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Original Article

Antibiotic resistance and biofilm formation in children with Enteropathogenic *Escherichia coli* (EPEC) in Brazilian Amazon

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Abstract

Introduction: Enteropathogenic *Escherichia coli* is an important causative agent of diarrhea in both developed and developing countries.
Methodology: We assessed the antibiotic resistance profile and the ability of 71 Enteropathogenic *Escherichia coli (EPEC)* isolates from children in the age group 6 years, or younger, to form biofilm. These children were hospitalized in Cosme and Damião Children Hospital in Porto Velho, Western Brazilian Amazon, between 2010 and 2012, with clinical symptoms of acute gastroenteritis.
Results: The highest frequency of atypical EPEC (aEPEC) isolates reached 83.1% (59/71). Most EPEC isolates presented Localized Adherence Like (LAL) pattern in HEp-2 cells (57.7% - 41/71). Biofilm production was observed in 33.8% (24/71) of EPEC isolates, and it means statistically significant association with *shf* gene ($p = 0.0254$). The highest antimicrobial resistance rates and a large number of multiresistant isolates 67.6% (48/71), regarded cefuroxime (CXM), ampicillin (AMP), trimethoprim-sulfamethoxazole (SXT) and tetracycline (TET), respectively, mainly in typical EPEC (tEPEC). Furthermore, 96% (68/71) of EPEC isolates in the present study were resistant to at least one antibiotic, whereas only 3 isolates were sensitive to all the tested drugs.
Conclusion: Based on our findings, there was increased aEPEC identification. EPEC isolates showed high resistance rate; most strains showed multiresistance; thus, they work as warning about the continuous need of surveillance towards antimicrobial use. Besides, the ability of forming biofilm was evidenced by the EPEC isolates. This outcome is worrisome, since it is a natural resistance mechanism of bacteria.

Key words: Antibiotic resistance; biofilm; enteropathogenic *E. coli*; Porto Velho-Rondônia.

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Introduction

Enteropathogenic *Escherichia coli* (EPEC) is an important causative agent of diarrhea in both developed and developing countries [1,2]. This diarrheagenic *E. coli* pathotype is capable of causing diarrhea due to specific virulence determinants found within a chromosomal pathogenicity island named Locus of Enterocyte Effacement (LEE) and to a plasmid named EPEC Adherence Factor (EAF) [3]. EPEC is divided into two categories, namely: typical EPEC (tEPEC) and atypical EPEC (aEPEC), which are based on EAF presence and absence - respectively, which is the main difference between the two groups [3].

EAF gives EPEC the ability to produce localized adherence (LA), which is a typical adherence model observed in cell cultures after 3-hour incubation. This phenotype results from "*bundle-forming pilus*" (BFP), which is a type IV fimbria found in tEPEC strains, only [4]. The main factor responsible for the pathogenic mechanism of EPEC is found in LEE. It is encoded into a Type III secretion system (TTSS) of proteins involved in the intimate adhesion process and in ESPs (EPEC secreted proteins) [5].

The development of these virulence factors enables EPEC to generate a typical histopathological lesion on the apical surface of the enterocyte known as "*Attaching and Effacing*" (A/E). It is characterized by the destruction of the intestinal microvilli and by cytoskeleton rearrangement. These processes form a structure similar to that of the pedestal bacteria are attached to [3].

Besides the aforementioned virulence factors, EPEC may also host genes found in other diarrheagenic

E. coli pathotypes, such as those enabling biofilm formation [6–8].

Biofilm formation has been linked to several human diseases, as well as to greater resistance to antimicrobial agents [9,10]. This process has been considered an important microbial survival strategy, since biofilm development allows bacteria to survive in hostile environments and to colonize new niches through the adoption of several dispersion mechanisms. According to Barnhart & Chapman, enteric bacteria such as *E. coli* and *Salmonella spp*. express protein fibers called Curli, which are responsible for the contact between the bacterium and the abiotic surface, and for the cell-cell contact [11]. These protein fibers play an important role in the early biofilm development stages; however, many structures expressed on the bacterial surface, such as Curli, flagella, pili and exopolysaccharides, act in different biofilm development stages.

