31.5%) of the control samples. Of the 90 isolated atypical EPEC, we found that 55 (61.1%) of the cases presented the LAL adhesion pattern after 6 h of incubation (Figure 1), while in the control samples 35 (38.9%) showed this adhesion profile. EAEC showed the AA adhesion pattern in 91 (55.5%) of the patients and 73 (44.5%) of the control samples (Figure 1). Diffuse adhesion was characterized

Table 2 - Amplification conditions for PCR reactions to identify adherent *Escherichia coli* obtained from children diarrhoea in the Brazilian Amazon.

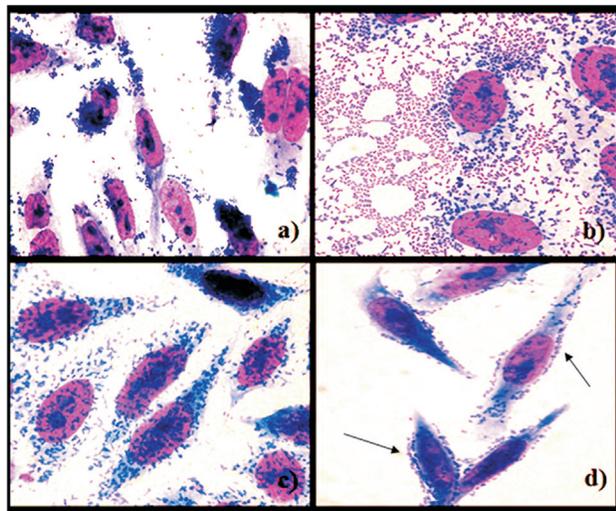
Target gene	First cycle	Intermediaries conditions	Final cycle
<i>eaeA</i>	94 °C, 5 min	25 cycles [94 °C, 2 min; 68 °C 1 min; 72 °C, 2 min]	72 °C, 5 min
<i>eagg</i>	94 °C, 5 min	25 cycles [94 °C, 2 min; 68 °C 1 min; 72 °C, 2 min]	72 °C, 5 min
<i>bfpA</i>	94 °C, 5 min	30 cycles [94 °C, 1 min; 56 °C 2 min; 72 °C, 1 min]	72 °C, 5 min
<i>daaE</i>	94 °C, 5 min	30 cycles [94 °C, 1 min; 56 °C 2 min; 72 °C, 1 min]	72 °C, 5 min
<i>aggA</i>	94 °C, 5 min	35 cycles [94 °C, 1 min; 60 °C 40 s; 72 °C, 30 s]	72 °C, 5 min
<i>aafA</i>	94 °C, 5 min	35 cycles [94 °C, 1 min; 60 °C 20 s; 72 °C, 15 s]	72 °C, 5 min
<i>astA</i>	94 °C, 5 min	30 cycles [94 °C, 1 min; 58 °C 1 min; 72 °C, 30 s]	72 °C, 5 min

Table 3 - Occurrence of enteropathogenic *E. coli* among children with and without diarrhoea from Porto Velho, RO, Brazil.

Pathogen (n° of colonies)	Virulence genes associated with Enteroadherent <i>E. coli</i>					
	<i>eaeA</i>	<i>bfp</i>	<i>eagg</i>	<i>aggA</i>	<i>aafA</i>	<i>daaE</i>
Typical EPEC (38)	38 (100%)	38 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Atypical EPEC (90)	90 (100%)	0 (0%)	10 (11.1%)	0 (0%)	0 (0%)	0 (0%)
EAEC (164)	0 (0%)	0 (0%)	41 (25%)	32 (19.5%)	16 (9.7%)	0 (0%)
DAEC (68)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6 (8.8%)

Table 4 - Adhesion pattern of *E. coli* isolated from children with and without diarrhea in Porto Velho, RO, Brazil.

Adhesion pattern	Total (n = 877)	No. of isolates (%) in children	
		With diarrhoea (n = 470)	Without diarrhoea (n = 407)
Typical EPEC (AL)	38 (4.3)	26 (5.5)	12 (2.9)
Atypical EPEC (ALL)	90 (10.2)	55 (11.7)	35 (8.5)
EAEC (AA)	164 (18.7)	91 (19.3)	73 (17.9)
DAEC (AD)	68 (7.7)	35 (7.4)	33 (8.1)
Total	360 (41.0)	207 (44.0)	153 (37.5)

**Figure 1** - Micrographs of adherence patterns on HEp2 cells of *Escherichia coli* obtained from faeces of children with diarrhoea and controls from Porto Velho-RO, Brazil: a) LA pattern after 3 h of incubation with typical EPEC; b) AA pattern after 3 h of incubation with EAEC; c) DA pattern after 3 h of incubation with DAEC; d) LAL pattern after 6 h of incubation with atypical EPEC (arrows).

in 35 (51.4%) of the patients and 33 (48.6%) of the control samples (Figure 1). When in association with EAEC, we observed that atypical EPEC showed an atypical phenotype (LAL) at a high frequency (Figure 1). We also found that the presence of the *aggA* (AAF/I) gene was significantly associated with diarrhoea, because 31 isolates having the *aggA* gene (6.5%) were related to patients, while only (0.2%) of controls had this gene ($p = 0.00006$). The presence of the *astA* gene in samples of EAEC showed no corre-

lation with diarrhoea, since it occurred in both patient and control isolates at almost the same frequency (Table 5).

