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Escherichia coli pathotypes associated with diarrhea in a Coastal Ecuadorian city

Maritza Gardenia Páez Llerena

Gabriel Trueba, Ph.D.
Director de Trabajo de Titulación

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***Escherichia coli* pathotypes associated with diarrhea in a Coastal Ecuadorian city**

Maritza Gardenia Páez Llerena

	Firmas
Gabriel Trueba, Ph.D. Director de la Maestría en Microbiología Director del Trabajo de Titulación	_____
Karen Levy, Ph.D. Miembro del Comité de Tesis	_____
Pablo Endara, M.Sc. Miembro del Comité de Tesis	_____
Hugo Burgos, Ph.D., Decano del Colegio de Posgrados	_____

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Firma del estudiante: _____

Nombre: Maritza Gardenia Páez Llerena

Código de estudiante: 00116516

C. I.: 1715425318

Fecha: Quito, 3 de octubre 2016

DEDICATORIA

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RESUMEN

La diarrea es la segunda causa de muerte en niños menores de cinco años en todo el mundo y *Escherichia coli* diarreogénica (DEC) es una de las responsables de esta enfermedad en los países en vías de desarrollo como el Ecuador. El diagnóstico de los patotipos de *E. coli* y el conocimiento de los perfiles de resistencia a antibióticos son necesarios para controlar la enfermedad. El presente estudio analizó DEC en 223 muestras de heces, recogidas del hospital Delfina Torres de Concha de Esmeraldas de Abril a Septiembre del 2014. El diagnóstico se realizó mediante PCR convencional utilizando cebadores específicos para cada patotipo y 12 antibióticos se utilizaron para determinar el perfil de resistencia clínica con el método de difusión en disco. Se encontró presencia de DEC en 46.84% de los casos de diarrea y el 28,57% en los controles, y una asociación con diarrea (Odds Ratio (OR) = 2.20, 95% IC: 1.22-3.98; P= 0.004). El patotipo más prevalente entre los casos y controles fue *E. coli* adherente difusa (DAEC), con presencia en 26,12% y el 12,5% respectivamente (OR = 2.47; 95% IC: 1.16-5.40, P= 0.009). Co-infecciones de DEC fueron encontradas en un 6,30% y un 4,46% de los casos y controles respectivamente; esta diferencia no fue estadísticamente significativa (OR = 1.44; 95% IC: 0.37-5.93, P= 0.542). Finalmente, los patotipos exhibieron resistencia clínica a 11 de 12 antibióticos analizados, sulfisoxazol (79,76%), seguidos de ampicilina (76,19%), trimetoprim-sulfametoxazol (73.80%), estreptomicina y tetraciclina (61,90%), cefalotina (48.80%), cloranfenicol (17,85%), amoxicilina-ácido clavulánico (9,52%), gentamicina (8,33%), cefotaxima (7,14%) y ciprofloxacina (5,95%). No se encontró resistencia al imipenem.

Palabras clave: E. coli patogénica, diarrea, Esmeraldas, Ecuador, resistencia a antibióticos, *E. coli* diarreogénica, co-infecciones, *E. coli* adherente difusa (DAEC).

ABSTRACT

Diarrhea is the second leading cause of death among children under five years old around the world and diarrheagenic *Escherichia coli* (DEC) is one of the causes of this disease in developing countries like Ecuador. Diagnosis of *E. coli* pathotypes and knowledge of antibiotic resistance profiles are necessary to control the disease. The present study analyzed DEC in 223 stool samples, collected from Esmeraldas hospital Delfina Torres de Concha from April to September 2014. The diagnosis was made by conventional PCR using specific primers for each pathotype and 12 antibiotics were used to determine the antibiotic resistance profile with disk diffusion method. The prevalence of DEC was 46.84% in cases of diarrhea and 28.57% in controls, and a statistically significant association with diarrhea (Odds Ratio (OR) =2.20, 95% CI: 1.22-3.98, P=0.004). The most prevalent pathotype in cases and controls was Diffuse Adherent *E. coli* (DAEC) with 26.12% in cases and 12.5% in controls and a statistically significant association with diarrhea (OR=2.47, 95% CI: 1.16-5.40 P=0.009). DEC co-infections were found in 6.30% cases and 4.46% controls; this difference was not statistically significant (OR = 1.44, 95% CI: 0.37-5.93, P=0.542). Finally, pathotypes exhibited clinical resistance to 11 of 12 antibiotics analyzed, sulfisoxazole (79.76%), followed by ampicillin (76.19%), sulfamethoxazole-trimethoprim (73.80%), streptomycin and tetracycline (61.90%), cephalotin (48.80%), chloramphenicol (17.85%), amoxicillin-clavulanic acid (9.52%), gentamicin (8.33%), cefotaxime (7.14%) and ciprofloxacin (5.95%). No resistance was observed to imipenem.

Key words: Pathogenic *E. coli*, diarrhea, Esmeraldas, Ecuador, antibiotic resistance, diarrheagenic *E. coli*, co-infections, Diffuse Adherent E. coli (DAEC).

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PART I

GENERAL INTRODUCTION

Escherichia coli is a motile, non-spore-forming, gram negative bacilli that typically ferments lactose and is present in the human colonic flora, usually colonizing the human gastrointestinal tract within few hours after birth (1) (2). *E. coli* classified as commensal bacteria coexist in the mucous layer of the mammalian colon with mutual benefit to the host and microorganism. However, some strains evolved into pathogenic variants through the acquisition of plasmids, phages and pathogenicity islands (3), allowing them to adapt to new niches and causing diseases like infections in urinary tract, sepsis/meningitis and diarrhea. It is important to mention that the most successful combination of virulence elements that once were motile, now remain permanently in their genome (chromosome and motile genome elements), resulting in the *E. coli* pathotypes (1).

***Escherichia coli* pathotypes:**

The *E. coli* pathotypes of public health importance worldwide related with diarrheal disease are: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shigatoxin producing *E. coli* (STEC), including Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), *E. coli* Shigellae (*Shigella)*, Enteroaggregative *E. coli* (EAEC) and Diffuse Adherent *E. coli* (DAEC). Extraintestinal infections are cause by Uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC) (1) (4). Pathotypes can cause disease by colonization of the mucosal membrane, evasion of host defenses, and multiplication in the infection site, leading to inflammatory response in gastrointestinal mucosa by release of cytokines, chemokines and recruitment of inflammatory cells (5).

