UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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***Escherichia coli* pathotypes associated with diarrhea in Borbon- Ecuador and antibiotic resistance.**

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DEDICATORIA

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SCIENTIFIC PAPER I:

***Escherichia coli* pathotypes associated with diarrhea in Borbon- Ecuador**

ABSTRACT

Diarrheagenic *E. coli* (DEC) are the important etiological agent of diarrhea caused by bacteria in children living in developing countries, and are an important problem of public health in Ecuador. Due to their heterogenic distribution according to zone and the studied time, it is critical to monitor the presence of different pathotypes and their association with cases of diarrhea, especially in rural areas. A case-control study using fecal samples of 501 individuals (256 cases and 245 controls) from which *E. coli* strains where isolated and pathotype specific genes were amplified. We found 141 pathotype strains; 127 samples were positive for one pathotype: 59 (24.1%) belonged to asymptomatic individuals and 68 (26.6%) to cases. Fourteen stool samples presented 2 pathotypes; 12 (4.69%) were from cases and 2 (0.82%) from controls (OR = 6.48, IC del 95%: 1.39-30.21, P = 0.017). Enterotoxigenic *E. coli* was the only pathotype associated with diarrhea (OR ajusted 2.35; IC95%=1.03-5.38; P=0.042). More frequency of enteropathogenic *E. coli* was also found; 25(10.2%) in controls and 23 (9%) in cases, but this pathotype was not associated with diarrhea.

Keywords: *E. coli* pathotype, case-control study, Borbón-Ecuador, co-infections.

RESUMEN

Diarrheagenic *E. coli* (DEC) es un importante agente etiológico de la diarrea causada por bacterias en los países en desarrollo, que afecta principalmente a los niños. Por lo tanto, este grupo de patógenos intestinales representa un problema importante de salud pública en Ecuador. Debido a su distribución heterogénea según la zona y el tiempo de estudio, es indispensable determinar la presencia de patotipos de *E. coli* y su asociación con los casos de diarrea, especialmente en las zonas rurales. Se llevo a cabo un estudio de casos y controles a partir de muestras fecales de 501 individuos (256 casos y 245 controles) de los cuales se obtuvieron cepas correspondientes a *E. coli* que fueron sometidas a amplificación para genes específicos de 7 diferentes patotipos de *E.coli*. Obtuvimos 141 cepas positivas: 59 (24.1%) pertenecientes a controles y 68 (26.6%) a casos. La mayoría de las muestras (n=127) fueron positivas a un patotipo mientras que 14 muestras presentaron diferentes colonias con patotipos distintos; 12 (4.69%) were from cases and 2 (0.82%) from controls (OR = 6.48, IC del 95%: 1.39-30.21, P = 0.017). *E. coli* enterotoxigénica (ETEC) fue el único patotipo que mostró una asociación significativa con la diarrea (OR ajustado 2.35; IC95% = 1.03-5.38; P= 0.042). También se encontró mayor frecuencia de *E. coli* enteropatógena; 25 (10.2%) en controles y 23 (9%) en casos pero este patotipo no estuvo asociado a diarrea.

Palabras clave: patotipo de *E. coli,* estudio de casos y controles, Borbón-Ecuador, coinfecciones.

SCIENTIFIC PAPER I: *ESCHERICHIA COLI* PATHOTYPES ASSOCIATED WITH DIARRHEA IN BORBON- ECUADOR.

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INTRODUCTION

Diarrheal diseases cause significant morbidity and mortality principally in infants and children in developing countries (Bryce *et al.,* 2005; Boschi-Pinto *et al.,* 2008). Some of the important etiological agents of these diseases are diarrheagenic *E. coli* (DEC) (pathovars or pathotypes) which include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffuse adherent *E. coli* (DAEC), *E. coli* Shigellae (*Shigella)* and enterohemorrhagic *E. coli* (EHEC) (Beauchamp & Sofos, 2010; Okeke, 2009), each one expresses different virulence factors (Croxen *et al.,* 2013).

ETEC may present 1 or 2 enterotoxins: thermolabile toxin (LT) and thermostable toxin (ST) which activate molecular pumps in enterocytes which eliminate water and ions (Ma, 2016; Pelkonen *et al.,* 2017). LT and ST are located in a plasmid which also codes for CFA (colonization factor antigens) a pili or fimbriae allowing the adherence to enterocytes. The majority of human CFAs are encoded on mobile elements such as transposable elements ISs (Insertion Sequences) and plasmids (Johnson & Nolan, 2009).

ETEC is transmitted mainly by water and food contamination and affects mostly infants (under two years), suggesting that a protective immune response occurs with age (Qadri *et al.,* 2005). Enterohemorrhagic *E. coli* or EHEC produces the Stxs cytotoxins *(*genes variants *stx*1 y *stx*2) from *stx* phages inserted in its genome (Gouali *et al.,* 2013; Torres *et al.,* 2005). Stx toxin attaches to the 60S subunit of the ribosomes in the intestinal or endothelial cells of the host, blocking the protein synthesis (Gouali *et al.,* 2013). Some EHECs also display

attaching and effacing ability coded by the chromosomal gene *eae* (also present in EPEC strains) which codes for the external membrane intimine protein (Campellone, 2010; Franzin & Sircili, 2015; Caprioli *et al.,* 2005). Enteroinvasive *E. coli* or EIEC invades colonic epithelial cells (Casalino *et al.,* 2003); EIEC also shows the ability to invade M-cells, macrophages, epithelial cells and production of enterotoxins (Campilongo *et al.,* 2014); EIEC and *E. coli* Shigellae are biochemically and genetically related (Lan *et al.,* 2001). Enteropathogenic *E. coli* or EPEC exhibits as main pathogenicity factor the intimate adherence (bacteria and cell membrane of intestinal epithelium cells); this pathotype uses a type III secretion system (T3SS: type III secretion system), encoded in the pathogenicity island LEE (locus of enterocyte effacement), for translocation of intracellular signals that allows the entry of various effector proteins into the enterocyte (Daniell *et al.,* 2001), and induces actin polymerization which destroys microvilli, this mechanism is known as attaching and effacing (A/E) (Hernandes *et al.,* 2009). The adherence is mediated by pili called BFP (bundle-forming pilus) coded in a plasmid: EAF (EPEC adherence factor de 50-70MDa) and some chromosomal genes (Bakhshi *et al.,* 2013). The EPEC strains are considered typical when they exhibit: *eae* genes (intimin) which is involved in A/E and the plasmid EAF which codes for *bfp* genes; on the other hand they are considered atypical when they present only *eae* genes and not the EAF plasmid (Croxen *et al.,* 2013). EPEC is associated with cases of diarrhea in infants (children between six months and two years), it can cause outbreaks or isolated cases of diarrhea (Croxen *et al.,* 2013, Bakhshi *et al.,* 2013), it can be also found in healthy and sick adults, mainly when there is a predisposing factor such as diabetes (Bakhshi *et al.,* 2013). Enteroaggregative *E. coli* or EAEC shows several pathogenicity mechanisms, in addition it has the capacity to increase the mucus production and secretion that traps the

bacteria allowing its autoagglutination in a film on top of the intestinal epithelium increasing the persistency in the intestine causing lengthy diarrhea (Nishi *et al.,* 2003). Adherence and hemo-agglutination of erythrocyte is due to the presence of an aggregative adherence (AAF/I) fimbria (fimbria I), codes by the *agg*A gene that is in a plasmid (60 MDa), it contains genes that code for the EASTI toxin. Furthermore, the fimbria AAF/II has been described immunologically different from the first one, coded by the *aaf*A gene; nonetheless, not all EAEC exhibit these fimbriae (Dudley *et al.,* 2006). EAEC can cause outbreaks or isolated cases of persistent diarrhea. In children, it can be severe and require intravenous rehydration (Nishi *et al.,* 2003; Dudley *et al.,* 2006). Diffuse Adherent *E. coli* or DAEC exhibits a diffuse adherence mechanism through a superficial fimbria (F1845), it can be coded by a chromosomal or plasmid gene (le Bouguénec & Servin, 2006). The diffuse adherence phenomenon has been associated with an external membrane protein, in a strain 0126:H27 serotype, whose genes have been found in a minority of isolations (Shazberg *et al.,* 2003). Moreover, DAEC has the capacity to induce the formation of protruding structures that confer protection to bacteria, however, these structures have not been demonstrated *in vivo* (Prorok-Hamon *et al.,* 2014).

