

Comparative study between diarrheagenic *Escherichia coli* virotypes from Mexico City and San Luis Potosí

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Abstract

The distribution and frequency of *Escherichia coli* virotypes can vary considerably from region to region, due to the geographical characteristics, the degree of social lag as well as the population of each region. Therefore, the objective of the present work was to compare *Escherichia coli* isolated from children with diarrhea obtained from two states with different levels of social lag and establish virotypes, adhesion to HEp-2 cells, biofilm formation, and antimicrobial resistance of this bacterium. In the present work, we compared *E. coli* isolated from children with diarrhea obtained from the City of San Luis Potosí (S.L.P.) with *E. coli* isolated from children with diarrhea of Mexico (City CDMX), with the purpose of identifying and establishing virotypes, adherence to HEp-2 cells, formation of biofilms, and antimicrobial resistance of these bacteria. The results showed that the percentage of virotypes found in S.L.P. were higher with respect to the virotypes found in the CDMX, being enterotoxigenic the most frequent virotype for both populations. The incidence of this virotype was higher for *E. coli* isolated from S.L.P., and the statistical analysis showed $p < 0.000$. The incidence of the enteroinvasive and EHEC virotypes was higher in the state of S.L.P., with $p < 0.000$. The adhesion phenotype, both aggregative and diffuse, was related to the presence of the *aat* and *astA* genes. The tests to determine the production of biofilms in strains of *E. coli*, allowed us to determine that the method in Congo red agar, were the most effective for its evaluation. In relation to the antimicrobials tested, a higher percentage of resistance of the *E. coli* strains of the CDMX was observed, in relation to the strains of S.L.P., with a statistically significant difference of $p < 0.05$.

Key words: Comparative study. Diarrheagenic *Escherichia coli*. Children. Virotypes. Antimicrobial resistance.

Introduction

Changes in society, technology, and microorganisms alone contribute to the emergence of new diseases, to the re-emergence of already controlled diseases, as well as to the development of antimicrobial resistance. At present, pathogens resistant to several antimicrobials have proliferated, both in humans and in veterinary medicine.

Gastrointestinal diseases in infants are one of the public health problems worldwide, especially in developing countries such as Mexico.

In 2015, the World Health Organization estimated that diarrheal diseases cause more than half of the global burden of foodborne diseases, with 550 million people sick and 230,000 people dying every year. Diarrhea is usually due to the ingestion of poorly cooked foods or dairy products contaminated with norovirus, *Campylobacter*, nontyphoidal *Salmonella*, and pathogenic *Escherichia coli*¹. In Mexico, intestinal infections represented the second cause of morbidity, with more than five million cases in 2011, which placed

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this condition as an important public health problem, since the national incidence increased for the third consecutive year, with the most vulnerable sector being children under 5 years of age².

In 2013, acute diarrheal disease (ADD) was the second cause of morbidity in the population under 5 years of age in the state of San Luis Potosí (S.L.P.), where the disease can present serious complications in children, due to some circumstances such as having a birth weight of < 2.5 kilos, have not received breastfeeding, have some degree of malnutrition, and do not have the complete vaccination schedule according to their age³. In 2013, the number of children under 5 years with ADD was approximately 40 thousand cases⁴. The incidence of ADD in this state may be due to the social backwardness, since according to the National Council for the Evaluation of Social Development Policy (CONEVAL) in the year 2012, SLP occupied tenth place in social backwardness at the national level, while Mexico City was located at 31 places, with a very low degree of social lag⁵.

Among the virulence factors of *E. coli* are adhesins and toxins. Pathogenic strains of *E. coli* have specific adhesion factors⁶, which allow them to colonize the intestine. These adhesions can be divided into two groups: fimbrial adhesins and afimbral adhesins⁷. The afimbral adhesins are outer membrane proteins such as intimin⁶.

Bacterial toxins are of two types, lipopolysaccharide endotoxins and exotoxins. Exotoxins are classified as enterotoxins, cytotoxins, and neurotoxins. Enterotoxins produce watery diarrhea and inflammatory diarrhea cytotoxins, whose bowel movements contain blood and mucus⁷. Once *E. coli* has colonized the intestine, the damage is caused by the production of enterotoxins, cytotoxins as well as by invasion of the target cell.

Based on their mechanism of damage, *E. coli* are classified into six pathotypes: Enterotoxigenic (ETEC), Enterohemorrhagic, also known as producers of STx toxin (EHEC), Enteropathogenic *E. coli* (EPEC) is classified in tEPEC (they have the bfpA + genes) eaeA) and aEPEC (possesses the eaeA gene), Enteroinvasive *E. coli* (EAEC), enteroinvasive (EIEC), and diffuse adherence *E. coli*⁶.

Savarino et al.⁸ have considered the astA gene, which codes for the EAST1 enterotoxin, as a characteristic of the EAEC group. On the other hand, Nataro et al.⁹ recognized two different phenotypes of *E. coli*, belonging to the enteroadherent strain: diffuse and enteroinvasive, this discovery is the first description of EAEC. The enteroinvasive adhesion is characterized by a

formation of "stacked brick" (stacked-brick in English), where bacteria adhere to both the cell surface and the glass of the preparation. The reproducibility presented by this trial has made it a standard test for the determination of the aggregative adhesion phenotype and thus the identification of the EAEC viotype. In addition to, molecular assays such as the production of the EAST1 toxin, encoded by the astA gene⁸, the aat gene, which codes for a protein called dispersion, as well as the chromosomal gene AggR present in a pathogenicity island of *E. coli* was identified¹⁰.