Virulence mechanisms differ in each pathotype: ETEC causes watery diarrhea, due to the colonization of the mucosa and production of two enterotoxins: a heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) (6). EIEC penetrates the membrane of epithelial cell by endocytosis, lyses the endocytic vacuole, multiplies intracellularly, and finally transmits through the cytoplasm and extension into adjacent epithelial cells. This pathotype is associated with colitis and occasionally dysentery (1). EPEC, the first pathotype described, causes attaching and effacing (A/E) lesions which are mediated by the product of the locus of enterocyte effacement (LEE) that encodes the intimin protein, where the bacteria attaches to intestinal epithelial cells (7). Typical EPEC strains have a plasmid called EPEC adherence factor (EAF), which encodes a type IV pilus called bundle-forming pilus (BFP). On the other hand atypical EPEC contains LEE but lacks the plasmid EAF (1) (4). DAEC is characterized by the cytopathic effects it causes; the majority of strains produce a fimbrial adhesion called F1845 (a member of Dr family of adhesins). DAEC can be divided into two groups: 1) those that possess the virulence factors afa/Dr adhesins associated with enteric and urinary tract infections; 2) includes a potential cause of diarrhea (adhesin AIDA-I) (8). EAEC adhere to HEp-2 cells using an autoaggregative pattern, in which bacteria adhere to each other. Some strains use an aggregative adherence fimbriae (AAFs) related to the Dr family of adhesins and others use a protein called dispersin, allowing spread across the mucosa surface and penetration; several virulence factors are regulated by a single transcriptional activator called aggR (1). Finally, EHEC infections can lead to hemorrhagic colitis and to uremic syndrome, this pathotype cause A/E lesions by LEE proteins and production of Shiga toxins responsible for vascular damage (9).

Prevalence of *E. coli* pathotypes in Ecuador:

Diarrheal diseases are a leading cause of preventable death, particularly in children under five in developing countries including Ecuador (10) and *E. coli* pathotypes are a potential cause of this disease.

In 1986 a sero-epidemiological analysis was carried out in 1620 serum samples from randomly selected Ecuadorian children (540 in urban and 540 in rural areas) for ETEC presence by LT and LPS specific ELISA. Naturally acquired ETEC diarrhea induces a serum antibody response to the homologous lipopolysaccharide (LPS) and to heat-labile enterotoxin (LT). ETEC at the time was the most studied pathotype because prior studies showed this pathotype and rotavirus as common pathogens associated with diarrhea in Latin America children (11) (12). Immunoglobulin G ELISA measuring antibodies to purified LT represented an effective tool for seroepidemiologic analysis of diarrheal in infections with LT-producing *E. coli*, also IgM measures from pooled LPS from the most common O serogropus identified in ETEC diarrhea. ETEC positive serum controls were obtained from volunteers who were orally administered ETEC strain O78:H11. Results showed ETEC presence in correlation with age: 15 of 113 Ecuadorian infants <6 months of age showed IgG antibodies to LT in ELISA. A gradual increase in prevalence was seen in children 6-18 months; 90% prevalence was reached in the second year of life and remained elevated through the maximum age of study participants (five years old). In concern of LPS ELISA, only 11 (10%) of 113 Ecuadorian infants <6 months of age showed IgM antibody to pooled ETEC LPS in ELISA, 50% prevalence was reached in 6 to 8 month old infants and 90% in 12 to 14 month old children (13).

The project Ecología Desarrollo Salud y Sociedad (EcoDess) was established in 2003, this project used a case-control design, in order to analyze the diarrheal disease and the spread of antibiotics in humans in 24 tropical-rain forest villages randomly selected in Canton Eloy Alfaro. All selected communities were located along the rivers: Cayapas, Santiago, Onzole, and Borbón (the biggest community) was also enrolled in the study (14). This project showed prevalence of different *E. coli* pathotypes. From August 2003 to July 2005 a community-based case-control study found EPEC, ETEC and EIEC was present in stool samples of 5097 individuals (915 stool samples). EIEC was the most abundant (3.2 cases/100 persons), followed by Shigellae (1.5 cases/100 persons), ETEC (1.3 cases/100 persons), and finally EPEC (0.9 case/100 persons), with higher prevalence in the community of Borbón (the economical center of the region). Only EIEC and ETEC were significantly associated with diarrhea. Pulsed field gel electrophoresis (PFGE) typing in EIEC isolates revealed that this pathotype was not associated with an outbreak (15).

A similar survey in the same region carried out (Bayas, *et al.* 2011) from August 2003 to December 2010 (4196 fecal samples; 916 cases and 3280 controls) found 275 pathogenic *E. coli* (130 cases and 145 controls). ETEC was the most prevalent pathotype (0.05 to 3.71 percent of the population), ETEC-LT was the most frequent, the second most prevalent pathotype was EIEC (0.97-4.44 cases per 100 persons), *E. coli* Shigellae (0-1.67 cases per 100 persons) and finally EPEC (0.02-1.28 cases per 100 persons). All four pathotypes were associated with diarrhea, *E. coli* Shigellae was most strongly associated with diarrhea (RR = 6.90, 95% CI: 3.76, 13.69), while EIEC was lower and not statistically associated (RR = 1.15, (95% CI: 0.61, 1.96). This study suggested that prevalence of *E. coli* pathotypes tend to vary

overtime, because between 2003 to 2005 EIEC was the most prevalent pathotype and from 2005 and 2010 ETEC showed higher prevalence. This changes could be due to outbreaks, environmental factors or may just be a function of under sampling (16). EIEC and ETEC prevalence patterns were characterized across space and time in the 16 communities mention above (17). In 2012 another survey in Borbón found that the most prevalent pathotype were EIEC (3.97%), ETEC LT and ST (3.31%) in single and co-infections and finally in co-infection presence *E. coli* Shigellae (1.32%), any pathotype were associated with diarrhea (18). These observations may also suggest the existence of genetically the different strains (belonging to the same pathotype) with different ability to infect or to transmit. Another phenomenon associated with the presence of diarrhea may be the presence of 2 or more pathogens (19).

Finally, studies carried out in Quito showed that among 200 people in the community of Guamaní the most prevalent pathotypes were *Shigella* (5.5%), the only pathotype significantly associated with diarrhea (OR = 23, 95% CI: 1.35, 390), followed of EIEC (4%), ETEC LT and ST (3%) and finally, STEC (1.5%) (18). On the other hand, in 233 samples from Enrique Garces hospital and local health center in a low income neighborhood in Quito from April to September 2014 the most prevalent pathotype was DAEC (15.3% in cases and 6.1% in controls), typical and atypical EPEC (3.4% in cases and 7.6% in controls), followed of ETEC (5.1% in cases and 3.5% in controls); EAEC (0.8% in cases and 3.5 in controls), EIEC (3.4% in cases) and *Shigella* (2.5% in cases). In this study, only DAEC was significantly associated with diarrhea (OR = 2.78, 95% CI: 1.11, 6.93, P=0.03) (20).