There are several risk factors associated with transmission and frequency of diarrheagenic *E. coli* infections such as lack of hygiene, poverty, malnutrition and poor sanitary infrastructure (Kaper *et al.,* 2004), which may partially explain variation in incidence and morbidity of *E. coli*'s pathotypes in time and space, in rural and urban communities in Ecuador (Vieira *et al.,* 2007; Bayas-Rea *et al.,* 2011; Bhavnani *et al.,* 2012; Vasco *et al.,* 2014). We studied the

prevalence of pathotypes of *E. coli* in rural communities of northern coastal Ecuador, a region deficient in health services and sanitary infrastructure.

MATERIALS AND METHODS

Human subjects and study design:

A case-control study was conducted, in which participants from 22 communities located in northern Coastal Ecuador: Sampling was performed through the Borbon Hospital or through ministry of health visits to the outlying communities. Previous studies in Borbon city presented higher number of pathotypes, possibly due to the concentration of people (commercial capital of the region) (Eisenberg *et al.,* 2006).

The “cases” were defined as those patients that attended the Hospital or ministry of health clinic visits, exhibiting acute diarrhea (three or more loose stools in a 24-hour period) and those people showing signs of diarrhea during home visits of the field team. Controls were patients that attended the hospital for other reasons different from diarrhea and did not showed diarrheic symptoms at least seven days before taking the sample. Demographic data from the patient were also registered (age, gender, sanitation, water consumption, contact with animals, trips during the last year, etc.) using electronic devices and the Open Data Kit software.

Borbon groups were designated as follows:

Borbon Hospital: these individuals were recruited directly from Borbon Hospital matching diarrheal cases with controls from the hospital (Population: Borbon hospital).

Therefore the **Borbon community** individuals were recruited by health care workers in the community (not from hospital admissions), because the recruitment of diarrheal cases in hospital was not sufficient, we sent health care workers to the field to recruit more participants. (Population: Borbon Community).

Borbon-Borbon Hospital and Borbon community groups were kept separate due to the potential bias associated with the severity of illness between those presenting to the hospital and those recruited at the community level who did not seek out formal medical attention for their symptoms. Finally, **Borbon river communities:** these individuals were recruited at the Borbon Hospital, however they actually live in the river communities and traveled to the hospital for medical attention. The Rios participants recruited from the MoH traveling clinic were combined with these Rios participants recruited at Borbon Hospital (Population: Rios).

Individuals from all ages could participate in the study, cases and controls were, for statistical analysis, paired in categories: 0-24 months, 25- 60 months, 61-180 months, and greater than 181 months. Before the inscription all participants signed a document of consent approved by the Institutional Review Board of Emory University and Universidad San Francisco de Quito. The Ministry of Health also approved of the study.

Individuals that reported to have taken antibiotics less than a week before taking the sample or that they have not lived in Borbon at least for six months were excluded from the study.

Bacterial identification, DNA extraction and PCR analysis:

Fecal samples were grown in MacConkey's lactose agar media (MKL), and then incubated at 37°C for 24 hours, once that colonies were obtained 5 lactose positive CFU (colony forming units) were randomly selected and non-lactose fermenting colony were also collected. Colonies were transferred to a Chromocult agar media (Merck, Darmsladt, Alemania) (CC) to determine β-glucoronidase activity (MUG); each of the colonies were cultured in nutrient agar (AN) and were frozen in Brain Heart Infusion (BHI) + 20 % glycerol broth (Belmonte *et al.,* 2009).

For DNA extraction 5 or 6 colonies from the same fecal sample were pooled together in a tube with 300 µl of sterile distilled water (colony pool) and boiled for 10 min to release the DNA, these tubes were centrifuged at 1.217 X *g*. for 1 min and the supernatant (DNA) was used in a polymerase chain reaction (PCR) for detection of the different pathotypes of *E. coli*. (Jin *et al.,* 2008; Seni, 2015).

Detection of *E. coli* pathotypes.

If the colony pool test was positive for any pathotype gene by PCR, each of the colonies (comprasing the pool) was grown separately in nutrient agar (from the colonies frozen in -80 and tested individually for each of the pathotypes (Jin *et al.,* 2008; Seni, 2015).

The target genes used to detect each pathotype were: *bfp* for typical EPEC; *lt* and *sta* for ETEC; *ipaH* for EIEC and *Shigella* (differentiation between *Shigella* and EIEC was done with API 20E gallery: BioMérieux, Marcy l'Etoile, France); *aggR* for EAEC; *afa* for DAEC, *eaeA* for

atypical EPEC, and *stx1* and *stx2* genes for detection of enterohemorrhagic *E. coli* (EHEC), for this last pathotype only colonies with positive results to *eaeA* were evaluated. If the colony pool from the sample (5 or 6 colonies from a sample) prove positive for any pathotype, PCR was conducted separately for each isolated colony. *E. coli* 25922 American Type Culture Collection (ATCC), was used as negative control.

For *ipaH*, *lt* and *bfp* genes PCR the protocol was carried out in a 25 µL mixture containing: 1x PCR Buffer; 1.5 mM MgCl2; 0.02 U GoTaq-DNA polimerase; 200 µM dNTPs; 0.2 µM (forward and reverse primers) and 2.5 µL of DNA suspension (Table 6). PCR amplification for *ipaH*, *lt* and *bfp* genes consisted of: initial denaturation at 94ºC (5:00 min), denaturation at 94°C (1:00), annealing at 56 ºC (2:00 min) and extention at 72°C (1:00 min) for 29 cycles (Table 7) (Toma *et al.,* 2003; Vieira *et al.,* 2007).

For *sta* gene PCR the protocol was carried out in a 25 µL mixture containing: 1x PCR Buffer; 1.5 mM MgCl2; 0.02 U GoTaq-DNA polimerase; 200 µM dNTPs; 0.2 µM (forward and reverse primers) and 2.5 µL of DNA suspension (Table 6). PCR amplification for *st* gene consisted of: initial denaturation at 94ºC (5:00 min), denaturation at 94°C (1:00), annealing at 57.3ºC (2:00 min), extention at 72°C (1:00 min) for 29 cycles and final elongation at 72°C (1:00 min) (Table 7) (Toma *et al.,* 2003; Vieira *et al.,* 2007).

For the *aggR* gene, PCR the protocol was carried out with 10 µl mixture containing: 1X PCR Buffer; 2 mM MgCl2; 0.02 U Go Taq DNA polimerase; 200 µM dNTPs; 0.4 µM (forward and reverse primers) and 3 µL of DNA (Table 6); PCR amplification for *aggR* gene consisted of:

initial denaturation at 94°C (5:00 min), denaturation at 94°C (0:30 min), annealing at 50 °C (1:00 min), extension at 72°C (1:30 min) for 24 cycles and final elongation at 72°C (5:00) (Table 7) (Vieira *et al.,* 2007).

For *eaeA* gene, PCR the protocol was carried out with 25 µL mixture containing: 1X PCR Buffer; 2 mM $MgCl_2$; 0.02 U GoTaq-DNA polimerase; 200 µM dNTPs; 0.25 µM forward and reverse primers, and 1.5 µL of DNA (Table 6). PCR amplification for *eaeA* consisted of: denaturation at 95ºC (1:00 min); annealing at 65ºC (2:00 min) and elongation at 72ºC (1:30 min) for 10 cycles; denaturation at 95ºC (1:00 min); annealing at 60ºC (2:00 min) and elongation at 72ºC (1:30 min) for 15 cycles; denaturation at 95ºC (0:30 min); annealing at 60ºC (2:00 min) and elongation at 72ºC (2:30 min) for 10 cycles (Table 7). (Paton & Paton, 1998).

For *stx1* and *stx2* genes, PCR the protocol was carried out with 25 µL mixture containing: 1X PCR Buffer; 1.5 mM of MgCl2; 0.02 U GoTaq-DNA polimerase; 200 µM dNTPs; 1 µM forward and reverse primers and 1.5 µL of DNA (Table 6). PCR amplification for *stx1* and *stx2* consisted of: initial denaturation at 94ºC (5:00 min); denaturation at 94ºC (2:00 min) annealing at 58ºC (1:00 min) and extention at 72ºC (1:00 min) for 29 cycles (Table 7). (Paton & Paton, 1998).