In nature, microorganisms rarely live in isolated colonies of a single species, generally, living in communities called biofilms¹¹. Biofilm is a term used to define biological systems or a matrix with bacterial populations adhered to each other and/or to a surface. This structure allows bacteria to take refuge from harmful factors such as the environment, antibiotics, and immune system, among others¹¹.

The architecture is not solid, on the inside microcolonies are communicated by microchannels that favor the entry of nutrients and elimination of waste products¹².

Recently, it was found that the moment when *E. coli* begins to form the biofilm, the bacteria become ovoid and smaller to eventually enter a stationary phase where they stop dividing. As this occurs, they begin to produce and excrete autoaggregation curli fibers. These curli fibers, at the molecular level, are proteins assembled in an amyloid structure, which allows their staining with Congo Red¹³.

The presence of biofilm produced by *E. coli* had been considered a very important virulence factor since it has been related to antimicrobial resistance. The formation of this structure was detected in catheters, medical instruments and heart valves, favoring the chronicity of the ailments and making their elimination difficult¹³.

For the study of the biofilm production of different microorganisms, several methodologies have been developed, among them are the Christensen Tube Method and the Congo Red in agar plates. Christensen et al.¹⁴ were recognized one of the first groups to develop methodologies to determine the production of biofilm. When conducting various investigations of intrahospital infections, they found a certain mucoid substance in catheters, heart valves, and prostheses, among others and were associated with infections caused by coagulase-negative staphylococci (usually *Staphylococcus epidermidis*) that produce what they called at that time, slime.

Ensuring that the Christensen method is not always successful in determining biofilm production and that variations in the culture medium affect the results,

Freeman et al.¹⁵ described an alternative methodology to detect the production of biofilm on a solid medium. This method is based on the coloration of the bacterial colonies, thanks to the Congo red dye present in the brain-heart infusion agar plates.

In both Latin America and North America, epidemiological studies were conducted and the purpose was to determine different pathotypes associated with diarrhea cases in children. These studies demonstrated that *E. coli* that belongs to the ETEC and EPEC pathotypes are the main isolated pathogens¹⁵⁻¹⁷. In regard to Mexico, since 1987, several studies showed the presence of EPEC in most of the isolates obtained from patients with a prevalence of 17-19% of cases of infant diarrhea¹⁸. In 2005, Estrada et al. reported the presence of four *E. coli* pathotypes in a community bordering Mexico City.

On the other hand, despite the efforts made for the prevention and control of infectious diseases, they continue to represent a global public health problem due to both emerging and re-emerging microorganisms.

Among the pathogens that are emerging and resistant to antimicrobials are *E. coli*, which affects humans and different animal species. Multiple investigations in both humans and animals show resistance to the most frequently used antimicrobials¹⁹⁻²². Bacteria are generally resistant to more than one antimicrobial²¹. Studies in Mexican children show that these bacteria cause diarrhea disease in hospitalized children and are resistant to trimethoprim-sulfamethoxazole (TMP-SMX) and ampicillin (AM). These drugs are mostly used in the treatment of pediatric diarrhea¹⁷. In the State of S.L.P., there are no studies in this regard.

For the aforementioned, the objective of the present work was to compare the *E. coli* isolated from children with diarrhea obtained from two states with different degree of social lag and establish the viotypes, adhesion to HEp-2 cells, biofilm formation and antimicrobial resistance of said bacteria.

Materials and methods

Bacterial strains and characterization

The study was conducted from 38 stool samples from children with diarrhea from the Dr. Ignacio Morones Prieto Central Hospital in the state of SLP and 27 stool samples from children from the National Institute of Pediatrics (INP) of Mexico City. The samples were taken by highly qualified personnel; later they were transported to the laboratory in the Cary Blair medium. Once in

Table 1. Percentage of genes found in *E. coli* strains from each population studied

¹ Genes	% of Strains Mexico City	% of Strains S.L.P.
<i>LT</i> *	30	57
<i>ST</i> *	14	37
<i>ST: LT</i> *	2	27
<i>ial</i> *	4	28
<i>St x 1</i> *	3	22
<i>St x 2</i>	0	9
<i>St x 1:St x 2</i>	0	5
<i>St x 1:St x 2:eaeA</i>	4	0
<i>eaeA</i>	9	9
<i>bfpA</i>	2	0
<i>astA</i>	14	13
<i>aat</i>	3	1
<i>astA:aat</i>	3	0

Genes that code for: *LT*: heat-labile toxin; *ST*: heat-stable toxin; *St x 1* and *St x 2*: "Shiga-like" cytotoxins; *eaeA*: gene that codes for intimina; *bfpA*: codifies for pili hair formers; *ial*: invasiveness; *astA*: enteroaggregative toxin heat stable EAST1; *aat*: gene that participates in the secretion of the protein called dispersin.

*p < 0.05; S.L.P.: San Luis Potosí.

the laboratory, they were planted on MacConkey agar medium (DIBICO[®]) and incubated at 37°C for 24 h. One to five colonies were selected, with morphological characteristics of *E. coli*. Subsequently, conventional biotyping tests were performed to confirm the presence of *E. coli*. From these tests, 100 strains of *E. coli* from S.L.P. and 100 strains of *E. coli* from Mexico City were obtained. In addition to these biochemical tests, the production of β hemolysis was evaluated. To do this, the strains of *E. coli* were seeded on 5% sheep blood agar, incubated at 37°C for 24 h and subsequently the β hemolysis was evaluated by observing the total destruction of the erythrocytes. Reference strains A panel of *E. coli* reference strains were used as positive controls for the different polymerase chain reaction (PCR) assays, antibiograms, and adhesion to HEp-2 cells. Strains TB334C (Stx1 +, Stx2 +, eaeA +), B-171 (BfpA +, eaeA +), EIEC O124: H30 (ial +), and O407: H1 (LT +, ST +) were kindly donated by Dr. Estrada García of the Research Center and Advanced Studies (CINVESTAV), EAEC 044: H18 (aat, astA) strain donated by Dr. Chataway of Public Health England. Finally, O42 and C600 (K12) strains were provided by the National Epidemiological Reference Institute of Mexico (INDRE).