Prevalence of *E. coli* pathotypes in Peru and Colombia:

Several investigations focused on *E. coli* pathotypes have also occurred in Peru. In a diarrhea surveillance study conducted from September 2006 to May 2007, researchers analyzed 557 stool samples from Peruvian children with diarrhea and 195 controls. The prevalence of DEC (diarrheagenic *E. coli*) was 29% in cases and 30% in controls; EAEC (14% in cases, 18% in controls) were the most prevalent, followed by EPEC (7% in cases, 7% in controls), DAEC (4% in cases, 3% in controls); ETEC (4% in cases, 2% controls) and STEC (1% cases, 0.5% controls). No EIEC strains were isolated, and any association with diarrhea were measured. Diarrheagenic *E. coli* were frequently resistant to ampicillin, cotrimoxazole, tetracycline, nalidixic acid and chloramphenicol and show higher frequency of resistance to all antibiotics in diarrheal samples than in controls (21). Age-related susceptibility to infection has also been studied, the same author in the same population, but for a longer period of time (between September 2006 and December 2007), analyzed 936 stool samples and 424 controls, the most common pathotypes isolated were EAEC (15.1%) and EPEC (7.6%). DAEC and ETEC were more frequently isolated in cases in older infants and all pathogens were more frequently isolated from infants > 6 months age (22).

Mosquito, *et al.* 2015 studied the antibiotic resistance in phylogroups from 369 *E. coli* isolates randomly selected (74 commensal and 94 DEC (diarrheagenic *E. coli*) from asymptomatic children and 201 from children with diarrhea) from 1032 Peruvian infants. The most prevalent pathotype were EAEC (94), followed of EPEC (87), ETEC (83) and DAEC (31). These authors found that DEC-control strains were more associated to the phylogroup A and DEC-diarrhea strains were more related to phylogroup D. Finally, antibiotic resistance

was higher in phylogroups related to extraintestinal pathotypes (B2 and D) than phylogenetic groups related to commensal *E. coli* strains or gastrointestinal tract strains (A and B1) (23).

In a DEC prevalence study carried out in two Northern Colombian cities (Sincelejo and Cartagena). Two hundred sixty seven stool samples were analyzed from children less than 5 years of age with diarrhea, and from them 139 *E. coli* isolated were recovered. Twenty (14.4%) *E. coli* strains were positive for DEC (diarrheagenic *E. coli*). The most frequent pathotype were ETEC, and low rates of STEC, EPEC, EAEC and DAEC were identified (24).

Another report of a study carried out from October 2006 to February 2007, designed to identify *E. coli* pathotypes in 108 stool samples from children with diarrhea attending six hospital in Colombia and 76 food products for human consumption (38 correspond to meat and 38 vegetables). One hundred eighty four *E. coli* strains from clinical samples and food products were analyzed, 18 (9.8%) were positive for any pathotype 12 (11.1%) among all clinical isolates and 6 (7.9%) among food products. The most common pathotype in clinical samples were atypical EPEC (9%), while STEC was more common in food products (7.1%). In clinical samples they detected STEC, ETEC, EAEC and atypical EPEC, and only STEC, EAEC and typical EPEC were detected in food products samples; these authors suggested, that meat and vegetables may be the source of STEC and EAEC in the community (25).

A case-control study was conducted to evaluate the association of *E. coli* pathotypes with diarrhea in children younger than 5 years of age in Cartagena, from May 2009 to May 2010.

Stool samples from 349 cases and 349 controls were subjected to polymerase chain reaction (PCR) analysis. A total of 38 (5.44%) diarrheagenic *E. coli* were isolated, the most prevalent pathotype were ETEC (3.58%), followed by EPEC (0.86%), EAEC (0.57%) and EIEC (0.14%). Only ETEC were associated with childhood diarrhea (26).

Summary matrix:

A summary of published data on the presence of *E. coli* pathotypes in Ecuador, Peru and Colombia is present in the following matrix.

Publication	Number of Tested sample	Prevalence rates or frequency (P%) and association with diarrhea (OR, RR, 95% CI)						
		DAEC	**EAEC**	**ETEC**	**EPEC**	**EIEC**	***Shigella***	**STEC**
Briissow, *et al*. 1990	1620 children <5 years old	Not tested	Not tested	IgG antibodies to LT: 13%<6 months, 90% second year of life and IgM antibodies to LPS: 10%<6 months, 90% 12-14 month old children	Not tested	Not tested	Not tested	Not tested
Vieira, *et al.* 2007	915 (236 cases, 679 controls)	Not tested	Not tested	P:1.3(7.6 cases, 1.2 controls) OR=6.9, 95% CI: 2.8-18.6	P:0.9 (1.7 cases, 0.9 controls) OR= 1.9, 95% CI: 0.4-8.2	P:3.2 (8.9 cases, 3.1 controls) OR=3.1, 95% CI: 1.6-6.0	P: 1.5 (0.9 cases, 1.5 controls) OR=0.6, 95% CI: 0.06-2.7	Not tested
Bayas, *et al.* 2010	4196 (916 cases, 3280 controls)	Not tested	Not tested	P:0.05-3.71 RR: 6.43, 95% CI: 0-52.69	P: 0.02-1.28 RR: 3.46, 95% CI: 1.60-8.06	P:0.97-4.44 RR: 1.15, 95% CI: 0.61-1.96	P: 0-1.67 RR: 6.90, 95% CI: 3.76-13.69	Not tested
Vasco, *et al.* 2014 Borbón	151 (39 cases, 112 controls)	Not tested	Not tested	P (ETEC ST+LT):3.31 OR ST all infection= 6, 95% CI: 0.53-68 OR LT= NA	Not found	P:3.31 OR all infection=1.46, 95% CI: 0.25-8.3	P:1.32 OR all infection=2.92, 95% CI: 0.18-48	Not found
Vasco, *et al.* 2014 Guamaní	200 (100 cases, 100 controls)	Not tested	Not tested	P (ETEC ST+LT):3 OR ST all infection= 7, 95% CI: 0.36-135 OR LT= 0.5, 95% CI:0.008-9.6	Not found	P: 4 OR all infection=5, 95% CI: 0.55-236	P: 5.5 OR all infection=23, 95% CI: 1.35-390	P: 1.5 OR all infection=3, 95% CI: 0.12-73
Montero, *et al.* 2016	233 (118 cases, 115 controls)	P:15.3 cases, 6.1 controls OR=2.78, 95% CI: 1.11-6.93, P=0.03	P: 0.8 cases, 3.5 controls OR=0.24, 95% CI: 0.03-2.15, P=0,20	P: 5.1 cases, 3.5 controls OR=1.49, 95% CI: 0.41-5.41, P=0.55	P: 3.4 cases, 7.6 controls OR=0.47, 95% CI: 0.14-1.30, P=0,23	P: 3.4 cases, 0 controls OR= NA	P: 2.5 cases, 0 controls OR= NA	Not found
Ochoa, Ruiz *et al.* 2009	752 (557 cases, 195 controls)	P: 4 cases, 3 controls	P: 14 cases, 18 controls	P: 4 cases, 2 controls	P: 7 cases, 7 controls	Not found	Not found	P: 1 cases, 0.5 controls