A total of 205/425 (48.2%) isolates were submitted to serotyping of somatic antigen (O), and the results are presented in Table 6. We observed that the classic serogroups of EPEC and EAEC were the most frequently isolated, in agreement with the results obtained with the virulence factors and especially with the phenotypes in cellular assays. The serogroups O125, O111, O44, O55 and O127 appeared as possibly associated with diarrhoea, because we found that most of these serogroups were isolated from children with acute gastroenteritis, in agreement with the distribution of categories of diarrhoeagenic *E. coli* isolated (Table 6). EAEC isolates were mostly in the O44, O55, O111, O125 and O127 serogroups (Table 6).

Discussion

The pathogenic profile of enteroadherent *E. coli* has been studied throughout the world. We studied the frequencies of EPEC, EAEC and DAEC in faecal samples from children with and without diarrhoea in Porto Velho, Rondonia Brazil. Screening for the genetic *eaeA* marker showed that 128 *E. coli* isolates possessed this characteristic factor of both typical and atypical EPEC. This number of EPEC represented 11.4% of the total isolates, showing a presence of EPEC approximately twice that found in previous study (Nakhjavani *et al.*, 2013) in a screen for this *E. coli* group among 412 isolates obtained from 612 stool samples of children with diarrhoea in Tehran, Iran. The 90 samples that only had the *eaeA* gene belonged to the group of atypical EPEC, representing about 70% of all EPEC, corroborating the proportion of atypical EPEC presented by previous study (Nakhjavani *et al.*, 2013).

Table 5 - Phenotypic and genotypic frequencies found in enteroaggregative *E. coli* analysed among children with (470) and without (407) diarrhoea from Porto Velho, RO, Brazil.

Virulence Markers	Target	With diarrhoea	Without diarrhoea	Value p
AA	Adherent Pattern	91 (19.4%)	73 (17.8%)	0.5
aggA	Fimbrial subunity AAF/I	31 (6.5%)	1 (0.2%)	0.00006
aafA	Fimbrial subunity AAF/II	8 (1.7)	8 (1.9%)	0.7
astA	East Toxin	9 (1.9%)	8 (1.9%)	0.9
Total		174 (37.0)	121 (29.7%)	0.02

Table 6 - Serotyping of somatic antigen (O) of enteroaggregative *E. coli* isolated from children with and without diarrhoea from Porto Velho, RO, Brazil.

Pathogen	Serotype	With diarrhoea (n = 252) %	Without diarrhoea (n = 173) %	Total (n = 425) %
Typical EPEC	O111	3 (1.2)	-	3 (0.7)
	O119	2 (0.8)	-	2 (0.5)
	O127	2 (0.8)	3 (1.6)	5 (1.2)
	O44	2 (0.8)	1 (0.6)	3 (0.7)
	O55	4 (1.5)	1 (0.6)	5 (1.2)
	O126	1 (0.4)	-	1 (0.2)
	O86	-	1 (0.6)	1 (0.2)
Subtotal		14 (5.5)	6 (3.5)	20 (4.7)
Atypical EPEC	O1	4 (1.5)	1 (0.6)	5 (1.2)
	O111	2 (0.8)	-	2 (0.5)
	O125	5 (1.9)	2 (1.1)	7 (1.6)
	O166	6 (2.4)	-	6 (1.4)
	O44	7 (2.8)	5 (2.9)	12 (2.8)
	O6	4 (1.5)	-	4 (0.9)
	O86	2 (0.8)	1 (0.6)	3 (0.7)
Subtotal		30 (11.9)	9 (5.2)	39 (9.1)
EAEC	O1	7 (2.8)	10 (5.8)	17 (4.0)
	O111	7 (2.8)	6 (3.4)	13 (3.0)
	O125	6 (2.4)	4 (2.3)	10 (2.2)
	O127	10 (3.9)	3 (1.7)	13 (3.0)
	O166	6 (2.3)	4 (2.3)	10 (2.2)
	O44	12 (4.8)	9 (5.2)	21 (4.8)
	O55	6 (2.3)	3 (1.7)	12 (2.8)
	O8	4 (1.5)	1 (0.6)	5 (1.2)
	O169	5 (1.9)	1 (0.6)	6 (1.4)
	O26	2 (0.8)	5 (2.9)	7 (1.6)
Subtotal		65 (25.8)	46 (26.6)	111 (26.2)
DAEC	O111	2 (0.8)	1 (0.6)	3 (0.7)
	O1	3 (1.2)	1 (0.6)	4 (0.9)
	O125	4 (1.5)	2 (1.1)	6 (1.4)
	O127	5 (1.9)	6 (3.4)	11 (2.5)
	O44	5 (1.9)	4 (2.3)	9 (2.1)
	O6	2 (0.8)	-	2 (0.5)
Subtotal		21 (8.3)	14 (8.1)	35 (8.2)
Total		130 (51.6)	75 (43.4)	205 (48.2)