Several studies conducted in Brazil, and abroad, showed that tEPEC and aEPEC are able to produce biofilm [12,13]. Studies have also shown that tEPEC and aEPEC are linked to high antibiotic-resistance levels [14,15]. Although EPECs cause diarrhea and are able to produce biofilm, EPEC infections are treated with therapies other than the antimicrobial one. Antibiotic administration is the therapy applied to persistent infections; therefore, choosing the effective antibiotic may be a critical issue for patient recovery and even for the achievement of good survival rates [16,17]. There is no research focused on evaluating biofilm profile and on assessing EPEC samples resistant to antibiotics in Northern Brazil. Thus, the aim of the current study was to investigate the ability of EPEC isolates to form biofilm and to develop an antimicrobial resistant profile. Previous studies have recorded EPEC resistance to multiple antibiotics, mainly the resistance shown by typical EPEC isolates [18].

Methodology

Study site and patients

Five hundred and ninety-one (591) fecal samples were collected from February 2010 to February 2012 from children in the age group 6 years, or younger, presenting clinical symptoms of acute gastroenteritis. These children were admitted to Cosme and Damian Children's Hospital (HICD - Hospital Infantil Cosme e Damião) in Porto Velho City, Rondônia State. Acute gastroenteritis cases were defined as those presenting three, or more, liquid or semi-liquid defecations within a 24-hour period. Sample collection was performed three times a week for two straight years. A single sample was collected from each child with sterile universal collector. The collected samples were recorded, labeled and stored at -80ºC in the Microbiology Laboratory of the Tropical Medicine Research Center. The study was approved by the Ethical Committee of Rondônia Tropical Medicine Research Center, process n. 77565.

Bacteriology

Escherichia coli strains were selected from MacConkey selective agar, which was provided by HiMedia USA. Colonies were processed through routine microbiological and biochemical tests - purchased at bioMérieux France (API20E system). Five colonies suggestive of *E. coli* were subjected to PCR testing in order to identify the virulence genes.

tEPEC and aEPEC analysis through multiplex polymerase chain reaction (PCR)

EPEC was characterized through the Multiplex PCR described by Müller *et al.* [19] as follows: tEPEC (*escV* and *bfpB*) and aEPEC (*escV*). Non-pathogenic *E. coli* strain HB101 was used as negative control and to monitor PCR contamination. Amplifications of the *shf* gene were conducted based on Mohamed *et al*. [20].

HEp-2 adherence test

All *E. coli* isolates were subjected to HEp-2 adherence tests for EPEC identification [21]. HEp-2 cells were grown overnight (up to 50% confluence) in Dulbecco's Modified Eagle's medium (Gibco BRL, Gaithersburg-MD, USA) with penicillin, streptomycin and 2% foetal bovine serum – samples were assembled on eight-well chamber slides. Bacteria were grown in Luria-Bertani broth (LB), (Himedia, Mumbai, MH, India) at rest, for 16 hours. HEp-2 cells were washed five times in Phosphate Buffer Saline (PBS); next, the medium was replaced by Dulbecco's Modified Eagle's medium with 1% mannose (Himedia, Mumbai, MH, India). Bacterial suspension (10 µL) was added to each well; slides were incubated at 37°C, in 5% CO_2, for 3 hours. Monolayers were washed five times in PBS and, subsequently, they were fixed in 70% methanol and Giemsa. The strains were stained for 1 minute in Panoptic Quick staining kit solutions (Newprov, Pinhais, PR, Brazil). Each strain was tested in duplicate; appropriate controls were included to the test. Strains adhering to the monolayers were recorded as localized (LA), localized-like (LAL), diffuse (DA) or as aggregative (AA) adherence. All adherence assays were performed from 3 to 6 incubation hours.

The HEp-2 adherence assay is useful to diagnose diarrheagenic *E. coli*. tEPEC produces the LA model by forming compact microcolonies in HEp-2 cells after 3-hour incubation, whereas aEPEC is unable to produce LA; it only forms loose bacterial microcolonies. These microcolonies are detected after prolonged adhesion assays (6-hour incubation) by the formation of the LAL pattern [3].

Biofilm detection through spectrophotometry

Biofilm in polystyrene was detected in 96-well polystyrene microtiter plates (Costar, USA), as previously described by Stepanović *et al.* with modifications [22].