Publication	Number of Tested sample	Prevalence rates or frequency (P%) and association with diarrhea (OR, RR, 95% CI)						
		DAEC	**EAEC**	**ETEC**	**EPEC**	**EIEC**	***Shigella***	**STEC**
Ochoa, Ecker, *et al*. 2009	1360 (936 cases, 424 controls)	P: 4.6 cases, 2.1 controls	P: 15.1 cases, 17.9 controls	P: 3.2 cases, 1.2 controls	P: 7.6 cases, 9.9 controls	Not found	P: 0.1 cases, 0.5 controls	P: 0.5 cases, 1.2 controls
Gómez-Duarte, *et al.* 2010	267 <5 years old from Sincelejo and Cartagena	P Sincelejo: 0.9 P Cartagena: 0	P Sincelejo: 1.8 P Cartagena: 0	P Sincelejo: 4.5 P Cartagena: 7.1	P Sincelejo: 0 P Cartagena: 3.6	Not found	Not found	P Sincelejo: 3.6 P Cartagena: 3.6
Rúgeles, *et al.* 2010	108 stool samples from children	Not found	P:4.5	P: 3.0	Atypical EPEC: 9.0 Typical EPEC: 0	Not found	Not found	P: 1.5
	76 food products: meat and vegetables	Not found	P: 3.6	P: 0	Atypical EPEC: 0 Typical EPEC: 10.7	Not found	Not found	P: 7.1
Gómez-Duarte *et al.* 2013	815 (349 controls and 466 cases) <5 years old	Not found	P: 0.57 (0.29 cases, 0.86 controls) OR=0.39, 95% CI: 0.04-3.83, P=0.42	P: 3.58 (4.87 cases, 2.29 controls) OR=2.51, 95% CI: 1.05-5.98, P=0.037	P: 0.86 (1.43 cases, 0.29 controls) OR=5.92, 95% CI: 0.68-51.23, P= 0,10	P: 0.14 (0.29 cases, 0 controls) OR= NA	Not found	Not found

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PART II

SCIENTIFIC PAPER

Escherichia coli pathotypes associated with diarrhea in a Coastal Ecuadorian city

AUTHORS

Maritza Páez[1], Gabriel Trueba[1], Pablo Endara[1], William Cevallos[2], and Karen Levy[3]

[1]Microbiology Institute, Universidad San Francisco de Quito, Quito, Ecuador; [2]Biomedical Center-School of Medicine, Universidad Central del Ecuador, Quito, Ecuador; [3]Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta

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#Address correspondence Karen Levy, Emory University, USA, Karen.levy@emory.edu

INTRODUCTION

Diarrhea is the second leading cause of death among children under five years old around the world, causing approximately, one in nine child deaths worldwide despite the availability of treatment (1). Diarrheagenic *E. coli* (DEC) is an important etiologic agent of diarrhea, and includes seven pathotypes: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shigatoxin producing *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Diffuse Adherent *E. coli* (DAEC) and *E. coli* Shigellae (*Shigella)* (2) (3).

Infectious diarrhea prevention is mainly focused on availability of safe water, sanitary infrastructure, breastfeeding and rotavirus vaccination (4). Esmeraldas Province is one of the poorest regions in Ecuador, and has deficient sanitary conditions (5). *E. coli* pathotypes have been detected in some rural communities in this province (6) (7) (8) (9) (10). The objective of this study was to determine the prevalence (or frequency) of seven *Escherichia coli* pathotypes and their association with diarrheal disease in an urban community of Esmeraldas Province. Additionally we determined the patterns of antibiotic resistance in these pathotypes.

MATERIALS AND METHODS

Human subjects and Study design:

We conducted a case-control study recruiting participants in the city of Esmeraldas, the capital of Esmeraldas Province (a community with deficient sanitary infrastructure). Subjects were recruited and enrolled in the study at Delfina Torres de Concha Hospital between April and September 2014. Esmeraldas is located in Ecuador's northern Pacific Coast region, at 15 m.a.s.l., the population was 189504 habitants in 2010 (5). Cases were defined as anyone who came to Delfina Torres de Concha Hospital suffering from acute diarrhea (three or more loose stools in a 24 hours period) and controls as somebody who came to the hospital for another reason and did not have diarrheal symptoms during the past seven days. Subjects were excluded if they reported having taken antibiotics anytime in the prior week, or if they had not lived in the city of Esmeraldas for at least six months. Prior to enrollment all participants signed a consent document approved by the Institutional Review Boards of Emory University and Universidad San Francisco de Quito. Surveys with demographic data (age, gender, sanitation, water consumption, contact with animals, travel in the last year, etc.) were carried out using electronic devices and using Open Data Kit program (http://opendatakit.org). Individuals of all ages were eligible to participate in the study, and cases were age-matched with controls using the following age categories: 0-24 months: ±6 months; 25-60 months: ±12 months; 61-180 months: ±24 months; >180 months: any age above 180 months.

Laboratory Procedures:

Bacterial Identification, DNA extraction and PCR Analysis.

Stool samples were cultured on MacConkey's agar media (MKL). After 24 hours of incubation, up to five lactose-positives isolates and one non-lactose fermenting isolates were randomly selected and cultured on Chromocult agar media (Merck, Darmsladt, Germany) (CC) for β-glucoronidase (MUG) activity; colonies classified by their lactose and MUG activity were frozen as previously described (11). In addition, the five selected colonies were cultured in nutrient agar and colonies from each isolate were pooled together in a tube containing 300 µl of sterile distilled water and boiled to release the DNA (12). Colonies unable to ferment lactose were identified by biochemical test as *Shigella* or *E. coli,* followed by DNA extraction (12).

Pooled DNA were centrifuged to 1,780 g for 1 min, and the supernatant was subjected to polymerase chain reaction (PCR) for the presence of *E. coli* pathotypes: Enteroinvasive (EIEC), Enterotoxigenic (ETEC), Typical and Atypical Enteropathogenic (EPEC), Diffusely adherente (DAEC), Enteroaggregative (EAEC). The target virulence genes used for each pathotype were: *bfp* for typical EPEC; *lt* and *sta* for ETEC; *ipaH* for EIEC and *Shigella*; *aggR* for EAEC; *afa* for DAEC and *eaeA* for atypical EPEC. Positive pools for *eaeA* gen were tested for *stx1* and *stx2* genes to detect potential enterohemorrhagic *E. coli* (EHEC). If the pooled resulted positive for any pathotype each isolate was then tested individually for that specific gene.