DNA extraction and PCR reaction

The genomic DNA of the *E. coli* isolates was extracted by the boiling method, previously described by Al-Gallas et al.²³. The PCR (single or multiple) allowed to detect genes that code for the toxins (LT, STa, Stx1, Stx2, and EAST1) and genes that code for adhesins (intimin, eae, pili Type IV, bfpA, for invasiveness and gene that codes for dispersin, aat). The PCR reactions used for the amplification of the different genes were carried out using Apex Taq DNA polymerase (Genesee Scientific, USA) and primers. The 50 μ l reaction mixtures contained the following: 1X buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 0.2 mM of each dNTP (Apex dNTP mixture, 10 mM), 0.25 μ M of each primer, 2.0 U of Apex Taq DNA polymerase, 3 μ l of tempered DNA, and 37 μ l of nuclease-free water. The multiplex PCR products (mPCR) were separated on a 2% Agarose gel (Amresco, Ohio, USA). The amplified PCR products were visualized with ethidium bromide under a UV illuminator and recorded using the Digidoc Imaging System (UVP, CA, U.S.A.).

Antimicrobial susceptibility assays

The antimicrobial susceptibility test was carried out to determine the profiles of susceptibility and resistance of the bacteria to the antibiotics tested. For this, the paper disc diffusion method was used. Impregnated antimicrobial discs with AM (10 μ g), amikacin (30 μ g), kanamycin (30 μ g), cephalothin (30 μ g), amoxicillin/clavulanate (30 μ g), cefotaxime (30 μ g), TMP/SMX (1.25 μ g/23.75 μ g), and cefoxitin (30/mg). These discs (6 μ m diameter) were placed in an automatic Sensi Disc dispenser (DB BBL Sensi Disc Antimicrobial Susceptibility Test Discs, Sparks MD, USA). The inoculated agar plates were incubated at 37°C for 18-24 h. After incubation, the diameters of the zone of inhibition of the antibiotic were measured. The results were used to classify the isolates as resistant, intermediate, or susceptible to a particular antibiotic, using standard reference values as shown in the National Committee for Clinical Laboratory Standards 2014. Multidrug resistance defined as resistance to three or more different classes of antibiotics.

Adhesion test in HEp-2 cells

One hundred strains of *E. coli* previously identified as EAEC (ast1 + and/or aat +) were tested for adhesion to HEp-2 cells, as described by Donnenberg and

Nataro²⁴, with modifications. In summary, 6.0 \times 10⁵ HEp-2 cells were cultured in DMEM medium with high glucose content (Biowest, Miami FL, USA) supplemented with 10% fetal bovine serum (Biowest, Miami FL, USA) and 1% antibiotic/antifungal solution (PAA, Pasching, Austria). The cells were placed in 24-well plates (TPP, Switzerland) containing a glass lentil and incubated until reaching a confluence of 80-90% (1-2 days). The HEp-2 cells were washed 3 times with sterile PBS, 500 μ l of fresh DMEM medium without fetal bovine serum or antibiotics were added.

One day before the experiment, the *E. coli* strains were grown overnight in 1.5 ml of LB medium with 1% D-mannose and the concentration was adjusted to 0.5 UMF (2 \times 10⁸ CFU/ml) with sterile PBS. The cultures were centrifuged and then resuspended in 1 ml of sterile PBS. 50 μ l of the bacterial suspension was mixed with 450 μ l of DMEM medium without fetal bovine serum or antibiotics and inoculated into cultures of HEp-2 cells. The infected cells were incubated for 3 h at 37°C under a 5% CO₂ atmosphere and washed 5 times with sterile PBS, fixed with absolute methanol for 1 min and stained with 10% Giemsa. Adherence patterns were assigned according to the description of Nataro and Kaper⁶.

Controls in each experiment included strains of *E. coli* O42 that showed aggregate adhesion and *E. coli* K12 that did not bind to HEp-2 cells.

Results

The present study was carried out with 200 strains of *E. coli*, 100 of them from the Central Hospital Dr. Ignacio Morones Prieto of the state of S.L.P., and 100 from the INP, from the Mexico city.

The β -hemolysis assay showed that of the 200 strains of *E. coli* studied, 25 had β -hemolysis, of which 14 were from Mexico city and 11 from S.L.P.

Identification of virulence genes of *E. coli*

Through the PCR assay, nine virulence genes were identified, of which five code for toxins (LT, ST, STX1, STX2, and astA), two for adhesins (bfpA and eaeA), one for invasiveness (ial), and a gene that participates in the secretion of the protein called dispersin, aat.