Finally, for *afa* gene*,* PCR the protocol was carried out with 25 µL mixture containing: 1X PCR Buffer; 1.5 mM $MgCl_2$; 0.02 U GoTaq-DNA polymerase; 200 µM dNTPs; 0.2 µM (forward and reverse primers) and 2.5 µL of DNA (Table 6). PCR amplifications consisted of: denaturation

at 94ºC (2:00 min), annealing at 65ºC (1:00 min), and extension at 72ºC (2:00 min) for 24 cycles (Table 7) (Le Bougunec *et al.,* 1992).

Electrophoresis was performed in 1.5% agarose gel, prepared with ethidium bromide. The expected sizes of each gene were: *aggR* (254 pb), *lt* (708 pb), *sta* (182 pb), *bfp* (324 pb), *eaeA* (384 pb), *ipaH* (424 pb), *afa* (750), *stx1* (180 pb) and *stx2* (255 pb) (Le Bougunec *et al.,* 1992). A positive control (positive for each gene) and two negative controls consisting of *E.coli* K12 and no DNA were carried out.

Statistical analysis:

Comparisons between cases and controls were made using Chi-square. The association between presence of *E. coli* pathotypes and diarrhea were calculated using odds ratio (OR) and adjusting for confounding variable susing StataMP 13 (StataCorp. LP, College Station, TX). Results were considering as statistically significant if p-value ≤ 0.05.

RESULTS

501 individuals were analyzed (256 cases and 245 controls), obtained from three different sites in Borbón (Borbon Hospital, Borbon community, and Borbon river communities) (Table1). Of all the demographic aspects evaluated in this study no showed significant difference was found in Borbon (Table 1). Moreover, the analysis by sectors indicated a significant difference in the analyzed sample for: Reported recent contact with animals in "Borbon Hospital" (p= 0.013) because most controls had no contact with animals and in

most cases they had contact with animals; finally sanitation at home for the sector “Borbon community” (p=0.025) varied in cases (greater use of latrine and diaper) and in controls (greater use of septic tank) (Table 1.1-1.3).

Pathotypes of *E. coli* were analyzed in 501 samples (256 cases and 245 controls) which were stratified according to their locations into: Borbon Hospital (111 cases and 107 controls), Borbon community (55 cases and 49 controls), and Borbon river communities (79 cases and 100 controls) (Table 1). Seven *E. coli* pathotypes were investigated, EPEC was found at higher frequency (n= 48 (9.58%); 25 in controls (52.08%) and 23 in cases (47.92%)) most of the EPEC (95.83%) were atypical EPEC (positive for the gene *eaeA* and negative for *bfp*); Enterotoxigenic *E. coli* was the only pathotype associated with diarrhea (OR ajusted 2.35, IC95%=1.03-5.38; P=0.042). Other pathotypes such as DAEC, EIEC and EAEC were also found but in low frequency (table 2).

Pathotypes of *E. coli*:

From 501 study subjects, 141 strains were positive for diarrheagenic *Escherichia coli* (DEC), 127 strains were pathotypes; 59 (24.1%) belonged to controls and 68 (26.6%) to cases (Table 2).

In Borbon, we found significant association of ETEC infection and diarrhea; 29 positives distributed in 9 (3.7%) controls and 20 (7.8%) cases (OR ajusted 2.35 with IC95%=1.03-5.38; *p*-value=0.042) (table 2). No other statistically significant associations were found betweem other pathotypes and presence of diarrhea.

Differences were found between the analyzed groups of Borbon: DEC diarrheagenic *Escherichia coli* (DEC) were significant association in Borbon Hospital (OR ajusted 2.11 with IC95%= 1.06-4.19; *p-value*= 0.033) and we found significant association of ETEC infection and diarrhea (OR ajusted 14.21 with IC95%= 1.66-121.3; *p-value*= 0.015). In Borbon community and Borbon river communities no statistically significant associations were found betweem other pathotypes and presence of diarrhea.

The most prevalent pathotype was EPEC with 48 positives (46 were aEPEC), of which 25 belonged to the control group (52.08%) and the remaining 23 (47.92%) to cases, followed by DAEC with 30 positives 12 (4.9%) controls and 18 (7%) cases; 23 EAEC positives 12 (4.9%) in controls and 11 (4.3%) in cases. Other pahtoypes detected at lower frequencies were EIEC with 8 positives, 2 (0.82%) in controls and 6 (2.3%) in cases, and 3 positives for EHEC (gene stx1) 2 (0.82%) in controls and 1 (0.4) in cases, none of which presented statistical significance to cases of diarrhea in Borbón (Table 2). There was no significative difference between location of the sample and outcome or frequency of any pathotype. (Table 2.1-2.3).

Additionally 14 strains had genes belonging to 2 different pathotypes 12 (4.69%) found in cases and 2 (0.82%) in controls showing statistically significant association with diarrhea (OR = 6.48, CI of 95%: 1.39-30.21, P = 0.017) (Table 4). These strains corresponded to the following pathotypes: 4 positive samples (28.57%) to DAEC and aEPEC pathotypes (genes: *afa* and *eaeA*) ; 3 positive samples (21.43%) to ETEC and ETEC pathotypes (2 samples with gene: *lt* and *sta,* 1 sample with gene *lt* and *st-lt*); 3 positive samples (21.43%) to EPEC and EAEA pathotypes (genes: *eaeA* and *aggR*); 1 positive sample were presented with ETEC and EIEC pathotypes (genes: *lt* and *ipaH*); 1 positive sample EIEC and EAEC pathotypes (genes:

ipaH and *aggR*), 1 positive sample DAEC and ETEC pathotypes (genes: *lt* and *afa*) and 1 positive sample ETEC and EAEC pathotypes (genes: *lt* and *aggR*) (Table 4).

Moreover, in this study we found 3 colonies (from different patients) whith genes belonging to two pathotypes, 2 isolates had EPEC and DAEC genes (*eaeA* and *afa*), and 1 isolate had EPEC and EAEC genes (*eaeA* and *aggR*).

DISCUSSION

Among the age categories a difference could be shown, with the majority of samples collected in children from 0-5 years (0-60 months) of age in cases as well as in controls (controls: 50.61%; cases: 51,17%) (Table 1). This age can be a factor of vulnerability to the infection with pathotypes of E. coli (Qadri et al., 2005). Consistantly, several studies register pathotypes causing diarrhea principally in children younger than 5 years (Croxen et al., 2013, Bakhshi et al., 2013). Therefore, for the present study the number of cases and controls at that age were considered to be ideal. However, the demographic analysis for each sector of study no showed significant differences in age category.

In addition, there were differences according to each sector indicate changes in the habits of sanitation and differences in the manners between areas which might have an influence in the transmission of *E. coli* pathotypes for each sector unless they all belong to the same region of “Borbón” (Croxen *et al.,* 2013). Concerning the contact to animals, data indicates a

different treatment and usage of domestic animals which could give rise to the transmission of bacterial species, virus or parasites causing diarrhea (UNICEF/WHO, 2009).

In Borbon, the present study found that ETEC carrying ST was significantly associated with clinical disease. Previous studies indicated that ETEC is present in Ecuadorian urban and rural communities. ETEC is also endemic in Latin America and the Caribean (Gomez-Duarte *et al.,* 2010; Paniagua *et al.,* 2007; Estrada-Garcia *et al.,* 2009; Regua-Mangia *et al.,* 2004).

The current finding is in agreement with recent reports indicating that ETEC is one of the main etiologic agents causing diarrhea in developing countries and accounting for 1.5 million deaths annually, together with: Rotavirus, *Vibrio cholerae* and *Shigella* spp. (von Mentzer *et al.,* 2014; Kotloff *et al.,* 2013; Platts-Mills *et al.,* 2015). Although ETEC previously was also associated with diarrhea during some years, other pathotypes (and not ETEC) caused were associated to clinical disease in other periods of time (Bhavnani *et al.,* 2016; Vasco *et al.,* 2014; Vieira *et al.,* 2007). This study corroborate previous reports indicating that pathotypes responsible for diarrhea tend to vary overtime in a region (Bhavnani *et al.,* 2016); this finding contradicts GEMS (Global Enteric MulticentreStudy) recommendations which indicate that efforts to treat diarrheic diseases should be focused in few pathogens such as ETEC, *Cryptosporidium* and *Shigella* (Kotloff *et al.,* 2013).