For *ipaH*, *lt* and *bfp* and *sta* the optimized protocol was carried out with 25 µl mixture containing: 1x PCR Buffer; 1.5 mM $MgCl_2$; 0.02 U Go Taq DNA polimerase; 200 µM

dNTPs; 0.2 µM (each primer) and 2.5 µl of DNA. The PCR cycles program were: Denaturation at 94^{o}C for 1 min, annealing at 56^{o}C for 2 min, and extension at 72^{o}C for 1 min, for 29 cycles, with an exception of s*ta* were the PCR program increases 2^{o}C in the annealing (13). *aggR* PCR reaction constitute of 25 µl mixture containing: 1x PCR Buffer; 2 mM $MgCl_2$; 0.02 U Go Taq DNA polimerase; 200 µM dNTPs; 0.4 µM (each primer) and 3 µl of DNA and subjected to denaturation at 94^{o}C for 0.5 min, annealing at 50^{o}C for 1 min, and extension at 72^{o}C for 1.5 min, for 24 cycles, and 72^{o}C for 5 min (12). For *eaeA* the 25 µl mixture PCR reaction contained: 1x PCR Buffer; 2 mM $MgCl_2$; 0.02 U Go Taq DNA polimerase; 200 µM dNTPs; 0.25 µM (each primer) and 1.5 µl of DNA, stx1 and stx2 contain the same reaction with a modification of 0.5 less $MgCl_2$ and the final concentration of 1 µM for each primer. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 95^{o}C; 2 min of annealing at 65^{o}C for the first 10 cycles, decrementing at 60^{o}C by cycle 15; and 1.5 min of elongation at 72^{o}C, incrementing to 2.5 min from cycles 25 to 35 (14). Finally, for *afa* gene, PCR was done in a 25 µl mixture containing: 1x PCR Buffer; 1.5 mM $MgCl_2$; 0.02 U Go Taq DNA polimerase; 200 µM dNTPs; 0.2 µM (each primer) and 2.5 µl of DNA. PCR amplifications consisted of 24 cycles of denaturation at 94^{o}C for 2 min, annealing at 65^{o}C for 1 min, and extension at 72^{o}C for 2 min (15).

PCR products were then electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized by UV transilumination. PCR sized were: *aggR* (254 bp), *lt* (708 bp), *sta* (182 bp), *bfp* (324 bp), *eaeA* (384 bp), *ipaH* (424 bp), *afa* (750 bp), *stx1* (180 bp) and *stx2* (255 bp).

Antibiotic Susceptibility Testing.

The antibiotic susceptibility of *E. coli* pathotypes was measured using disk diffusion method according Clinical and Laboratory Standards Institute (CLSI) 2015 guidelines. Antibiotics analyzed included: ampicillin (AM, 10 µg), amoxicillin-clavulanic acid (AmC, 20/10 µg), cefotaxime (CTX, 30 µg), cephalothin (CF, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), sulfisoxazole (G, 200 µg), gentamicin (CN, 10 µg) streptomycin (S, 10 µg), tetracycline (Te, 30 µg), imipenem (IPM, 10 µg). The interpretive criteria to determine resistance vs. susceptible cut-off values of zone diameters was taken from CLSI 2015 guidelines (16).

Statistical analyses:

Statistical analyses were performed using Microsoft Office Excel 2013, and StataMP 13 (StataCorp. LP,College Station, TX). We calculated odds ratios (OR) to compare presence of *E. coli* pathotypes between case and control samples and antibiotic resistance by pathotype. Chi-square were used for group comparisons and logistic regression was used to calculate adjusted OR for confounding variables. Statistical significance was consider if P-value ≤0.05.

RESULTS

Risk factors for diarrhea:

A total of 223 individuals were enrolled in this study (111 cases and 112 controls). We observed no differences in cases versus controls with respect to age or gender of the subjects (Table 1). The majority of cases (67.57%) and controls (53.57%) reported drinking tap water and people who don't carry out additional treatment to the tap water were twice as likely to develop diarrhea than people that treat (boiling or filtering) water before drinking it (OR=1.91, 95% CI: 1.06-3.44, P-value=0.024). Similarity, people that travel during the last year were ~2 times more likely to develop diarrhea (OR=2.20, 95% CI: 1.20-4.06, P-value=0.006). We did not observe any increased risk or protection from reported sanitation type or recent contact with animals.

***Escherichia coli* pathotypes:**

A total of 307 *E. coli* strains were obtained from the 223 subjects of study. A total of 84 strains were positive for diarrheagenic *Escherichia coli* (DEC), with 52 (46.84%) of them present in cases (41 children and 11 adults) and 32 (28.57%) in controls (26 children and 6 adults). There was a statistically significant association of DEC presence with diarrhea (OR=2.20, 95% CI: 1.22-3.98, P-value=0.004). The most prevalent pathotype was: DAEC with 26.12% in cases and 12.5% in controls, followed by EAEC (6.30% in cases and 7.14% in controls); atypical EPEC (*bfp*-, *eaeA*+, *stx1*-, *stx2*- genes) with 8.10% in cases and 4.46% in controls; ETEC (3.60% in cases and 4.46% in controls); EIEC (1.80% in cases and no presence in controls) and finally, typical EPEC (*bfp+,eae+*) with

0.90% in cases a no presence in controls. DAEC was the only pathotype that had a significant association with diarrhea (OR=2.47, 95% CI: 1.16-5.40, P-value=0.009). OR adjusted for home water treatment and travel in the last year (OR=2.33, CI 95% 1.16-4.69, P-value=0.0006). ETEC and EAEC were found more often in controls than in cases, but this difference did not reach the level of statistical significance. No STEC, EHEC or *Shigella* were detected. (Table 2).

In addition, we found no association between having any *E. coli* pathotype and travel in the last year, home water treatment and contact with animals (Table 5).

Finally, the possibility that diarrhea is explained by other microorganisms such as rotavirus or diarrheic parasites is unlikely in this study, because no statistical differences for this microorganisms were present between the two groups (Table 8).

Co-infections:

More than one pathotype in one stool sample were found 7 (6.30%) cases and 5 (4.46%) controls. Six cases and 4 control had 2 pathotypes; 1 case and 1 control had 3 pathotypes (Table 3). The majority of co-infections were found in children (Table 4). We did not find any association between having a co-infection and any of the potential risk factors assessed.