As can be seen in Table 1, of the strains of *E. coli* isolated from the Mexico city, 30% presented the LT gene; 14% the ST gene, 14% the astA gene, and 4% the ial gene. While for the state of S.L.P., 57% of the strains presented the LT gene; 37% presented TS; 27%

Table 2. Type of adherence and genes that carry strains of *E. coli*

Location	Number of strains	Genes	Type of adherence		
			AA	AD	N
CDMX	14	<i>astA+</i>	1	10	3
	0	<i>aat+</i>	0	0	0
	3	<i>astA+:aat+</i>	0	2	1
Total Strains	17				
S.L.P.	13	<i>astA+</i>	4	4	5
	1	<i>aat+</i>	0	0	1
	0	<i>astA+:aat+</i>	0	0	0
Total strains	14				

AA: aggregate adherence; AD: diffuse adhesion; N: negative adhesion; S.L.P.: San Luis Potosi.

presented the LT: ST genes; 28% presented the ial gene, 22% presented the STX1 gene, 13% the antler gene, and 9% presented the eaeA gene. These being the most prevalent among the nine genes studied. The statistical analysis showed significant differences ($p < 0.05$) for the LT, ST, LT: ST, ial, and STX1 genes.

Virotypes

The identification of virulence genes allowed us to classify *E. coli* into viotypes. This classification was made according to what was published by Estrada-García et al.¹⁷ (Fig. 1) and obtained the following results. For Mexico City: ETEC was 49% and in SLP was 67%. The statistical analysis showed a significant difference with a $p = 0.009$. EAEC in Mexico City was presented in 14% and in SLP was 14%. EIEC in Mexico City with 4% and in SLP was 28%, and the statistical analysis showed a significant difference of $p = 0.000$. EHEC for Mexico City with 3% and in SLP was 30%, and the statistical analysis showed a significant difference with a $p = 0.000$. The percentage of strains that were aEPEC in Mexico City was 9% and for SLP was 5%.

Adherence assay to HEp-2 cells

Adherence is one of the first factors for pathogenic *E. coli* strains to cause diarrheal infections. For this purpose, a gold standard test was considered for the identification

of the EAEC viotype. This test consists of adherence of the bacteria to a monolayer of HEp-2 cells.

As can be seen in table 2, of the 17 strains of *E. coli* that were evaluated for Mexico City, only one strain presented aggregative adherence (AA) and it was *astA+*. Twelfth strains showed diffuse adherence (AD) and of which two presented both *astA* and *aat* genes. The remaining four strains did not present any adherence pattern. Patterns of adherence of *E. coli* to HEp-2 cells (Fig. 2). Figure 2A shows negative control of adhesion of *E. coli* and we used the strain K-12. Figure 2B shows the positive control of aggregate adhesion and we used the strain O:42. Figures 2C, D, E and F, show different patterns of adherence, these strains of *E. coli* were isolated of samples from the CDMX and SLP; C) shows diffuse adherence, the *E. coli* strain comes from SLP; E) Shows aggregative adherence, the *E. coli* strain comes from the CDMX; F) shows aggregative adherence and the *E. coli* strain comes from SLP.

For SLP, 13 strains presented the *astA* gene of which four presented AA, four presented AD, and five strains did not present adherence. Regarding the strain that possesses the *aat* gene, it did not show adherence (Table 2).

Biofilm production

To evaluate the biofilm production, the Congo red and Christensen agar assays were used for which the strains that showed mobility were taken into account. Of the *E. coli* isolated from the CDMX, 60% were mobile and 84% for SLP.

Congo red agar assay

Of the 60 strains of flagellated *E. coli* isolated from the CDMX, only 42 strains formed biofilm and of which 11 were strong producers of biofilm (++)+, 31 were considered weak producers of the biofilm (+), and 18 strains were negative. For SLP, of the 84 strains of flagellated *E. coli*, only 64 strains formed biofilm, of these 28 were strong producers (++)+, and 36 strains were weak producers of biofilm (+), and 20 of them were negative (Table 3). In figure 3, the staining acquired by the colonies when producing biofilm is observed.

Christensen method (tube method)

According to the Christensen method, of 60 flagellate strains of *E. coli* for CDMX, 11 strains were biofilm

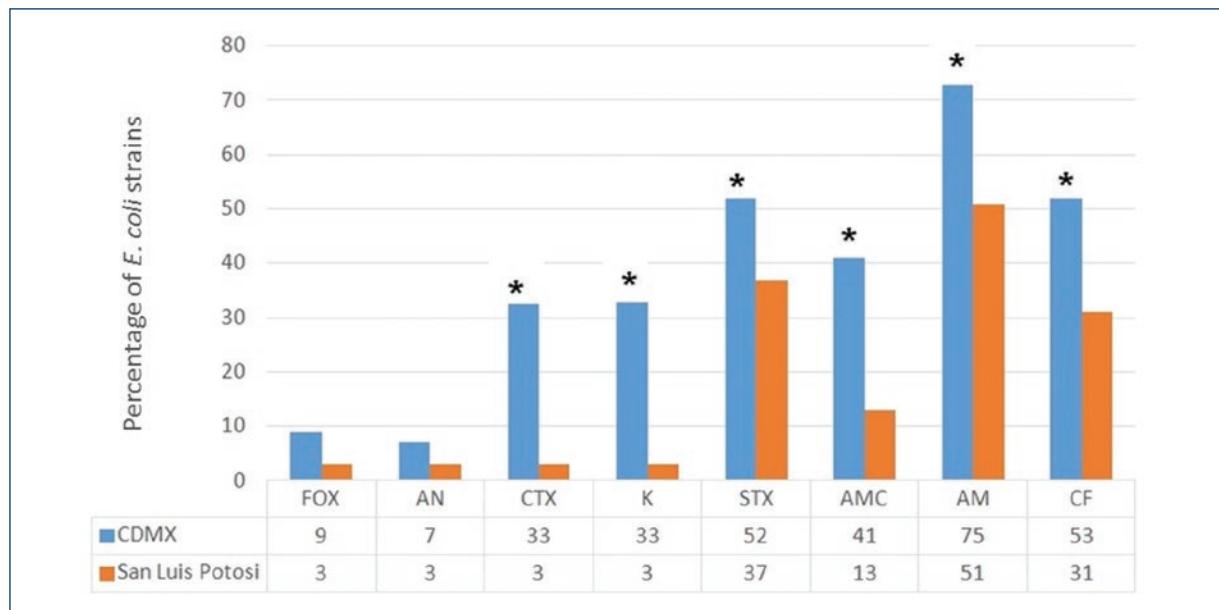


Figure 1. Cefoxitin, amikacin, cefotaxime, kanamycin, sulfamethoxazole/trimethoprim, amoxicillin/clavulanic acid, Ampicillin, cefalotin. *p < 0.00.