In this study the ST toxin gene was more common than LT gene and also ST is more frequently associated with diarrhea (Qadri *et al.,* 2005). The ETEC isolates were isolated mainly from children ages 0 to 5 (70%) as well as in controls (77.8%) coinciding with other studies in Latin America where rates of up to 20% in symptomatic and asymptomatic

children were reported (Rodas *et al.,* 2011; Ochoa *et al.,* 2009). We found 3 cases presented colony positive for ETEC with *lt* and *sta* genes.

Another important result of the present study in Borbon was the high prevalence of aEPEC, however there was no association with diarrhea (Table 2, 2.1-2.3). Similar results have been found in Ecuador (Vasco *et al.,* 2014). Current studies undertaken in Brazil found aEPEC in rates which varied among 0.05-12% in patients with diarrhea versus 0-14% in healthy people (Gomes *et al.,* 2016). In Chile and Colombia aEPEC was not associated with clinical disease (Gomez-Duarte *et al.,* 2010; Assis *et al.,* 2014). Contrastingly, other repors showed aEPEC is clearly associated to diarrhea (Dias *et al.,* 2016; Franzolin *et al.,* 2005; Scaletsky *et al.,* 2010; Vidal *et al.,* 2005; Bakhshi *et al.,* 2013; Afset *et al.,* 2003; Nguyen *et al.,* 2006).

Concerning typical EPEC (tEPEC), we did not find association with clinical disease, resistance to this pathotype may be due to loss of specific receptors and immunity (Cieza *et al.,* 2012). Association of tEPEC with symptomatic patients has been found in developed countries (Alikhani *et al.,* 2006).

DAEC was found in 30 samples, 4.9% corresponded to controls and 7% to cases which indicate that DAEC was nos associated with diarrhea (Table 2). Additionlly, 61.11% of DAEC isolates originated in children ages 0 and 5 years (58.33% of which were controls). Other reports found that DAEC affects children between 2 and 5 years (Mansan-Almeida *et al.,* 2013). In contrast to the present study, in other regions of Ecuador and other countries in Latin America, DAEC has been associated with diarrhea (Scaletsky *et al.,* 2010; Mansan-Almeida *et al.,* 2013). (Paéz *et al.,* 2014; Montero *et al.,* 2016).

We found 23 EAEC positive samples: 4.9% controls and 4.3% cases. This pathogen is an emergent cause of diarrhea in the whole world (Rüttler *et al.,* 2002). Other studies in developing countries find association of EAEC with persistent diarrhea in developing and industrialized countries (Villaseca *et al.,* 2005*;* Pereira *et al.,* 2008; Araujo *et al.,* 2007; Huang *et al.,* 2004; Rüttler *et al.,* 2002). Nevertheless EAEC strains could very in virulence (Bernier *et al.,* 2002; Jenkins *et al.,* 2007; Nüesch-Inderbinen *et al.,* 2013; Dallman *et al.,* 2014).

Pathotypes detected at lower frequency were: EIEC in controls n=8 (0.82%) and cases n=6 (2.3%); STEC (gen *stx1*) control n=2 (0.82%) and cases in and n=1 (0.4%). In previous studies in Ecuador, EIEC was significantly associated with diseases and elevated prevalence in the same communities (Vieira *et al.,* 2007). In this study only one sample presented *E. coli* Shigellae (*Shigella*), nevertheless, *Shigella* is a pathotype has been associated with diarrhea and higher prevalences in this regions (Vasco *et al.,* 2014).

In this study fourteen stool samples presented genes belonging to 2 different pathotypes, the most frequent was DAEC and aEPEC; in others studies (Esmeraldas-Ecuador) these co-infections of DAEC and EPEC were registered predominantly in cases (Paéz *et al.,* 2014;), Several studies describe the importance of interactions between diarrheagenic *Escherichia coli* with others bacterial pathogens, virus or parasites in diarrheal illness (Gomez-Duarte *et al.,* 2010, Bhavnani *et al.,* 2012; Vasco *et al.,* 2014; Lima *et al.,* 2017).

In this study we found 3 colonies (from different patients) whith genes belonging to two pathotypes, 2 isolates had EPEC and DAEC genes (*eaeA* and *afa*), and 1 isolate had EPEC and EAEC genes (*eaeA* and *aggR*). These findings are in agreement with studies of Croxen and collaborators (2013), who showed that isolates which combine main characters of virulence

of different pathotypes are potentially more virulent. In the same way, Sidhu and colaborators (2013) assert that, the presence of a single or multiple virulence genes in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of virulence genes to cause disease in the host.

Differences were found between the analyzed groups of Borbon: DEC diarrheagenic *Escherichia coli* (DEC) were significant association in Borbon Hospital (OR ajusted 2.11 with IC95%= 1.06-4.19; *p-value*= 0.033) and we found significant association of ETEC infection and diarrhea (OR ajusted 14.21 with IC95%= 1.66-121.3; *p-value*= 0.015). In Borbon community and Borbon river communities no statistically significant associations were found betweem other pathotypes and presence of diarrhea. This difference between Borbon Hospital and other groups can be explained because the severity of illness between groups, those presenting to the hospital and those recruited at the community level who did not seek out formal medical attention for their symptoms. Another reason was the number of patients per group (lost statistical association strength). Also, we found these differences probably due to environmental factors and particular biological factors of the circulating pathotype (transmission, infectious dose, environmental tolerance, host immunity, etc) (Vasco *et al.,* 2014; Gomes *et al.,* 2016).

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TABLES

Table 1. Demographic data of Borbon, study subjects according to cases and controls.

Borbon	Control	Case	Total	P-value*
Number of individuals	245	256	501	
Gender				
Female	122 (49.8%)	111 (43.36%)	233 (46.51%)	0.153
Male	123(50.20%)	145 (56.64%)	268 (53.49%)	
Mean age (standard deviation) (rank)	137.2 (169.56) (0 -857)	178.73 (256.41) (0- 1186)	158.42 (219.07) (0- 1186)	0.464
Age categories (months)				
0-24	71 (28.98)	80 (31.25%)	151 (30.14%)	0.649
25-60	53 (21.63)	51 (19.92%)	104 (20.76%)	
61-180	50 (20.41)	43 (16.80%)	93 (18.56%)	
≥180	71 (28.98)	82 (32.03%)	153 (30.54%)	
Sanitation at home				
Latrine	32 (13.11%)	32 (12.50%)	64 (12.80%)	0.328
Septic tank	121 (49.59%)	108 (42.19%)	229 (45.80%)	
Field or hole	31 (12.70%)	49 (19.14%)	80 (16.00%)	

Diaper	57 (23.36%)	62 (24.22%)	119 (23.80%)	
Flush toilet	2 (0.82%)	4 (1.56%)	6 (1.20%)	
River	1 (0.41%)	1 (0.39%)	2 (0.40%)	
Reported home water treatment				
No	200 (81.97%)	204 (79.69%)	404 (80.80%)	0.571
yes	44 (18.03%)	52 (20.31%)	96 (19.20%)	
Reported recent contact with animals				
No	157 (64.34%)	156 (60.94%)	313 (62.60%)	0.518
yes	87 (35.66%)	99 (38.67%)	186 (37.29%)	
Unknown	0 (0%)	1 (0.39%)	1 (0.20%)	
Reported travel in the last year				
No	196 (80.33%)	201 (78.52%)	397 (79.40%)	0.659
Yes	48 (19.67%)	55 (21.48%)	103 (20.60%)	

Table 2. Frequency and percentage of diarrheagenic *E. coli* and association with clinical disease in people in Borbon Hospital, Borbon community and Borbon river communities.