Antibiotic Resistance:

The dominant clinical resistance were to sulfisoxazole (79.76%), followed by ampicillin (76.19%), sulfamethoxazole-trimethoprim (73.80%), streptomycin and tetracycline (61.90%), cephalotin (48.80%), chloramphenicol (17.85%), amoxicillin-clavulanic acid (9.52%), gentamicin (8.33%), cefotaxime (7.14%), ciprofloxacin (5.95%). No isolates were resistant to imipenem. A higher frequency of resistance (to all antibiotics) was

observed in controls with the exception of amoxicillin-clavulanic acid, chloramphenicol and streptomycin, but none of these differences were statistically significant (Table 6 and 7). Among 84 DEC, 65 (77.38%) were multi-drug resistance, multidrug-resistance was not statistical lower in isolates from cases than in isolates from controls (75% vs. 81.25%, P-value=0.506) adjusted for gender and medicine intake OR=0.42, CI 95% 0.10-1.74, P-value=0.233 (Table 8).

Most pathotypes had resistance to 6 antibiotics (23.81%) and only 4 isolates (4.76%) were resistant to 4 antibiotics. The most common resistance antibiotic patterns among diarrheagenic *E. coli* was: G-Amp (75%) and G-SXT (73.8%) in two antibiotic combination; G-Amp-SXT (70.2%) and G-Amp-S (59.5%) in three antibiotic combination; finally, in four antibiotic combination G-Amp-SXT-S and G-Amp-SXT-Te (55.9%).

DISCUSSION

In this study we found that DEC strains are significantly associated with diarrhea case status (OR=2.20, 95% CI: 1.22-3.98, P=0.004). The most prevalent pathotype was DAEC (26.12% in cases and 12.5% in controls), and this pathotype was the only one associated with diarrhea in our study (OR=2.47, 95% CI: 1.16-5.40, P=0.009). These results are similar to a recent sister study carried out in Quito which found that this pathotype was also associated with diarrhea and had the highest prevalence (17). DAEC has been associated with diarrhea (usually in children or age-dependent diarrhea) in different geographic regions, including Bangladesh, United Kingdom, Mexico, Brazil and Peru (18) (19) (20) (21) (22) (23).

To our knowledge, this is the first report of DAEC and EAEC presence in Esmeraldas. Diffuse adherence pattern of *E.* coli to enterocytes was discovered using human epithelial cell (Hep-2 cell), and led to the posterior division into two categories: diffuse adherent *E. coli* (DAEC), which adhere to the entire surface of human epithelia and enteroaggregative *E. coli* (EAEC) adhering to Hep-2 cells in a “stacked brick” appearance. EAEC has been reported as an emerging enteric pathogen, and has gained importance due to pathogenesis: adherence to intestinal mucosa, deposit of a mucus biofilm and mucosal toxicity due to cytokine release, but little is known about DAEC. While, it is still not well-understood, it has been suggested that DAEC strains should be categorized within the EAEC pathotype, because the predominant adhesins of DAEC are related to fimbril adhesins encoded on EAEC plasmids (24) (25).

Our reports also showed lower prevalence of EPEC, ETEC and EIEC, and no presence of *E. coli* Shigellae (*Shigella).* There was no association of these pathotypes with diarrhea, although this might be a function of the small sample sizes obtained for these pathotypes. These results agree with a case a control study developed in 2012 in a rural community of Esmeraldas province (Borbón), where neither ETEC, EIEC nor *Shigella* were significally associated with diarrhea (9). These findings suggest that prevalence of EIEC and ETEC could change due to two possible reasons: 1) environmental factors like climate including rainfall, humidity; different geographical areas, period of investigation, socioeconomic levels, etc. (6) (7) (10) (26) (27), 2) virulence of EIEC and ETEC strains circulating in 2005 and 2010 may have been different from those isolated in 2014, or that people in rural communities have less immunity than those in urban communities as suggested previously (9) (28) (29).

Additionally, we found co-infections with two or three pathotypes, the most frequent was DAEC and EAEC and all co-infections were more frequent in cases 6.30% than in controls 4.46%. Several authors describe the importance of synergistic interactions between enteric pathogens like diarrheagenic *Escherichia coli* (DEC), rotavirus and parasites in diarrheal illness (7) (8) (9) (28) (30) (31).

We found dominant clinical resistance to sulfisoxazole (79.76%), followed by ampicillin (76.19%), sulfamethoxazole-trimethoprim (73.80%), streptomycin and tetracycline (61.90%), cephalotin (48.80%), chloramphenicol (17.85%), amoxicillin-clavulanic acid (9.52%), gentamicin (8.33%), cefotaxime (7.14%) and ciprofloxacin (5.95%). Our results concur with a previous study of 3317 strains of pathogenic *Escherichia coli* obtained

from distinct hospitals in Ecuador which showed resistance to ampicillin (85%), sulfamethoxazole-trimethoprim (70%), amoxicillin-clavulanic acid (46%), cefotaxime (67%), gentamicin (38%), ciprofloxacin (63%) and very little resistant to imipenem (2%); they did not investigate resistance to chloramphenicol, streptomycin, cephalotin and tetracycline. Additionally they found resistance to cefazolin (55%), amikacin (5%). (32) (33). In addition, a study in Peru revealed the same result where ampicillin was the most resistant antibiotic (85% in cases, 70% in controls) in diarrheagenic *Escherichia coli* (DEC), followed of cotrimoxazole or sulfamethoxazole-trimethoprim (79% cases, 61% in controls), tetracycline (65% in cases, 58% in controls), nalidixic acid (28% cases, 16% controls) and chloramphenicol (26% cases, 28% controls) (34). A prior study developed between 2003 and 2008 in 150 villages in Esmeraldas province reveal that ampicillin and sulfamethoxazole-trimethoprim were present in three of the most frequently resistant patterns in *E. coli* from people with diarrhea (cases) and controls (35).

We were unable to see any difference of antibiotic resistance in *E. coli* pathotypes from cases vs. controls. These results disagree with other studies that found higher frequency of resistance to antibiotic in diarrheal samples than in controls (34) (17). This higher rates of antibiotic resistant in both groups are probably explain to higher rates of resistance in the region. Prior studies evidence a significant association between antibiotics resistance (ampicillin and sulfamethoxazole-trimethoprim) and lack of remoteness, close villages have higher prevalence than far villages, probably explained by an easy access of antibiotics and previous consumption of it. Moreover,

higher rates of antibiotic resistance organisms have been reported in sites with greater anthropogenic influence like an urban scenario (35) (36).

Finally, DAEC exhibit higher resistance levels (more resistance in 9 of 12 antibiotics) than EPEC, ETEC and EAEC, in agreement with Ochoa, *et al.* 2009, potentially explained by higher exposure of this pathotype to antimicrobials, due to asymptomatic carriers and longer persistence in the human host (34) (3) (22).