Tested strains were grown overnight in LB (broth), at 37ºC; the culture was adjusted to optical density (OD) 600 nm. Three replicate wells were added with 100 µL of dilution 1/100. Plates were statically incubated at 37ºC for 24 hours to allow biofilm development. After incubation, the supernatant was removed through aspiration, and the plates were washed three times in 200 μL/well of sterile distilled water. Plates were dried (at 30ºC for 15 minutes) and each well was stained with 100 µL of 0.1% crystal violet (CV) for 15 minutes.

Wells were thoroughly rinsed three times with 200 µL sterile distilled water and air-dried; CV was solubilized in 200 µL of 1% Sodium Dodecyl sulfate (SDS). Finally, the amount of extracted CV was determined by measuring the OD640 in enzyme-linked immunosorbent assay (ELISA) plate reader (BioRad, Hercules, Ca, USA). The mean OD of the control wells (ODc) was subtracted from the OD595 nm of all tested wells. The microtiter plate biofilm assay was carried out in triplicate with all assessed strains; means and standard deviations of all experiments were calculated.

According to the results, strains were classified as follows: no biofilm producer (NBP) (DO ≤ 0.120 nm), weak biofilm producer (WBP) (DO 0.120 to 0.240 nm) and strong biofilm producer (SBP) (DO ≥ 0.240 nm). Enteroaggregative *E. coli* 042, which is a strong biofilm producer, and the non-pathogenic *E. coli* strain HB101, which was used as negative control, were the strains used to assure the quality of the biofilm assay.

Antimicrobial Sensitivity Test

Antimicrobial susceptibility tests were conducted in Mueller-Hinton agar (HIMEDIA) and on commercial antimicrobial disks (Oxoid, Basingstoke, Hants, UK) based on the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute [23]. The herein tested antibiotics were Cefuroxime (CXM 30µg), Gentamicin (GEN, 10µg), Imipenem (IPM, 10µg), Piperacillin / Tazobactam (TZP, 100/10µg), Tetracycline (TET, 30µg), Trimethoprim / sulfamethoxazole (SXT, 1.25/23.75 µg), Amoxicillin / clavulanic acid (AMC, 20 / 10µg), Amikacin (AMK, 30µg), Ampicillin (AMP, 10µg), Chloramphenicol (CHL, 30µg), Ciprofloxacin (CIP 5µg) and Ceftazidime (CAZ 30µg).

Multidrug resistance (MDR) was defined by the acquisition of non-susceptibility to at least one agent in three or more antimicrobial categories [20]. The ATCC 25922 *E. coli* strain was used for quality control in all tests.

Table 1. Characterization of typical EPEC and atypical EPEC profiles as epidemiological and biological factors.

Information *n* (%)	**Typical n (%)**	**Atypical n (%)**	**p-value**
Samples	12 (16.9)	59 (83.1)	
Gender			1
Male *42* (59.2)	7 (58.3)	35 (59.3)	
Female *29* (40.8)	5 (41.7)	24 (40.7)	
Mean age			
17.52 months	18.6	17.3	0.7881
Adherence Pattern			
Localized *21* (29.6)	5 (41.7)	16 (27.1)	0.3203
Localized Like *41* (57.7)	6 (50)	35 (59.3)	0.7498
Aggregative *9* (12.7)	1 (8.3)	8 (13.6)	1
Biofilm			1
Producer *24* (33.8)	4 (33.3)	20 (33.9)	
Strong producer	2 (50)	11 (55)	
Weak producer	2 (50)	9 (45)	
Non-Producer *47* (66.2)	8 (66.7)	39 (66.1)	
***shf* gene**			0.0511
Positive *57* (80.3)	7 (58.3)	50 (84.7)	
negative *14* (19.7)	5 (41.7)	9 (15.3)	