BORBON	CONTROL (N=245)	CASE (N=256)	Fischer's exact test	raw OR (95%IC)	P-value	adjusted OR	P-value*
	N (%)	N (%)					
ETEC	9 (3.7)	20 (7.8)	**0.056**	2.2 (0.99-4.98)	**0.05**	2.35 (1.03-5.38)	**0.042***
lt[b]	6 (2.47)	8 (3.12)	0.45	1.6 (0.58-4.51	0.358	1.76 (0.61-5.1)	0.297
st[c]	3 (1.23)	8 (3.12)	**0.046**	3.22 (1.03-10.02)	**0.043**	3.51 (1.1-11.1)	**0.03***
st and *lt* [a]	0 (0.0)	4 (1.56)	0.89	1.34 (0.36-11.52)	0.86	1.44 (0.46-11.79)	0.85
DAEC	12 (4.9)	18 (7)	0.35	1.47 (0.69-3.12)	0.317	1.24 (0.57-2.71)	0.587
EAEC	12 (4.9)	11 (4.3)	0.83	0.87 (0.38-2.01)	0.748	0.93 (0.39-2.2)	0.870
EIEC[d]	2 (0.82)	6 (2.3)[d]	0.29	2.92 (0.58-14.59)	0.19	3.5 (0.7-18)	0.140
EPEC	25 (10.2)	23 (9)	0.65	0.87 (0.48-1.58)	0.64	0.99 (0.5-1.8)	0.98
atypical EPEC[e]	24 (9.8)	22 (8.6)	0.65	0.87 (0.48-1.58)	0.64	1.0 (0.54-1.9)	0.98
typical EPEC[f]	1 (0.4)	1 (0.4)	1	0.96 (0.06-15.38)	0.98	0.74 (0.04-14)	0.843
EHEC	2 (0.82)	1 (0.4)	0.62	0.48 (0.04-5.29)	0.55	0.56 (0.05-6.4)	0.64
TOTAL	59 (24.1)	68 (26.6)	0.54	1.14 (0.76-1.7)	0.524	1.2 (0.79-1.8)	0.38

[a] 20% of ETEC positives to both genes (*lt* and *st*) in cases; [b] 66.67% of ETEC in contrels and 40% in cases; [c] 33.33% of ETEC in controls and 40% in cases; [d] 1 isolate accounting for *E. coli* Shigellae (*Shigella);* * statistically significant: P-value ≤ 0.05; ; [e] 96% of EPEC in controls and 95.65% in cases; [f] 4% of EPEC in cases and 0.4% in cases.

Table 2. 1. Frequency and percentage of diarrheagenic *E. coli* and association with clinical disease in people from Borbon Hospital.

BORBON HOSPITAL	CONTROL (N=111) N (%)	CASE (N=107) N (%)	Fischer's exact test	raw OR (95%IC)	P-value	adjusted OR	P- value*
ETEC	1 (0.90)	9 (8.41)	**0.009**	10.10 (1.26-81.17)	**0.030**	14.21 (1.66-121.3)	**0.015***
lt[b]	0 (0.00)	6 (5.61)	**0.006**	1		omitted	omitted
st[c]	1 (0.90)	1 (0.93)	0.206	4.27 (0.469-38.85)	0.197	6.47 (0.69-61.09)	0.103
st and *lt* [a]	0 (0.00)	2 (1.87)	1	1	omitted		omitted
DAEC	5 (4.50)	9 (8.41)	0.278	1.95 (0.63-6.01)	0.247	1.52 (0.46-4.98)	0.493
EAEC	5 (4.50)	3 (2.80)	0.722	0.61(0.14-2.62)	0.508	0.75 (0.16-3.41)	0.712
EIEC[d]	1 (0.90)	5 (4.67)[d]	0.114	5.39 (0.62-46.94)	0.127	5.25 (0.54-50.8)	0.152
EPEC	10 (9.01)	12 (11.21)	0.656	1.28 (0.53-3.09)	0.589	1.43 (0.55-3.74)	0.460
atypical EPEC[e]	9 (8.11)	12 (11.21)	0.496	1.43 (0.58-3.55)	0.439	1.58 (0.59-4.26)	0.357
typical EPEC[f]	1 (0.90)	0 (0.00)	1	1	omitted		omitted
EHEC	0 (0.00)	1 (0.93)	0.491	1	omitted		omitted
TOTAL	22 (19.8)	33 (30.8)	0.06	1.8 (0.97-3.35)	0.063	2.11 (1.06-4.19)	**0.033***

[a] 22.22% of ETEC positives to both genes (*lt* and *st*) in cases; [b] 66.67% of ETEC in cases; [c] 100% of ETEC in controls and 11.11 % in cases; [d] 1 isolate accounting for *E. coli* Shigellae (*Shigella);* * statistically significant: P-value ≤ 0.05; ; [e] 90% of EPEC in controls and 100% in cases; [f]10% of EPEC in controls.

Table 2. 2. Frequency and percentage of diarrheagenic *E. coli* and association with clinical disease in people from Borbon community.

BORBON COMMUNITY	CONTROL (N=55)	CASE (N=49)	Fischer's exact test	raw OR (95%IC)	p-value	adjusted OR	P-value
	N (%)	N (%)					
ETEC	2 (3.64)	5 (10.20)	0.250	3.01 (0.56-16.28)	0.201	3.56 (0.66-21.15)	0.162
lt[a]	1 (1.82)	0 (0.00)	1.00	1		omitted	omitted
st[b]	1 (1.82)	5 (10.20)	0.097	6.14 (0.69-54.48)	0.103	7.39 (0.78-71.02)	0.083
st and *lt*	0 (0.00)	0 (0.00)	1	1	omitted		omitted
DAEC	2 (3.64)	4 (8.16)	0.417	2.36 (0.41-13.46)	0.335	1.51 (0.46-4.98)	0.493
EAEC	4 (7.27)	2 (4.08)	0.681	0.54 (0.09-3.10)	0.492	2.42 (0.40-14.49)	0.332
EIEC	1 (1.82)	0 (0.00)	1.00	1		omitted	omitted
EPEC	6 (10.91)	4 (8.16)	0.746	0.73 (0.19-2.74)	0.636	0.75 (0.18-3.08)	0.689
atypical EPEC[c]	6 (10.91)	4 (8.16)	0.746	0.73 (0.19-2.74)	0.636	0.75 (0.18-3.08)	0.689
typical EPEC	0 (0.00)	0 (0.00)	1	1		omitted	omitted
EHEC	0 (0.00)	0 (0.00)	1	1	omitted		omitted
TOTAL	14 (25.5)	14 (28.6)	0.82	1.17 (0.49-2.8)	0.72	1.23 (0.49-3.09)	0.65

[a] 50% of ETEC in contrels; [b] 50% of ETEC in controls and 1000% in cases; ; [c] 100% of EPEC in controls.

Table 2. 3. Frequency and percentage of diarrheagenic *E. coli* and association with clinical disease in people from Borbon river communities.

BORBON RIVER COMMUNITIES	CONTROL (N=79)	CASE (N=100)	Fischer's exact test	raw OR (95%IC)	P-value	adjusted OR	P-value
	N (%)	N (%)					
ETEC	6 (7.59)	6 (6.00)	0.767	0.78 (0.24-2.51)	0.672	0.94 (0.27-3.22)	0.929
lt[b]	4 (5.05)	2 (2.00)	0.304	0.46 (0.11-1.98)	0.295	0.62 (0.13-2,87)	0.543
st[c]	1 (1.27)	3 (3.00)	0.695	1.60 (0.29-8.99)	0.591	2.25(0.36-13.91)	0.381
st and *lt* [a]	1 (1.27)	1 (1.00)	1	1	omitted		omitted
DAEC	5 (6.33)	5 (5.00)	0.751	0.78 (0.22-2.79)	0.701	0.42 (0.09-1.79)	0.240
EAEC	3 (3.80)	6 (6.00)	0.733	1.62 (0.39-6.68)	0.507	0.42 (0.09-1.79)	0.240
EIEC	0 (0.00)	1 (1.00)	1	1		omitted	omitted
EPEC	9 (11.39)	7 (7.00)	1	0.58 (0.21-1.65)	0.311	0.61 (0.19-1.85)	0.380
atypical EPEC[d]	9 (11.39)	6 (6.00)	0.277	0.49 (0.17-1.46)	0.203	0.61 (0.19-1.85)	0.380
typical EPEC[e]	0 (0.00)	1 (1.00)	1	1		omitted	omitted
EHEC	2 (2.53)	0 (0.00)	0.193	1	omitted		omitted
TOTAL	23 (29.10)	21 (21.00)	0.23	0.65 (0.33-1.28)	0.21	0.63 (0.30-1.34)	0.234

[a] 16.67% of ETEC positives to both genes (*lt* and *st*) in controls and 16.67% in cases; [b] 66.66% of ETEC in contrels and 33.33% in cases; [c] 16.67% of ETEC in controls and 50% in cases; [d]100% of EPEC in controls and 85.71% in cases; [e]14.29% of EPEC in cases.