In conclusion, we found that DAEC was associated with diarrhea and it was the most prevalent pathotype, found in 26.12% in cases and 12.5% in controls. This results shows the importance of this pathotype in Ecuador.

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Authors', addresses: Maritza Páez, Gabriel Trueba, Pablo Endara. Microbiology Institute, Universidad San Francisco de Quito, Quito, Ecuador; William Cevallos, Centro de Biomedicina, Universidad Central del Ecuador, Quito, Ecuador; Karen Levy,

Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta.

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PART III

TABLES AND FIGURES

Table 1			
Demographic data of Esmeraldas study subjects			
Parameter	Case (n=111)	Control (n=112)	*P-value
Age(year)			
Mean(SD)	12.24 (17.52)	12.14 (16.90)	0.575
Age categories			
<1year	17 (15.32%)	11 (9.82%)	
1-15year	66 (59.46%)	74 (66.07%)	
16-30year	16 (14.41%)	9 (8.04%)	
>30year	12 (10.81%)	18 (16.07%)	0.179
Gender			
Male	68 (61.26%)	57 (50.89%)	
Female	43 (38.74%)	55 (49.11%)	0.119
Sanitation at home			
Flush toilet	47 (42.34%)	51 (45.54%)	
Diaper	30 (27.03%)	35 (31.25%)	
Latrine*	7 (6.31%)	3 (2.68%)	
Septic tank	22 (19.82%)	22 (19.64%)	
Without registration	5 (4.50%)	0 (0%)	
Community latrine and septic tank	0 (0%)	1 (0.84%)	0.148
Reported home water treatment			
No	75 (67.57%)	60 (53.57%)	
Yes	34 (30.63%)	52 (46.43%)	
Unknown	2 (1.80%)	0 (0%)	**0.024**
Reported recent contact with animals			
No	55 (49.55%)	66 (58.93%)	
Yes	55 (49.55%)	46 (41.07%)	
Unknown	1 (0.90%)	0 (0%)	0.247
Reported travel in the last year			
No	64 (57.66%)	84 (75.00%)	
Yes	47 (42.34%)	28 (25.00%)	**0.006**

*Chi square test was used to the comparison between cases and controls ($p \leq 0.05$)

Table 2				
Frequency and percentage of diarrheagenic *E. coli* and association with clinical disease (odds ratio; OR) in people from Esmeraldas				
	Case (n=111)	Control (n=112)	OR(95%CI)	*P-value
All pathotypes	52 (46.84%)	32 (28.57%)	**2.20(1.22-3.98)**	**0.004**
Enterotoxigenic Escherichia coli	4 (3.60%)	5 (4.46%)	0.80(0.15-3.83)	0.744
Diffusely adherent Escherichia coli	29 (26.12%)	14 (12.5%)	**2.47(1.16-5.40)**	**0.009**
Enteroaggregative Escherichia coli	7 (6.30%)	8 (7.14%)	0.87(0.25-2.87)	0.80
Enteroinvasive Escherichia coli	2 (1.80%)	0 (0.0%)	NA	NA
Atypical Enteropathogenic Escherichia coli	9 (8.10%)	5 (4.46%)	1.88(0.54-7.40)	0.262
Typical Enteropathogenic Escherichia coli	1 (0.90%)	0 (0.0%)	NA	NA

*Chi square test was used to the comparison between cases and controls (p≤0.05)

Table 3				
Frequency and percentage of co-infections and association with clinical disease (odds ratio; OR) in people from Esmeraldas				
	Case (n=111)	Control (n=112)	OR(95%CI)	*P-value
Total of Co-infections	7 (6.30%)	5 (4.46%)	1.44 (0.37-5.93)	0.542
Co-infections with 2 Pathotypes	6 (5.40%)	4 (3.57%)	1.54 (0.35-7.63)	0.508
Co-infections with 3 Pathotypes	1 (0.90%)	1 (0.89%)	1.00 (0.01-79.91)	0.994

*Chi square test was used to the comparison between cases and controls (p≤0.05)

Table 4		
Co-infections in cases and controls classified by age and gender in Esmeraldas city		
Total of Co-infections	Case (n=7)	Control (n=5)
Age		
Children	7 (100%)	4 (80%)
Adults	0 (0.0%)	1 (20%)
Gender		
Male	4 (57.14%)	1 (20%)
Female	3 (42.85%)	4 (80%)

Table 5

Association between risk factors and presence of *E. coli* pathotypes (cases, n=52) and (controls, n=32) in people from Esmeraldas city

Travel in the last year	**DAEC, *n* (%)**			
	Case (*n*=29)	Control (*n*=14)	OR (95%CI)	*P-value
	12(41.38)	5(35.71)	1.270 (0.28-6.0)	0.721
	EPEC •, *n* (%)			
	Case (*n*=10)	Control (*n*=5)	OR (95%CI)	*P-value
	3(30.0)	3(60.0)	0.285(0.016-4.29)	0.263
	ETEC, *n* (%)			
	Case (*n*=4)	Control (*n*=5)	OR (95%CI)	*P-value
	2(50.0)	1(20.0)	4(0.11-293.82)	0.348
	EAEC, *n* (%)			
	Case (*n*=7)	Control (*n*=8)	OR (95%CI)	*P-value
	3(42.85)	1(12.5)	5.25(0.26-314.08)	0.184
	Any Pathotype, *n* (%)			
	Case (*n*=52)	Control (*n*=32)	OR (95%CI)	*P-value
	22(42.30)	10(31.25)	1.61(0.58-4.60)	0.310
Home water treatment	**DAEC, *n* (%)**			
	Case (*n*=29)	Control (*n*=14)	OR (95%CI)	*P-value
	9(31.03)	5(35.71)	0.81 (0.17-4.02)	0.758
	EPEC •, *n* (%)			
	Case (*n*=10)	Control (*n*=5)	OR (95%CI)	*P-value
	3(30.0)	2(40.0)	0.64(0.043-11.91)	0.698
	ETEC, *n* (%)			
	Case (*n*=4)	Control (*n*=5)	OR (95%CI)	*P-value
	2(50.0)	4(80.0)	0.25(0.003-9.077)	0.342
	EAEC, *n* (%)			
	Case (*n*=7)	Control (*n*=8)	OR (95%CI)	*P-value
	2(28.57)	4(50.0)	0.4(0.025-5.026)	0.398
	Any Pathotype, *n* (%)			
	Case (*n*=52)	Control (*n*=32)	OR (95%CI)	*P-value
	17(32.69)	15(46.87)	0.55(0.202-1.49)	0.193
Contact with animals	**DAEC, *n* (%)**			
	Case (*n*=29)	Control (*n*=14)	OR (95%CI)	*P-value
	12(41.38)	6(42.86)	0.94(0.21-4.23)	0.926
	EPEC •, *n* (%)			
	Case (*n*=10)	Control (*n*=5)	OR (95%CI)	*P-value
	4(40.0)	3(60.0)	0.44(0.026-6.23)	0.464
	ETEC, *n* (%)			
	Case (*n*=4)	Control (*n*=5)	OR (95%CI)	*P-value
	2(50.0)	2(40.0)	1.5(0.054-39.79)	0.764
	EAEC, *n* (%)			
	Case (*n*=7)	Control (*n*=8)	OR (95%CI)	*P-value
	3(42.85)	2(25.0)	2.25(0.16-37.19)	0.464
	Any Pathotype, *n* (%)			
	Case (*n*=52)	Control (*n*=32)	OR (95%CI)	*P-value
	23(44.23)	13(40.62)	1.15(0.43-3.12)	0.745