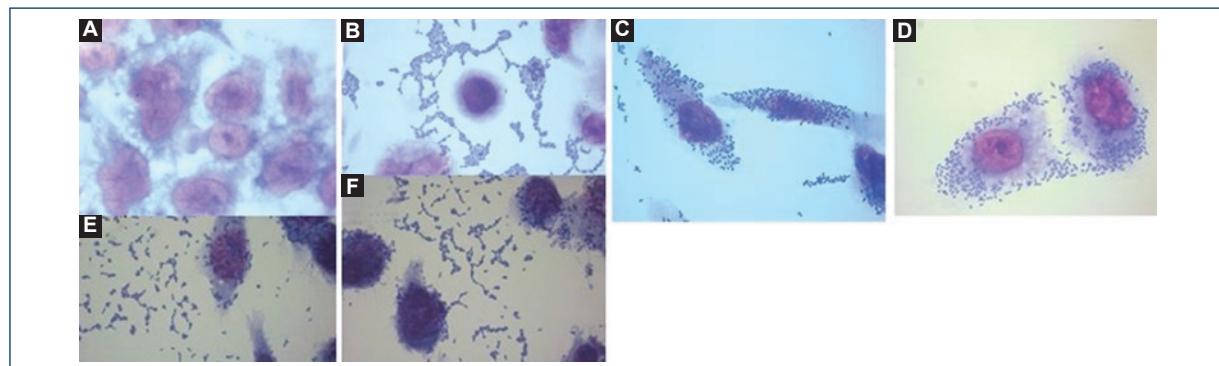


Figure 2. **A:** Negative control *Escherichia coli* strain K:12 $\times 100$; **B:** Positive control aggregate adhesion strain *E. coli* 0:42 $\times 100$; **C:** Diffuse adhesion strain $\times 100$ Mexico City; **D:** Diffuse adhesion strain $\times 100$ San Luis Potosi (S.L.P.); **E:** Aggregates adhesion strain $\times 100$ Mexico City; **F:** Aggregates adherence strain $\times 100$ S.L.P.

Table 3. Biofilm evaluation of *E. coli* strains by the Congo red agar method (ARC)

	Red agar	Congo
	CDMX n (%)	S.L.P. n (%)
(++) ¹	11 (19)	28 (33)
(+) ¹	31 (51)	36 (43)
Total positive strains	42 (70)	64 (76)
(-) ¹ Total negative strains	18 (30)	20 (24)
Total strains evaluated	60 (100)	84 (100)

¹(++) strong biofilm-producing strains, (+) weak biofilm-producing strains.
(-) non-biofilm-producing strains. S.L.P.: San Luis Potosí.

producers, while for S.L.P. of 84 flagellate strains of *E. coli*, 18 strains were biofilm producers. The number of strains that were not biofilm producers for CDMX was 48 strains and 66 strains for S.L.P. (Table 3). Figure 4 shows the staining of the tube wall inoculated with a biofilm-producing strain.

Antibiogram

To evaluate the resistance and sensitivity to certain antibiotics, antibiogram tests were performed. As we can see in figure 5, the strains of *E. coli* isolated from Mexico City showed 75% resistance to

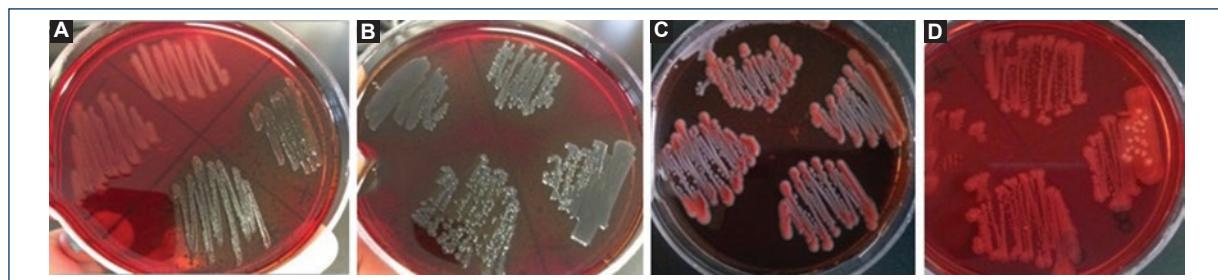


Figure 3. **A:** Upper strain O:42 negative control (colorless colonies), lower part K strain: 12 positive control (crystalline dry black colonies); **B:** (++) dry black crystalline colonies; **C:** Pink colonies with the black center (+); **D:** Colorless colonies, negative control; **(B-D)** Belong to strains of San Luis Potosí.

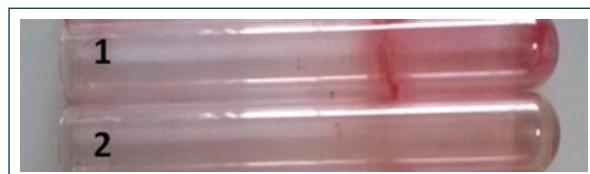


Figure 4. Tube 1, *Escherichia coli* K12 positive control strain. Tube 2 (–), O: 42 negative control strain.