SUPPLEMENTARY INFORMATION

Table 1. 1. Demographic data of "Borbon Hospital" study subjects according to cases and controls.

Borbon Hospital	Control	Case	Total	P- value*
Number of individuals	111	107	218	
Gender				
Female	56 (50,45%)	44 (41.12%)	100 (45.87%)	0.177
Male	55 (49.55%)	63 (58.88%)	118 (54.13%)	
Mean age (standard deviation) (rank)	158.47 (180.03) (4- 849)	204.33 (246.11) (0- 1160)	180.98(215.74) (0- 1160)	1.00
Age categories (months)				
0-24	37 (33.33%)	37 (34.58%)	74 (33.93%)	0.892
25-60	13 (11.71%)	14 (13.08%)	27 (12.39%)	
61-180	21 (18.92%)	16 (14.95%)	37 (16.97%)	
≥180	40 (36.04%)	40 (37.38%)	80 (36.70%)	
Sanitation at home				
Latrine	6 (5.45%)	8 (7.48%)	14 (6.45%)	0.393

Septic tank	59 (53.64%)	58 (54.21%)	117 (53.92%)	
Field or hole	12 (10.91%)	15 (14.02%)	27 (12.44%)	
Diaper	32 (29.09%)	22 (20.56%)	54 (24.88%)	
Flush toilet	1 (0.91%)	4 (3.74%)	5 (2.30%)	
River	NA	NA	NA	
Reported home water treatment				
No	92 (83.64%)	93 (86.92%)	185 (85.25%)	0.568
Yes	18 (16.36%)	14 (13.08%)	32 (14.75%)	
Reported recent contact with animals				
No	74 (67.27%)	56 (50.47%)	128 (58.99%)	**0.013**
Yes	36 (32.73%)	53 (49.53%)	89 (41.01%)	
Reported travel in the last year				
No	86 (78.18%)	77 (71.96%)	163 (75.12%)	0.347
Yes	24 (21.82%)	30 (28.04%)	54 (24.88%)	

*statistically significant: P-value ≤ 0.05

Table 1. 2. Demographic data of "Borbon community" study subjects according to cases and controls

Borbon community	Control	Case	total	P-value*
Number of people	55	49	104	
Gender				
Female	27 (49.09%)	22 (44.90%)	49 (47.12%)	0.698
Male	28 (50.91%)	27 (55.10%)	55 (52.88%)	
Mean age (standard deviation) (rank)	125.90 (184.94) (3-857)	181.65 (261.49) (7- 1186)	152.17 (224.90) (3- 1186)	0.853
Age categories (months)				
0-24	14 (25.45%)	15 (30.61%)	29 (27.88%)	0.779
25-60	19 (34.55%)	13 (26.53%)	32 (30.77%)	
61-180	10 (18.18%)	8 (16.33%)	18 (17.31%)	
≥180	12 (21.82%)	13 (26.53%)	25 (24.04%)	
Sanitation at home				
Latrine	2 (3.64%)	5 (10.20%)	7 (6.73%)	**0.025**
Septic tank	43 (78.18%)	26 (53.06%)	69 (66.35%)	
Field or hole	2 (3.64%)	2 (4.08%)	4 (3.85%)	
Diaper	7 (12.73%)	16 (32.65%)	23 (22.12%)	

Flush toilet	1 (1.82%)	0 (0.00%)	1 (0.96%)	
River	NA	NA	NA	
Reported home water treatment			217[a]	
no	43 (78.18%)	35 (71.43%)	78 (75.0%)	0.499
yes	12 (21.82%)	14 (28.57%)	26 (25.0%)	
Reported recent contact with animals				
no	32 (58.18%)	32 (65.31%)	64 (62.54%)	0.546
yes	23 (41.82%)	17 (34.69%)	40 (38.46%)	
Reported travel in the last year				
No	47 (85.45%)	39 (79.59%)	86 (82.69%)	0.450
Yes	8 (14.55%)	10 (20.41%)	18 (17.83%)	

[a]A person did not respond to: water treatment, contact with animals and recent travel; * statistically significant: P-value ≤ 0.05

Table 1. 3. Demographic data of "Borbon river communities" study subjects according to cases and controls.

Rivers	Control	Case	Total	P-value*
Número de personas	79	100	179	
Gender				
Female	39 (49.37%)	45 (45.0%)	84 (46.93%)	0.651
Male	40 (50.63%)	55 (55.0%)	95 (53.07%)	
Mean age (standard deviation) (rank)	115.16 (139.04) (0- 66)	149.92 (264.16) (527- 1024)	134.58 (218.13) (527- 1024)	0.250
Age categories (months)				
0-24	20 (25.32%)	28 (28.00%)	48 (26.82%)	0.762
25-60	21 (26.58%)	24 (24.00%)	45 (25.14%)	
61-180	19 (24.05%)	19 (19.00%)	38 (21.23%)	
≥180	19 (24.05%)	29 (29.00%)	48 (26.82%)	
Sanitation at home				
Latrine	24 (30.38%)	19 (19.00%)	43 (24.02%)	0.336
Septic tank	19 (24.05%)	24 (24.00%)	43 (24.02%)	
Field or hole	17 (21.52%)	32 (32.00%)	49 (27.37%)	
Diaper	18 (22.78%)	24 (24.00%)	42 (23.46%)	
Flush toilet	NA	NA	NA	

River	1 (1.27%)	1 (1.00%)	2 (1.12%)	
Reported home water treatment				
no	65 (82.28%)	76 (76.0%)	141 (78.77%)	0.360
yes	14 (17.72%)	24 (24.0%)	38 (21.23%)	
Reported recent contact with animals				
no	51 (64.56%)	70 (70.0%)	121 (67.6%)	0.512
yes	28 (35.44%)	29 (29.0%)	57 (31.84%)	
no sabe	0 (0.0%)	1 (1.0%)	1 (0.56%)	
Reported travel in the last year				
No	63 (79.75%)	85 (85.0%)	148 (82.68%)	0.427
yes	16 (20.25%)	15 (15.0%)	31 (17.32%)	

* statistically significant: P-value ≤ 0.05

Table 3. Demographic data of Borbon study subjects

	Borbon Hospital	Borbon community	Borbon river communities	Total (Borbon)
Number of individuals	218	104	179	501
Gender				
Female	100 (45.9%)	49 (47.1%)	84 (46.9%)	233 (46.5%)
Male	118 (54.1%)	55 (52.9%)	95 (53.1%)	268 (53.5%)
Mean age (standard deviation) (rank)	180.98 (215.74) (0 - 1160)	152.17 (224.90) (3 - 1186)	134.58 (218.13) (0- 1024)	158.42 (219.07) (0- 1186)
Age categories (months)				
0-24	74 (33.94%)	29 (27.88%)	48 (26.82%)	151 (30.14%)
25-60	27 (12.39%)	32 (30.77%)	45 (25.14%)	104 (20.76%)
61-180	37 (16.97%)	18 (17.31%)	38 (21.23%)	93 (18.56%)
≥180	80 (36.70%)	25 (24.04%)	48 (26.82%)	153 (30.54%)

Table 4. Co-infection in the pacient.