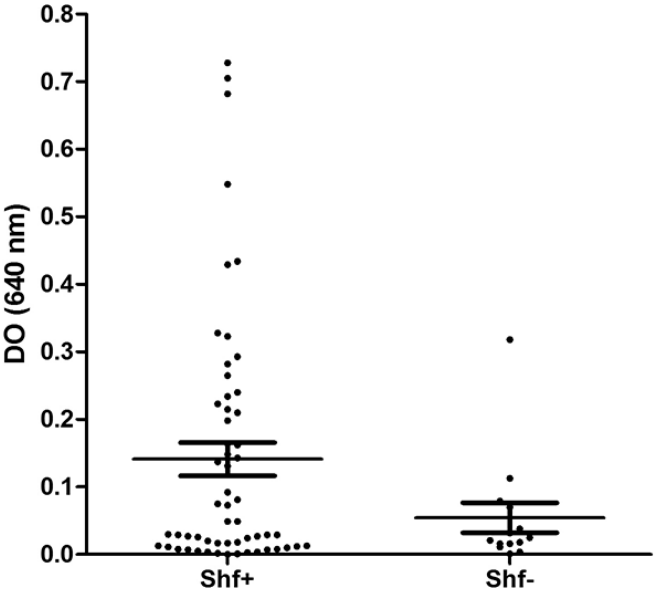
Results

In total, 1,625 *E. coli* specimens were isolated from samples collected from 591 children with diarrhea. Five (5) colonies suggestive of *E. coli,* from each child, were subjected to PCR testing and to the HEp-2 adherence test - these procedures allowed the isolation of 71 EPEC colonies. tEPEC was identified in 16.9% (12/71) of the strains, and aEPEC in 83.1% (59/71) of them. Children of both sexes were equally affected: 59.2% (42/71) boys and 40.8% (29/71) girls. The mean age of the children was approximately 17 months. The same pattern was observed in both EPEC categories. Neither age, nor sex, presented statically differences between both EPEC categories. Most EPEC isolates presented localized adherence-like (LAL) pattern in HEp-2 cells (57.7% (41/71). This value was followed by 29.6% (21/71) localized adherence (LA) and by 12.7% (9/71) aggregative adherence (AA) (Table 1).

Biofilm production was observed in 33.8 % (24/71) of EPEC strains; 33.3% (4/12) of them were tEPEC, and 33.9% (20/59) were aEPEC. In addition, 15.5% (11/71) of the isolates were weak biofilm producers, 18.3% (13/71) were strong producers and 66.2% (47/71) were not biofilm producers (Table 1). No statistically significant relation was observed when the association between adherence patterns in HEp-2 cells and biofilm production was assessed.

EPEC isolates were highly resistant to several antibiotics. Results of the antibiotic-susceptibility test showed that 78.9% (56/71) of isolates were cefuroxime resistant, 66.2% (47/71) were resistant to ampicillin, 63.4% (45/71) to trimethoprim / sulfamethoxazole and 43.7% to tetracycline (31/71). Furthermore, 19.7% (14/71) of the EPECs isolates and 22% of aEPEC isolates (13/59) were Imipenem resistant. There was significant statistical difference in ampicillin resistance between typical and atypical EPECs (p = 0.0488; OR = 7.028; CI 95% = 0.8491 to 58.17) (Table 2). EPEC isolates were sensitive to ceftazidime, ciprofloxacin and amikacin, they recorded sensitivity frequency higher than 90% (Table 2).

Figure 1. Correlation between biofilm production and *shf* gene presence in EPEC.



Biofilm producers presented DO ≥ 0.120; There was significant relation between the presence of the *shf* gene and biofilm production (p = 0.0254; OR = 8.794; CI 95% = 1.075 to 71.97).

In total, 96% (68/71) of EPEC isolates were resistant to at least one antibiotic, whereas only 3 isolates were sensitive to all tested antibiotics. Multidrug resistance was recorded in tEPEC (75% (9/12)) and in aEPEC (66.1% (39/59)) isolates (Table 3).

There was no statistically significant relation between antibiotic resistance and biofilm production; however, EPEC strains were biofilm producers and

Table 2. Resistance of EPEC isolates to 12 antimicrobials from Porto Velho-RO.