Data on EIEC are not presented; due to small number of samples

*Chi square test was used to the comparison between cases and controls (p≤0.05)

• Isolates of Typical EPEC and atypical EPEC

Table 6

Frequency and percentage of clinical antibiotic resistance in diarrheagenic *E. coli* isolates (cases, n=52) and (controls, n=32) from Esmeraldas city

ANTIBIOTICS	Case *n* (%)	Control *n* (%)	OR(95%CI)	*P-value
Ampicillin	38(73.08%)	26(81.25%)	0.62(0.17-2.03)	0.393
Amoxicillin-clavulanic acid	5(9.62%)	3(9.38%)	1.02(0.18-7.11)	0.970
Cefotaxime	2(3.85%)	4(12.5%)	0.28(0.02-2.12)	0.134
Cephalothin	24(46.15%)	17(53.13%)	0.75(0.28-2.00)	0.534
Chloramphenicol	11(21.15%)	4(12.50%)	1.87(0.48-8.85)	0.314
Ciprofloxacin	3(5.77%)	2(6.25%)	0.91(0.09-11.6)	0.927
Sulfamethoxazole-trimethoprim	38(73.08%)	24(75%)	0.90(0.28-2.73)	0.84
Gentamicin	4(7.69%)	3(9.38%)	0.80(0.12-5.90)	0.786
Streptomycin	33(63.46%)	19(59.38%)	1.18(0.43-3.21)	0.708
Tetracycline	31(59.62%)	21(65.63%)	0.77(0.27-2.11)	0.581
Imipenem	0(0%)	0(0%)	NA	NA
Sulfisoxazole	40(76.92%)	27(84.38%)	0.61(0.15-2.16)	0.409
Multiresistance •	39(75%)	26(81.25%)	0.69(0.19-2.27)	0.506

*Chi square test was used to the comparison between cases and controls ($p \leq 0.05$)

• More than 3 antibiotics is resistant

Table 7•								
Clinical antibiotic resistance among the different diarrheagenic *E. coli* in isolates from cases (n=52) and controls (n=32) in Esmeraldas city								
	DAEC, *n*(%)		**EPEC*, *n*(%)**		**ETEC, *n*(%)**		**EAEC, *n*(%)**	
ANTIBIOTICS	**CASE(*n*=29)**	**CONTROL(*n*=14)**	**CASE(*n*=10)**	**CONTROL(*n*=5)**	**CASE(*n*=4)**	**CONTROL(*n*=5)**	**CASE(*n*=7)**	**CONTROL(*n*=8)**
AM	24(82.76)	13(92.86)	7(70.00)	3(60.00)	2(50.00)	3(60.00)	4(57.14)	7(87.50)
AmC	3(10.34)	0(0)	1(10.00)	1(20.00)	0(0)	1(20.00)	1(14.29)	1(12.50)
CTX	2(6.90)	3(21.43)	0(0)	1(20.00)	0(0)	0(0)	0(0)	0(0)
CF	15(51.72)	9(64.29)	5(50.00)	2(40.00)	2(50.00)	3(60.00)	2(28.57)	3(37.50)
C	5(17.24)	1(7.49)	5(50.00)	0(0)	0(0)	1(20.00)	1(14.29)	2(25.00)
CIP	2(6.90)	2(14.29)	1(10.00)	0(0)	0(0)	0(0)	0(0)	0(0)
SXT	25(86.21)	13(92.86)	7(70.00)	3(60.00)	2(50.00)	4(80.00)	3(42.86)	4(50.00)
CN	3(10.34)	3(21.43)	1(10.00)	0(0)	0(0)	0(0)	0(0)	0(0)
S	22(75.86)	13(92.86)	6(60.00)	1(20.00)	0(0)	1(20.00)	4(57.14)	4(50.00)
Te	23(79.31)	9(64.29)	5(50.00)	3(60.00)	2(50.00)	5(100.00)	1(14.29)	4(50.00)
IPM	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
G	25(86.21)	14(100)	7(70.00)	3(60.00)	2(50.00)	4(80.00)	4(57.14)	6(75.00)
Multiresistance	25(86.21)	14(100)	7(70.00)	3(60.00)	2(50.00)	4(80.00)	4(57.14)	5(62.50)

*Isolates of Typical EPEC and atypical EPEC together, due the small amount of Typical EPEC
Data on EIEC are not presented; due to small number of samples
• No statistically significant differences were detected between cases and controls for any of the antibiotics and pathotypes tested, by Fisher exact test.

PART IV

ANNEXES

Table 8				
Risk factors associated with the presence of pathotypes in case (n=52) and control (n=32) study population				
Factors	Case (n=52)	Control (n=32)	OR, CI 95%	P-value
Age (children)	41	26	0.86(0.23-2.92)	0.79
Gender male	37	15	2.79(1.01-7.73)	**0.02**
Medical use	22	6	3.17(1.02-10.94)	**0.02**
Trip in the last year	22	10	0.61(0.58-4.60)	0.31
Trip in the last week	4	1	2.58(0.23-131.24)	0.39
Use flush toilet	18	13	0.77(0.28-2.12)	0.57
Use diaper	17	13	0.70(0.25-1.96)	0.46
Use latrine	3	1	1.89(0.14-102.79)	0.58
Use of water purchased	14	14	0.47(0.16-1.32)	0.11
Internal water supply at home	20	13	0.91(0.33-2.48)	0.84
Water treatment before consumption	17	15	0.55(0.20-1.49)	0.19
Boil the water	17	15	0.55(0.20-1.49)	0.19
Contact with animals	23	23	1.15(0.43-3.12)	0.74
Presence of diarrheic parasites*	5	6	0.39(0.085-1.80)	0.155
Presence of rotavirus •	8	0	NA	NA

* Parasites could not be identified in 7 cases and 7 controls

• Rotavirus could not be identified in 1 cases and 2 controls

Chi square test was used to the comparison between cases and controls ($p \leq 0.05$)