	PATHOTYPE									STRAIN						
	CODE	EPEC (eaeA o *bfp*)	ETEC (*lt*)	ETEC (*sta*)	EIEC (*ipaH*)	DAEC (*afa*)	EAEC (*aggR*)	EHEC (*stx1*)	EHEC (*stx2*)	*eaeA*	*lt*	*sta*	*ipaH*	*Afa*	*aggR*	*stx1*
1	B37	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	B37.3 +/+				B37.1 +/+		
2	B42	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	B42.3 +/+				B42.1 +/+		
3	B66	NEG	NEG	POS	NEG	NEG	POS	-	-			B66.4 +/+			B66.1 -/+	
4	B75 L-/M+	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	B75.5 -/ +				B75.5 - /+		
	B75 L+/M+	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG					B75.4+/+		
5	B119 L- M+	NEG	NEG	NEG	NEG	NEG	POS	-	-						B119.1 -/+	
	B119 L+ M+	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	B119.1+ /+						
6	B84	NEG	POS	NEG	POS	NEG	NEG	-	-		B84.2		B84 .3			
7	C14 L-/M+	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	C14.2 -/+				C14.2-/+		
	C14 L+/M+	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG					C14.2 +/+		
8	C21 L+ M-	NEG	NEG	NEG	NEG	NEG	POS	-	-						C21.2 +/-	
	C21 L+ M+	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	C21.2; C21.4						
9	B201 L- M+	POS	NEG	NEG	NEG	NEG	POS	-	-	B201.5 -/+					B201.5 -/+	
	B201 L+ M+	POS	NEG	NEG	NEG	NEG	NEG	-	-	B201.3 +/+						
10	B202	NEG	NEG	NEG	POS	NEG	POS	-	-				B202.1		B202.2	
11	B244	NEG	POS	POS	NEG	NEG	NEG	-	-		B244.3 B244.5	B244 .3				
12	B295	NEG	POS	POS	NEG	NEG	NEG	-	-		B295.2 B295.3	B295.2				
13	R113 L- M+	NEG	NEG	NEG	NEG	POS	NEG	-	-					R113.1 -/+		
	R113 L+ M+	NEG	POS	NEG	NEG	NEG	NEG	-	-		R113.3 +/+					
14	R119 L+ M+	NEG	POS	POS	NEG	NEG	NEG	-	-		R119.3 +/+	R119.4 +/+ R119.5+/+				

POS= positive; NEG=negative

Table 4.1. Association of co-infections with cases and controls.

co-infections	**Control** (N=245) **N (%)**	**Case** (N=256) N (%)	**Total** (N=501) N (%)	**Fischer's exact test**	**raw OR (95%IC)**	**p-value**	**Adjusted OR (95%IC)**	**P-value***
Negative	243 (99.18)	244 (95.31)	487 (97.21)		1		1	
Positive	2 (0.82)	12 (4.69)	14 (2.79)	**0.012**	5.97 (1.32-26.98)	**0.020**	6.48 (1.39- 30.21)	**0.017***

* statistically significant: P-value ≤ 0.05

Tabla 5. Final concentration Master Mix

	GENE				
	bfp, lt, ipaH, st	*aggR*	*afa*	*stx1, stx2*	*eaeA*
Reaction Buffer 10x	1X	1X	1X	1X	1X
MgCl2 25mM	1,5 mM	2 mM	1,5 mM	1,5 mM	2 mM
dNTPs 10mM	200 µM	200 µM	200 µM	200 µM	200 µM
Primer 1 1µM	0.2 µM	0.4 µM	0.2 µM	1 µM	0.25 µM
Primer 2 1µM	0.2 µM	0.4 µM	0.2 µM	1 µM	0.25 µM
Taq Pol	0.02 U	0.02 U	0.02 U	0.02 U	0.02 U
ADN	2.5 µl	3 µl	2.5 µl	2.5 µl	3 µl
final volume	25 µl	25 µl	25 µl	25 µl	25 µl

Tabla 6. PCR program.

		GENE					
		bfp, lt, ipaH		*sta*		*aggR*	
Steps	**Program**	**temperature**	**duration**	**temperature**	**duration**	**temperature**	**duration**
1	**Initial denaturation**	94°C	5:00 min	94°C	5:00 min	94°C	5:00 min
2	**Denaturation**	94°C	1:00 min	94°C	1:00 min	94°C	0:30 min
3	**Anneling**	56 °C	2:00 min	57.3 °C	2:00 min	50 °C	1:00 min
4	**Extention**	72°C	1:00 min	72°C	1:00 min	72°C	1:30 min
5	**Cycles program**	Step 2-4; 29 cycles		Step 2-4; 29 cycles		Step 2-4; 24 cycles	
6	**Final elongation**			72°C	1:00 min	72°C	5:00 min

		GENE					
		eaeA		*stx1, stx2*		*afa*	
Steps	**Program**	**temperature**	**duration**	**temperature**	**duration**	**temperature**	**duration**
1	**Initial denaturation**			94°C	5:00 min		
2	**Denaturation**	95°C	1:00 min	94°C	2:00 min	94°C	2 min
3	**Anneling**	65°C	2:00 min	58C°	1:00 min	65 °C	1 min
4	**Extention**	72°C	1:30 min	72°C	1:00 min	72°C	2 min
5	**cycles program**	Step 2-4; 10 cycles		Step 2-4; 29 cycles		Step 2-4; 24 cycles	
6	**Denaturation**	95°C	1:00 min				
7	**Anneling**	60°C	2:00 min				
8	**Extention**	72°C	1:30 min				
9	**cycles program**	Step 6-8; 15 cycles					
10	**Denaturation**	95°C	0:30 min				
11	**Anneling**	60°C	2:00 min				
12	**Extention**	72°C	2:30 min				
13	**Cycles program**	Step 10-12; 10 cycles					
14	**Final elongation**			72°C	7:00 min		

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SCIENTIFIC PAPER II:

Antibiotic resistance in *Escherichia coli* pathotypes in rural Ecuador.

ABSTRACT

Antibiotic resistance is a public health concern worldwide, it has increased over the last years mainly due to the positive selection of multiresistant strains generated by the inadequate use of antibiotics in animals and humans; antimicrobial resistance is kept through time and space due to the mechanism used by bacteria to acquire, retain and transmit bacterial resistance. *E. coli* is considered a reservoir and transmitter of resistance genes to other members of the human and animal microbiota. Antibiotic resistance is a problem in pathogenic and opportunistic bacteria. We studied the antibiotic resistance of DEC in a Coastal rural community and compare with other resistance reported for other communities and during other periods of time in Ecuador. We analyzed 141 DEC isolates using the Kirby-Bauer method and 11 antibiotics: ampicillin, amoxicillin-clavulanic acid, cefotaxime, cephalothin, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin, streptomycin, tetracycline, and imipenem. Resistance was detected for cephalothin in 78.72% of analyzed strains, ampicillin in 75.18%, streptomycin in 70.92%, trimethoprim/sulfamethoxazole in 67.38%, tetracycline in 60.28, and amoxicillin-clavulanic acid in 46.10%. Multiresistance was observed in 112 (79.43%) of the isolates. Finally, bacterial resistances were compared to urban areas (Quito, Esmeraldas) and we found significantly more resistance in Borbon with than in urban areas.

Keywords: *E. coli* pathotype, Borbón-Ecuador, multiple antibiotic resistances.

RESUMEN

La resistencia a los antibióticos es un problema de salud pública en todo el mundo, ha aumentado en los últimos años principalmente debido a la selección positiva de cepas multirresistentes generadas por el uso inadecuado de antibióticos en animales y humanos; la resistencia antimicrobiana se mantiene a través del tiempo y el espacio debido al mecanismo utilizado por las bacterias para adquirir, retener y transmitir la resistencia bacteriana. *E. coli* se considera un reservorio y transmisor de genes de resistencia a otros miembros de la microbiota humana y animal. Las resistencias a los antibióticos son un problema en bacterias patógenas y oportunistas. Mediante este estudio se pretendió detectar las resistencias a los antibióticos de DEC en una comunidad rural costera y compararla con otras resistencias reportadas para otras comunidades y durante otros períodos de tiempo en Ecuador. Analizamos 141 aislamientos DEC utilizando el método Kirby-Bauer y 11 antibióticos: ampicilina, amoxicilina-ácido clavulánico, cefotaxima, cefalotina, cloranfenicol, ciprofloxacina, trimetoprima / sulfametoxazol, gentamicina, estreptomicina, tetraciclina e imipenem. Se detectó resistencia para cefalotina en 78,72% de las cepas analizadas, ampicilina en 75,18%, estreptomicina en 70,92%, trimetoprima/ sulfametoxazol en 67,38%, tetraciclina en 60,28 y amoxicilina-ácido clavulánico en 46,10%. Se observó multirresistencia en 112 (79.43%) de los aislamientos. Finalmente se comparó resistencias bacterianas en zonas urbanas (Quito, Esmeraldas) y se obtuvo significativamente mayor resistencia en Borbon que en las zonas urbanas.

Palabras claves: Patotipo de *E. coli*, Borbón-Ecuador, múltiples resistencias antibióticas.

INTRODUCTION

The progressive increase in antibiotic resistance in every geographic region is considered one of the most relevant problems worldwide, threatening human health and food safety around the globe (OMS, 2017). Antibiotic resistance is a natural phenomenon, however excessive use of drugs in both human and animals is selecting multiresistant strains, and this prolongs hospital stays, increases medical costs and mortality (Samie *et al.,* 2012).