Antimicrobials	EPEC	tEPEC[a]	aEPEC[b]
Cefuroxime (CXM)	56 (78.9)	8 (66.7)	48 (81.4)
Gentamicin (GEN)	11 (15.5)	2 (16.7)	9 (15.3)
Imipenem (IPM)	14 (19.7)	1 (8.3)	13 (22)
Piperacillin / Tazobactam (TZP)	9 (12.7)	1 (8.3)	8 (13.6)
Tetracycline (TET)	31 (43.7)	8 (66.7)	23 (39)
Trimethoprim/sulfamethoxazole (SXT)	45 (63.4)	10 (83.3)	35 (59.3)
Amoxicillin / clavulanic acid (AMC)	27 (38)	6 (50)	21 (35.6)
Amikacin (AMK)	7 (9.9)	2 (16.7)	5 (8.5)
Ampicillin (AMP)*	47 (66.2)	11 (91.7)	36 (61)
Chloramphenicol (CHL)	9 (12.7)	2 (16.7)	7 (11.9)
Ciprofloxacin (CIP)	6 (8.5)	1 (8.3)	5 (8.5)
Ceftazidime (CAZ)	3 (4.2)	2 (16.7)	1 (1.7)

[a]Typical EPEC; [b]Atypical EPEC; *Statistical difference between Typical and Atypical EPEC (p = 0.0488; OR 7.028; CI 95% = 0.8491 to 58.17).

resistant to piperacillin / tazobactam antibiotics. They showed high Odds Ratio value (p = 0.0531; OR = 4.889; CI 95% = 1.101 to 21.71).

Results in the present study evidenced high frequency of putative Enteroaggregative *E. coli* (EAEC) virulence of the *shf* gene (cryptic open reading frame) in 80.3% (57/71) of EPEC isolates (table 1). Besides, there was statistically significant relation between the presence of the *shf* gene and biofilm production (p = 0.0254; OR = 8.794; CI 95% = 1.075 to 71.97). The same outcomes were recorded for aEPEC (p = 0.0218; OR = 12.77; CI 95% = 0.7033 to 231.9), but not for tEPEC (Figure 1).

Discussion

EPEC is one of the main etiological agents among the enteric pathogens responsible for diarrhea cases. Results in the present study showed 12% (71/591) EPEC, 10% (59/591) aEPEC and 2% (12/591) tEPEC. The highest aEPEC frequency observed in the present study was in compliance with numbers in the literature. Some epidemiological studies about *E. coli* suggested

Table 3. Multidrug-resistance of EPEC isolates from Porto Velho-RO.

Antimicrobial resistance profile	Number of isolates	Frequency (%)
Typical EPEC		
CXM/AMK/AMP/	1	8.3
TET/SXT/AMP/CIP	1	8.3
TET/SXT/AMP/CHL	1	8.3
CXM/TET/SXT/AMP	1	8.3
CXM/TET/SXT/AMK/AMP	2	16.7
CXM/TZP/TET/SXT/AMK/AMP	1	8.3
CXM/GEN/TET/SXT/AMC/AMK/AMP/CHL/CAZ	1	8.3
CRX/GEN/IPM/TET/SUT/AMC/AMI/AMP/CAZ	1	8.3
Atypical EPEC		
CXM/SXT/AMP	3	5.1
CXM/TET/SXT	2	3.4
GEN/SXT/AMP	1	1.7
CXM/IMP/TET	1	1.7
CXM/AMC/AMP	1	1.7
TET/SXT/AMP	3	5.1
CXM/IPM/TZP	1	1.7
CXM/SXT/AMC/AMP	4	6.8
TET/SXT/AMC/AMP	1	1.7
TET/SXT/AMP/CHL	1	1.7
CXM/GEN/AMP/CHL	1	1.7
CXM/TET/SXT/AMP	1	1.7
CXM/IPM/AMC/AMP	1	1.7
CXM/TZP/SXT/AMC/AMP	1	1.7
CXM/TET/SXT/AMC/AMP	1	1.7
CXM/IPM/TET/SXT/AMP	1	1.7
CXM/TET/SXT/AMP/CIP	1	1.7
CXM/TET/SXT/AMP/CHL	1	1.7
CXM /GEN/TZP/AMC/AMP	1	1.7
CXM/GEN/IPM/AMC/AMP/CHL	1	1.7
CXM /TET/SXT/AMC/AMP/CHL	2	3.4
GEN/TET/SXT/AMC/AMP/CIP	1	1.7
CXM /TZP/TET/SXT/AMC/AMP	1	1.7
CXM /IPM/SXT/AMC/AMK/AMP	1	1.7
CXM/IPM/TZP/TET/SXT/AMC/AMP	1	1.7
CXM/TZP/TET/SXT/AMC/AMK/AMP	1	1.7
CXM/GEN/IPM/TET/SXT/AMC/AMP/CIP	1	1.7
CXM/GEN/TET/SXT/AMC/AMK/AMP/CHL	1	1.7
CXM /GEN/TZP/TET/SXT/AMC/AMP/CIP	1	1.7
CXM /GEN/IPM/TET/SXT/AMK/AMP/CIP	1	1.7