AM, while the strains from SLP showed 51% and the statistical analysis showed a statistically significant difference with $p = 0.000$. About 53% of the strains of Mexico City were resistant to cefalotin (CF) and in S.L.P., only 31% with $p = 0.001$. About 52% of the Mexico City strains showed resistance to SMX/TMP in relation to 37% of strains of S.L.P. with $p = 0.03$. 41% of the strains of Mexico City showed resistance to amoxicillin/Clavulanic acid in relation to 13% of strains of S.L.P. with a statistically significant difference of $p = 0.000$. About 33% of the strains were resistant to kanamycin in Mexico City, in relation to 3% of the strains of SLP and the statistical analysis showed $p = 0.000$. 33% strains in the CDMX and 3% in SLP presented resistance to cefotaxime, with $p = 0.000$.

Discussion

The bacterium *E. coli* is the etiological agent of diseases of the digestive tract, urinary tract, and central nervous system. At present, it is known that there are several factors related to genotypic changes of this bacterium, from which derives the importance of determining not only the bacterium as the causative agent of diarrhea but also to identify the virulence genes related to the disease.

Although there are reports in which the prevalence of different pathotypes was determined, these studies come from populations from different geographical areas, different development conditions and different age groups, which makes it difficult to carry out comparative studies.

In the present work, to compare the *E. coli* viotypes from two states of the Mexican Republic and evaluate both genotype and phenotype, stool samples were obtained from children with diarrhea from SLP and Mexico City. The population are different both in geographical characteristics and in their population composition. According to data obtained from the National Institute of Statistics and Geography (INEGI), 36% of the population in SLP and only 0.5% in Mexico City are considered rural and with differences in the degree of social lag.

The results showed a higher incidence of *E. coli* viotypes isolated from stool samples from SLP in relation to the viotypes of this bacterium identified from Mexico City.

The viotype that presented a higher prevalence in both study groups was ETEC. In Mexico City 49% and SLP. About 67% of the strains and the statistical analysis showed a significant difference ($p = 0.009$). The data obtained in this work agree with that published by Nataro and Kaper⁶ Paniagua et al.³ and Qadri et al.²⁵ who affirm that ETEC is the most frequently found pathotype in developing countries as well as in developed countries, and the high prevalence of ETEC is related to its capacity for colonization⁶.

Regarding the virulence genes identified for ETEC, the genes that code for LT and ST toxins were those that occurred most frequently in the strains studied. The prevalent gene for both groups was the one that codes for the LT toxin. The strains isolated from Mexico City presented 30% and SLP 57% of the strains. This

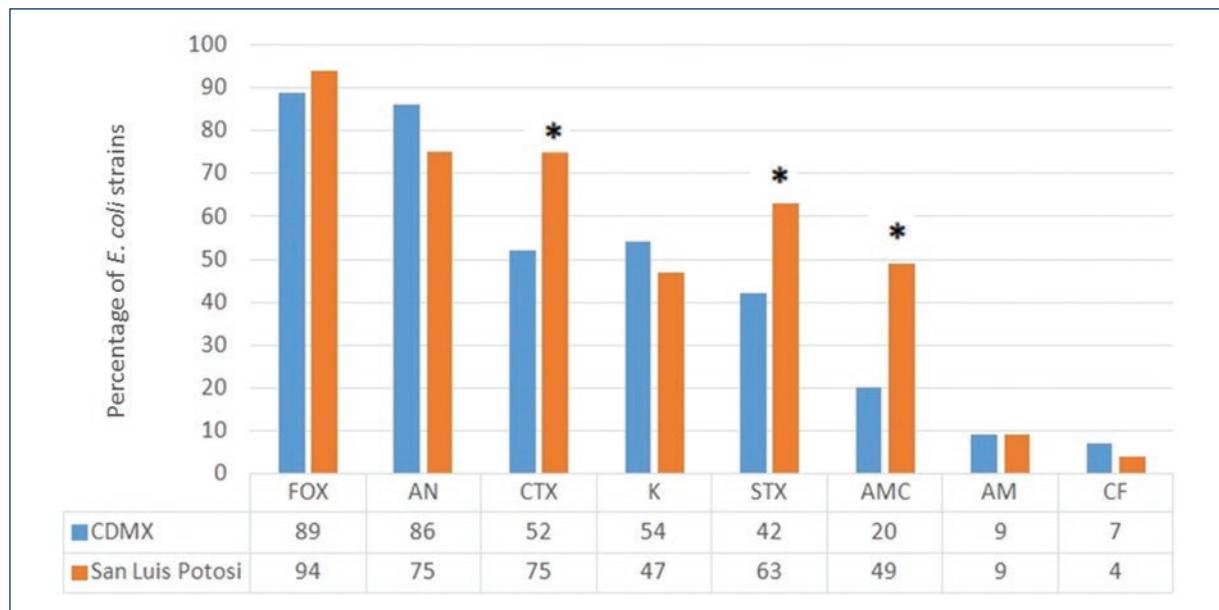


Figure 5. AM: ampicillin; AMC: amoxicillin/clavulanic acid; AN: amikacin; CF: cefalotin; CTX: cefotaxime; FOX: cefoxitin; K: kanamycin; STX: sulfamethoxazole/trimethoprim. * $p < 0.00$.

is concordant with Cortes et al.²⁶, Jiang et al.²⁷, Paniagua et al.³, Estrada-García et al.²⁸, and Qadri et al.²⁵.