The screen for the EAEC adherence factor showed that among 164 samples with the AA pattern, only 41 isolates were presented positive for at least one EAEC virulence marker; however, those 123 that were negative for these markers, were positive for the AA adhesion pattern in HEp-2 cells, demonstrating the specificity of the test cells. Within the EAEC samples, 32 possessed the *aggA* gene and 16 possessed the *aafA* gene.

We did not find an association between DAEC and diarrhoea. In contrast, studies conducted in the state of Espírito Santo and Northeast Brazil showed the diarrhoeagenic *E. coli* most prevalent in children with acute diarrhoea was DAEC (Scaletsky *et al.*, 2002b; Spano *et al.*, 2008).

Regarding the DA adhesion pattern, the results showed low specificity for the *daaE* gene. Studies have compared different methods to characterize enteroadherent *E. coli*. With regard to tests carried out for detection of DAEC, the use of a probe for detection of the *daaC* gene sequence showed low sensitivity (64.3%), confirming frequencies found by other investigators (Scaletsky *et al.*, 2002a).

This study identified a large number of isolates that did not amplify with specific markers; however, the adhesion phenotypic test was able to identify the different adherence profiles, although more tests must be performed to identify possible adhesins associated with this pattern of adherence. The association between atypical EPEC and EAEC indicates a possible phenotype only observed when the two groups are associated with diarrhoea. In this study, the presence of the *aggA* (AAF/I) gene was associated with diarrhoea. We found 31 isolates positive for the *aggA* gene (6.5%) linked to patient cases, while only one patient (0.2%) of controls had this gene ($p = 0.00006$). A recent study has shown similar results, finding a higher frequency of the *aggA* (AAF/I) gene isolated from cases of diarrhoea (Piva *et al.*, 2003). We observed that the presence of the fimbriae AAF/I in our EAEC isolates is statistically associated with diarrhoea. Studies performed in Spain showed that the presence of fimbriae AAF/I was not observed in EAEC samples; however, AAF/II was detected in 8.7% of the EAEC isolated (Vila *et al.*, 2000). Studies in southwest Nigeria showed the presence of AAF/I in 63% of isolated EAEC; 35% had AAF/II (Okeke *et al.*, 2000). Similar results were found in studies in India (Kahali *et al.*, 2004). In contrast other study (Elias *et al.*, 1999) detected AAF/I and AAF/II in 19% and 8%, respectively, of EAEC samples in São Paulo. Studies performed in Gabon showed that the presence of the AAF I and AAF II fimbriae was more likely in EAEC than non-EAEC isolates (Presterl *et al.*, 2003). From these data we conclude that the prevalence of AAF seems to vary in relation to geographic region (Flores and Okhuysen, 2009).

The distribution of the serogroups found revealed a diversity of strains, which did not allow us to infer the ac-

tual prevalence of a specific serogroup in the population studied. In this study, we observed a higher frequency of the O111, O125, O44, O55 and O127 serogroups (Table 6); we emphasize the importance of serogroups since they are described as associated with pathogenic strains of *E. coli*.

In conclusion, EAEC are the main cause of diarrhoea in children from Porto Velho, RO, Brazil and the presence of *aggA* is highly correlated with this disease. EPEC is the second most common cause and when the same child is infected by both EAEC and atypical EPEC, a higher number of isolates of the latter present the LAL adherence pattern in HEp2 cells. In contrast to other regions in Brazil, DAEC was a significant contributor to diarrhoea cases in this city in the Brazilian western Amazon. In the studied city a more specific prevalent serogroup in paediatric diarrhoea was not identified. Therefore, the need to understand the mechanisms involved in the pathogenicity of these strains should be emphasized, especially when we consider the associations identified, recalling that bacterial pathogenicity is a property contributed to by multiple factors that are expressed in their natural environment, including horizontal gene transmission. Going forward, other experimental models should be used for complementary phenotypic and genomic studies.

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