Only typical EPEC and atypical EPEC MDR were taken into consideration for analyses. AMC (Amoxicillin / clavulanic acid); AMK (Amikacin); AMP (Ampicillin); CAZ (Ceftazidime); CIP (Ciprofloxacin); CHL (Chloramphenicol); CXM (Cefuroxime); GEN (Gentamicin); IPM (Imipenem); SXT (Trimethoprim/sulfamethoxazole); TET (Tetracycline); TZP (Piperacillin / Tazobactam).

the increased identification of this pathogen [24]. Hernandes and collaborators reported increased aEPEC identification either in developed or in developing countries; they observed 10.5% aEPEC prevalence in Germany, 7.6% in Mexico, 5.4% in São Paulo, and 5.5% in Espírito Santo [24].

The study conducted by Orlandi *et al.* in Rondônia State about the etiology of diarrheal infections in children younger than 72 months admitted to Hospital Infantil São Cosme e Damião, Porto Velho- RO showed that aEPEC was more frequent than tEPEC in this sample group - 4.0% aEPEC frequency and 2.1% tEPEC frequency [25]. Based on this outcome, aEPEC strains are important enteropathogens associated with diarrhea cases.

In total, 41.7% (5/12) of tEPEC isolates presented LA, and this number features this pathotype. LAL adherence pattern was observed in 59.3% (35/59) of aEPEC isolates after 6-hour incubation. Many authors have reported that LAL is the most frequent among aEPEC strains; however, several adherence profiles (DA, AA, LA and LAL) were described due to aEPEC heterogeneity [24,26]. Pitondo-Silva *et al.* conducted a study in São Paulo (Brazil) with 60 EPEC isolates; they found 18 aEPEC isolates that presented LAL pattern in the HEp-2 cells assay [15]. Results in the present study show that 27.1% (16/59) of aEPECs present LA pattern; this number corroborates the study by Hernandes *et al*., who characterized and confirmed the ability of 9 aEPEC samples to adhere to HeLa cells and to form compact microcolonies similar to those formed by tEPECs [27].

Results of antimicrobial resistance tests were consistent with the literature about resistance emergence; 66.2% (47/71) resistance to ampicillin, 63.4% (45/71) resistance to trimethoprim / sulfamethoxazole and 43.7% (31/71) resistance to tetracycline. The 78.9% (56/71) resistance to cefuroxime was higher than that recorded for diarrheagenic *E. coli* strains isolated in samples from children in the age group 5 years in China (57.4%) [28]. According to Arenas Hernández *et al*., EPEC strains are more often resistant to ampicillin, tetracycline, streptomycin and sulfonamides [18]. In addition, the mean resistance recorded for EPEC was similar to that recorded for other diarrheagenic *E. coli*, which recorded more than 23% resistance to ampicillin, tetracycline and trimethoprim / sulfamethoxazole [29].

Mosquito *et al*. investigated the molecular mechanism of antibiotic-resistance in diarrheagenic *E. coli* isolated in samples from children. They found high resistance to ampicillin (83%), trimethoprim / sulfamethoxazole (78%) and tetracycline (55%) [30]. Studies conducted in Recife, Brazil, about *E. coli* frequency and the antimicrobial susceptibility of children (5 years old, or younger) hospitalized with acute diarrhea showed high resistance to ampicillin (88.9%) and trimethoprim / sulfamethoxazole (44.4%) [31]. Another Brazilian study evidenced high resistance of EPEC isolates to ampicillin (70%), cephalothin (65%), sulfonamide (61.7%) and tetracycline (36.7%) [15].

In total, 96% of EPEC isolates were resistant to at least 1 antibiotic; only 3 isolates were sensitive to the 12 tested antibiotics. Antimicrobial resistance has significantly increased in recent years, both in developed and developing countries. Thus, it has become one of the main public health issues, because the emergence of antimicrobial-resistant strains reduces the therapeutic options [29].