Given that, *E. coli* strains were from fecal samples taken during the spring and summer seasons, it is likely that the incidence of the ETEC pathotype is seasonal, and this is in line with that published by Estrada-García et al.²⁸.

The EHEC viotype was the second most frequent in the state of S.L.P., with 30% of the positive strains and in the CDMX with 3%. The statistical analysis showed a significant difference ($p = 0.000$). The high prevalence of this viotype in the S.L.P. population is striking compared to both Mexico City and that published by other authors. Estrada-García et al.²⁸ showed that the frequency of EHEC in Mexico City was 2.5%. Vilchez et al.²⁹ in a study conducted in Nicaragua reported 2.1%, Nataro and Kaper⁶ mentioned that this viotype is isolated less frequently in the underdeveloped population compared to the ETEC and EPEC viotypes. However, according to Clarke et al.³⁰, EHEC has become an important viotype due to the increase in the number of outbreaks that have appeared in both developed and developing countries and mentions that this increase may be due to the high consumption of badly cooked food.

Despite the high prevalence of EHEC in S.L.P., there is no report from the health authorities of the state regarding the outbreaks of hemolytic uremic syndrome, as occurred in Germany in the year 2011³¹.

Like EHEC, the prevalence of the EIEC viotype was higher in the state of S.L.P. (28%) and for Mexico City (4%) finding a statistically significant difference ($p = 0.000$). Estrada García et al.²⁸ found 5% of this viotype, which coincides with our data in Mexico City and differs with the results obtained in S.L.P.

Clarke et al.³⁰ as well as Escher et al.³² considered the EIEC viotype as the causal agent of morbidity and mortality in both children from developed and developing countries due to poor sanitary and hygiene conditions. It is likely that the high prevalence of this viotype in S.L.P. is related to that published by Clarke et al.³⁰ given that S.L.P. presents a high degree of social lag.

The EPEC viotype was presented in 9% of Mexico City and 5% of SLP strains. Paniagua, et al.³, in a study in children with diarrhea in Mexico City determined the presence of EPEC with a percentage of 9.3% similar to that found in this work and to that of other countries, which ranges from 5 to 10%³³. The EPEC viotype is classified as typical (tEPEC) and atypical (aEPEC). According to the results obtained in this study, only aEPEC strains were identified for both study groups, and these results coincide with what was published by Ochoa et al.³⁴ who affirms that aEPEC is the most commonly associated viotype of diarrhea in children.

The EAEC viotype was found in 14% of the strains for both study groups, data similar to that published by Aslani et al.³⁵ who conducted a study to identify the EAEC viotype in samples of children with diarrhea

presenting in 10% of the study population³⁶. Jenkins et al., (2006) out of 500 total samples were able to identify 8% of EAEC.

It has been considered that EAEC is an emergent pathotype³⁷ given that it has been presented in both developing and developed countries as in the case of the United Kingdom³⁷. Furthermore, this pathotype has been associated as one of the causative agents of traveler's diarrhea²⁹.

The genes identified for this viotype were aat and astA, which have been related to the aggregative adhesion phenotype. The astA gene found in greater numbers with respect to the aat gene, in both study groups, which agrees with that published by Jenkins et al.³⁶

Once these genes were identified, the adherence test was performed to evaluate the AA in *E. coli* strains. In Mexico City, of 17 strains that carried some of the two genes, only one strain showed AA and 12 diffuse adhesion strains. While for S.L.P., of 14 strains evaluated, four strains showed diffuse adherence and four strains AA. It should be mentioned that all the strains that had some type of adherence carried the astA gene. These data show that the astA and aat genes are present in *E. coli* strains with a not only aggregative adhesion phenotype but are also present in strains of *E. coli* that adhere to the HEp-2 cells in a diffuse form. These data agree with that published by Jenkins et al.³⁶, who performed the HEp-2 cell adhesion assay and found no relationship between the presence of genes and the aggregative adhesion phenotype.

In relation to the higher prevalence of *E. coli* viotypes in SLP, compared to the *E. coli* viotypes identified in the CDMX, this may be due to the living conditions of the population belonging to a former state. According to INEGI, SLP is considered one of the states with the greatest social backwardness, since 53 of the 58 municipalities have a high degree of poverty such as lack of services, education, health, basic services, and spaces deficient housing. This favors that gastrointestinal diseases are a serious health problem for this population.

Another factor of virulence that has recently been considered of great clinical importance has been the production of biofilm in various bacteria, including *E. coli*¹². In the present work, biofilm production was evaluated only in the mobile *E. coli* strains based on the work of Pratt and Kolter¹¹, Reisner et al.¹², and Wood et al.³⁸ who showed that the flagellum is related to biofilm formation. The formation of this was evaluated by two methods: Congo red agar (ARC) and the Christensen method (in tube). By the Congo red method

in Mexico City, 70% of the strains were biofilm producers and for S.L.P. 76% with the Christensen method, for Mexico City, 19% of the strains formed biofilm and for S.L.P. 21%. The results obtained showed that, although all the strains evaluated showed mobility, not all of them were biofilm producers. This is why we consider that in addition to the flagellum, there are other factors that are related to the formation of said structure. Other factors related to biofilm formation include cellulose production³⁹, as well as the expression of pili Type 1, which has been considered a determining factor in the first steps in biofilm production⁴⁰.

The results showed that the most effective method to evaluate biofilm production was the ARC method. These data agree with that published by Serra et al.¹³ Among the advantages of biofilm formation are resistance by bacteria to environmental conditions, evading the immune response and generating resistance to antibiotics⁴¹.