There was MDR in 67.6% (48/71) of the 71 tested EPECs. Zhou *et al.*, showed similar MDR frequency (66.7%) in diarrheagenic *E. coli* strains [25]. No statistically significant MDR differences were found between tEPEC (75% (9/12)) and aEPEC (66.1% (39/59)) (Data not shown). According to Arenas-Hernández *et al*., (2012), there is high percentage of multiresistant tEPEC strains, but the resistance rate recorded for aEPEC is low, or not reported at all [18]. Pitondo-Silva *et al*. found that 63.3% (38/60) of the EPEC isolates were resistant to three, or more, of the tested antimicrobials; moreover, they found that tEPEC was more resistant to antibiotics than aEPEC [15]. Scaletsky *et al.* conducted a study about the antimicrobial resistance of 70 tEPEC and 79 aEPEC isolates, and found increased resistance in tEPEC isolates - 43% of these isolates were resistant to three, or more, antibiotics [17].

The ability of EPEC isolates to form biofilm was observed in 33.8% (24/71) of isolates assessed in the present study; therefore, they are an additional virulence factor and a natural resistance mechanism in bacteria [8,26]. Biolfim is also related to bacterial persistence in infection cases, because the exopolysaccharide matrix formed in the biofilm avoids the penetration of antimicrobial agents and hinders bacterial destruction in the biofilm [32,33].

Moreira *et al*. conducted a study on biofilm formation by EPEC (E2348/69) and suggested that BFP and EspA (adhesions that play an important role in the formation of compact microcolonies during EPEC pathogenesis) are involved in biofilm formation on abiotic surfaces [34]. However, the mechanism used by

EPEC to form the biofilm, as well as the adhesions involved in the process, remain unknown.

Weiss-Muszkat *et al*. demonstrated through mutagenesis analysis applied to transposons that curli fibers and the *crl* regulator play an important role in biofilm development in aEPEC (O55:H7 serotype) at low temperatures [7]. Nascimento *et al*. (2014) suggested that type 1 fimbriae and diguanylate cyclase may be involved in biofilm formation in clinical aEPEC isolates [8].

Adherence patterns in HEp-2 cells due to biofilm production were observed in the present study; but, there was no association among the three adhesion profiles - this outcome is consistent with the study by Culler *et al*. [26]. These authors showed the ability of aEPEC strains to adhere to, and to form biofilm on, abiotic surfaces (55 isolates – 60.4%); however, there was no association between the four adherence patterns and biofilm formation. Thus, based on this result, these strains form biofilm, regardless of adhesions involved in adherence pattern establishment.

Interestingly, there was high frequency of *shf* gene (80.3%) in the EPEC isolates. Although this gene is often found in EAEC [35], there are publications reporting *shf* presence in atypical EPEC (O51:H40) samples when the putative properties of pathogenic *E. coli* are investigated [34]. It is possible recording the emergence of different combinations of virulence genes, mainly in aEPEC strains, because genes encoding virulence factors are located in plasmids, pathogenicity islands, transposons or in bacteriophages. These strains can carry genes encoding virulence factors from other DEC pathotypes more often than tEPEC strains [24].

Besides, there was statistically significant association between the presence of *shf* gene and biofilm production ($p = 0.0254$; OR = 8.794; CI 95% = 1.075 to 71.97). This association has been related to EAEC 042, and such relation suggests that this gene is required for biofilm formation, and that its transcription depends on *AggR* [35]. The protein encoded by the *shf* gene shows 25% similarity to the *Staphylococcus epidermidis* IcaB protein, which plays a crucial role in the exopolysaccharide modification of bacterial biofilm formation [35,36]. To the best of our knowledge, the literature has no report about the potential role of the *shf* gene in the ability of EPEC to form biofilm. The current study is the first to assess the association between biofilm formation by EPEC and the presence of the *shf* gene.

Conclusion

Based on the present results, there was increased aEPEC identification. EPEC isolates recorded high resistance rate (only 3 isolates were sensitive to the 12 tested antibiotics). Most strains evidenced MDR; thus, they work as a warning about the continuous need of surveillance over antimicrobial use. The ability of EPEC isolates to form biofilm was evidenced by the results. There was statistically significant association between the presence of the *shf* gene and biofilm, which demonstrates that additional studies are necessary to reveal other mechanisms involved in biofilm development, since this mechanism is natural in bacteria.

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