Interestingly, it was observed that strains of *E. coli* belonging to a certain pathotype carried genes belonging to other pathotypes. In figure 6, we can observe the distribution of genes in strains isolated from both Mexico City and SLP. In addition, these data show that the origin of *E. coli* strains behaves differently when sharing genes between different viotypes, that is, the strains of SLP share genes between different viotypes in relation to *E. coli* strains of the Mexico City. This heterogeneity can be due to the transfer of genetic material that occurs between the bacteria, which can later lead to a new classification of pathotypes.

In the antibiogram tests, resistance, and sensitivity to the antibiotics tested were determined. The results showed a greater number of strains of *E. coli* resistant to different antibiotics in Mexico City compared to strains of *E. coli* from SLP, with a statistically significant difference for AM, cephalothin, SMX/TMP, amoxicillin/clavulanic acid, cefotaxime, and kanamycin. Regarding the percentage of strains resistant to AM, the results coincide with that published by Jiang et al.⁴² Ampicillin as well as CF belong to the group of β -lactams, the high resistance of the strains against this antibiotic is probably related to the development of β -lactamases. It has been observed that bacteria that had developed β -lactamases, also show resistance to amoxicillin and some first-generation cephalosporins, as is the case with CF⁴³.

The results obtained in this work showed a higher percentage of resistance to antibiotics of the strains of *E. coli* belonging to Mexico City, with respect to the *E. coli* strains of SLP. According to Guan et al.⁴⁴, the resistance can be modified due to geographical location, agricultural management, as well as the levels and

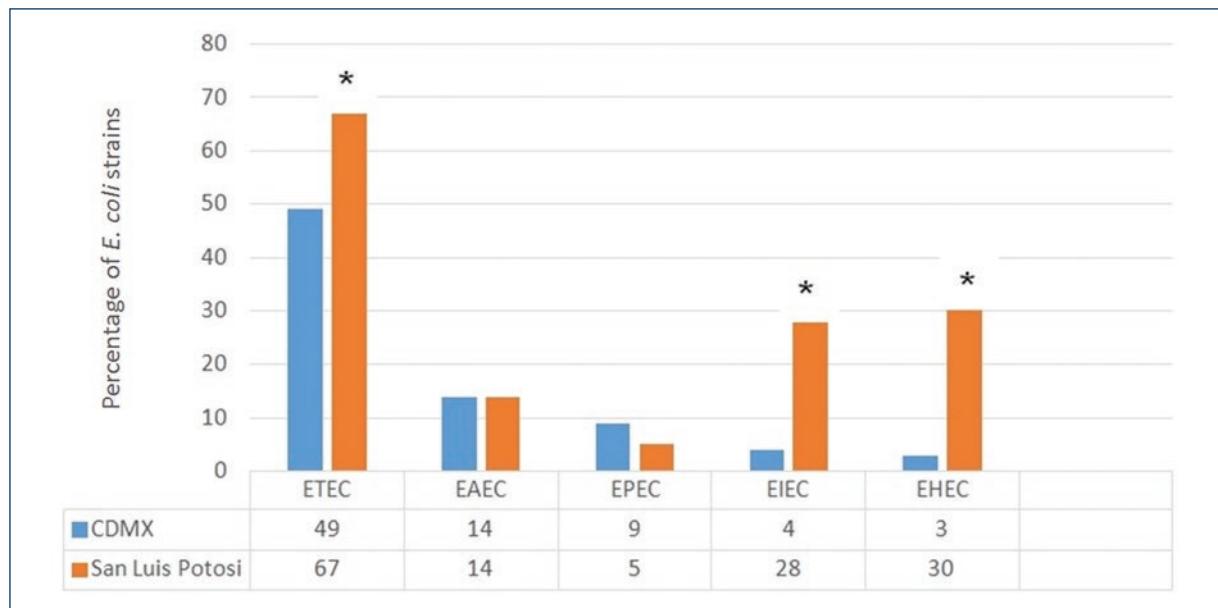


Figure 6. ETEC: enterotoxigenic *Escherichia coli*; EAEC: enteroaggregative *Escherichia coli*; EPEC: enteropathogenic *Escherichia coli*; EIEC: enteroinvasive *Escherichia coli*; EHEC: enterohemorrhagic *Escherichia coli*. * $p < 0.00$.

types of antibiotics used in the local population. In relation to the sensitivity to the antimicrobials tested, the highest percentage of sensitive strains occurred in *E. coli* isolated from S.L.P., with statistically significant differences for cefoxitin, SMX/TMP, and amoxicillin/clavulanic acid. This is probably due to better management of antibiotics in S.L.P.

This is the first comparative study performed with diarrheagenic *E. coli* in Mexico, with stool samples from the federal entities Mexico City and S.L.P. In these strains, both genotypic and phenotypic aspects of the bacteria were analyzed, showing a high prevalence of the ETEC virotype in both populations. This is in contrast to the EIEC and EHEC virotypes, which showed a high prevalence of *E. coli* strains from SLP, result that is striking since such a high prevalence of these virotypes had not been documented in Mexico. Likewise, a great heterogeneity of genes present in the strains of *E. coli* was observed, since these strains share genes that allow them to be classified in more than one virotype as the strains from S.L.P. being more heterogeneous.

Both the biofilm formation and the adhesion to HEp-2 cells were presented in greater numbers in strains of S.L.P. All these factors related to the virulence of *E. coli*, allow us to consider that the strains from S.L.P. are more virulent than the strains isolated in the Mexico City.

Regardless of the fact that *E. coli* is one of the most studied bacteria, the present work showed that this bacterium still has a lot of information to contribute.

Conflicts of interest

The authors declare that they have no conflicts of interests.

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Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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