Control of antibiotic resistance is complex, due to the variety of mechanisms used by the bacteria to acquire, preserve and disseminate these antibiotic resistances (Peter *et al.,* 2017). The increasingly growing antibiotic resistance in enterobacterias is due mainly to mobile genetic elements that can propagate easily through bacterial populations (Ozgumus *et al.,* 2007; Salman *et al.,* 2017). *E. coli* is considered a reservoir and transmitter of resistance genes to other members of the human or animal microbiota, turning the gastrointestinal tract in an ideal niche for the transference of antimicrobial resistance and pathogenicity genes factors (Adefisoye & Okoh, 2016).

Multiple antibiotic resistant DEC isolated from human, animals and the environment has been reported (Vila *et al.,* 2001). Maintenance of the antibiotic resistance increases by antibiotic selection and it is mediated by genes in genetic mobile elements such as: integrons, plasmid, etc. (Ozgumus *et al.,* 2007).

Plasmids are extra chromosomal auto-replicative elements responsible for virulence, antimicrobial resistance, substrate usage, etc. (Orden *et al.,* 2007). In *E. coli*, a variety of plasmids associated with virulence and antibiotic resistance have been detected; all diarrheagenic *E. coli* (DEC) pathotypes owe their virulence to plasmids (Johnson & Nolan, 2009).

Most commensal and pathogenic bacteria associated to animals (including humans) display resistance to antibiotics (tetracycline, streptomycin, amoxicillin, cephalothin, ticarcillin, and trimethoprim/sulfamethoxazole **(**Orden *et al.,* 2007)); this restrict their use of some antibiotics in severe diarrheas with persistent symptom such as those caused by EAEC o

ETEC (traveler's diarrhea) (Mendez *et al.,* 2009). These resistances are coded by different genes present in plasmid which confer antibiotic resistance (Orden *et al.,* 2007; Vila *et al.,* 2001).

In Ecuador, the antibiotic resistance in DEC varies in time and according to location (Bhavnani *et al.,* 2016; Vasco *et al.,* 2014; Montero *et al.,* 20016; Paéz *et al.,* 2016) although some resistances remain overtime (ampicillin, cephalothin, trimethoprim/sulfamethoxazole, amoxicillin, clavulanic acid and streptomycin) (Bhavnani *et al.,* 2016; Vasco *et al.,* 2014; Montero *et al.,* 2016; Paéz *et al.,* 2016). This study seeks to evaluate the antibiotic resistance in DEC, in a Coastal community.

MATERIALS AND METHODS

Human subjects and study design:

A case-control study was conducted, in which in Borbon. Sampling was performed through the Borbon Hospital or through ministry of health visits to the outlying communities. A case-control using fecal samples of 501 individuals (256 cases and 245 controls) from which *E. coli* strains where isolated and pathotype specific genes were amplified. We obtained 141 pathotype strains from which: bacterial susceptibility was analyzed.

The "cases" were defined as those patients that attended the Hospital or ministry of health clinic visits, exhibiting acute diarrhea (three or more loose stools in a 24-hour period) and those people showing signs of diarrhea during home visits of the field team. Controls were patients that attended the hospital for other reasons different from diarrhea and did not showed diarrheic symptoms at least seven days before taking the sample.

Laboratory Procedures:

Fecal samples were grown in MacConkey's lactose agar media (MKL), and then incubated at 37°C for 24 hours, once that colonies were obtained 5 lactose positive CFU (colony forming units) were randomly selected and non-lactose fermenting colony were also collected. Colonies were transferred to a Chromocult agar media (Merck, Darmsladt, Alemania) (CC) to determine β-glucoronidase activity (MUG); each of the MUG + colonies

were cultured in nutrient agar (AN) and were frozen in Brain Heart Infusion (BHI) + 20 % glycerol broth (Belmonte *et al.,* 2009).

For ADN extraction 5 or 6 colonies from the same fecal sample were pooled together in a tube with 300 µl of sterile distilled water (colony pool) and boiled for 10 min to release the DNA (supernatant was used for PCR testing) (Jin *et al.,* 2008; Seni, 2015).

If the colony pool test was positive for any pathotype gene by PCR, each of the colonies (comprising the pool) was grown separately in nutrient agar (from the colonies frozen in -80), DNA was released from three to five colonies by re-suspending them in 300 µl of sterile water and boiling them for 10 minutes (Jin *et al.,* 2008; Seni, 2015). Tubes were centrifuged at centrifuged at 1.217 X *g*. for 1 min and the supernatant (DNA) was used in a polymerase chain reaction (PCR) for detection of the different pathotypes of *E. coli*.

If the colony test was positive for any pathotype gene by PCR, each of the colonies were grown in nutrient agar (from the colonies frozen in -80) and an antibiogram was performed to determine antimicrobial susceptibility of the isolated colonies.

Antibiotic Susceptibility Testing:

Antibiotic susceptibility analysis were carried out to: 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), gentamicin (CN, 10 µg) streptomycin (S, 10 µg), tetracycline (Te, 30 µg), imipenem (IPM, 10 µg). In order to determine antibiotic resistance or susceptibility the Kirby-Bauer disk diffusion method was used according to the Clinic and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017).

Frozen cultures were grown in Nutrient agar (AN), and 2 to 3 colonies were resuspended in 3 ml of saline solution (0.1%) until 0,5 McFarland turbidity, plated in Mueller-Hinton agar with antibiotic disks and incubated for 24 hours at 37 ºC. Inhibition growth diameters were measured using a Digital Caliber (MarCla, MAHR).

Statistical analysis:

The resistance to antibiotics by pathotypes was calculated using odds ratio (OR) and OR adjusted for confusing variables through StataMP 13 (StataCorp. LP, College Station, TX) and Microsoft Office Excel 2013. Chi-square obtained in the analysis allowed comparisons between cases and controls, considering as statistically significant if P value ≤ 0.05.

RESULTS

The clinical resistance found in Borbon was cephalothin (CF) (n=111, 78.72%); ampicillin (AM) (n=106, 75.18%), streptomycin (S) (n=100, 70.92%), trimethoprim/sulfamethoxazole (SXT) (n=95, 67.38%), tetracycline (TE) (n=85, 60.28%), other clinical resistances found in this study were: amoxicillin-clavulanic acid (AMC) (n=65, 46.10%), chloramphenicol (C) (n=32, 22.69%), cephotaxim (CTX) (n=12, 8.51%), ciprofloxacin (CIP) (n=11, 7.80%), gentamycin (GM) (n=11, 7.80%), and imipenem (IPM) (n=6, 4.25%) (Table 1).

Additionally, multiple resistance reached 78.87% in this study, considered from resistance for at least three differents antibiotics.

Of all 141 DECs analyzed, only 8 (5.63%) presented sensibility to the 11 tested antibiotics, resistance to one single antibiotic (n=7, 4.93%), and multiresistance, starting from 3 antibiotic resistances, were present in 112 (79.43%) (Table 3.1).

A major frequency of antibiotic resistance was observed in cases (isolates from diarrheal cases), resistance to ampicilina, cefotaxime, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole streptomycin and imipenem but none of these differences were statistically significant (Table 2).

However, when separating the analysis by pathotypes, we found only difference statistically significant (OR with IC95% 7.99 (1.24-51.5); p-value= 0.029) for amoxicillin-clavulanic acid in controls against cases for EIEC pathothype (Table 3).

In Borbon, a higher proportion of antibiotic resistances in comparison with Quito or Esmeraldas (rural) was registered to: ampicillin (P= 0.033), amoxicillin-clavulanic acid (P= 0.000), cephalothin (P= 0.003), ciprofloxacin (P= 0.048), streptomycin (P= 0.000) y tetracycline (P= 0.016) (Table 4).

DISCUSSION

Clinical resistance in Borbon was found for: cephalothin (CF) (n=111, 78.72%); ampicillin (AM) (n=106, 75.18%), streptomycin (S) (n=100, 70.92%), trimethoprim/sulfamethoxazole (SXT) (n=95, 67.38%), tetracycline (TE) (n=85, 60.28%), amoxicillin-clavulanic acid (AMC) (n=65, 46.10%), chloramphenicol (C) (n=32, 22.69%), To a lower extend resistance was found for: cephotaxime (CTX) (n=12, 8.51%), ciprofloxacin (CIP) (n=11, 7.80%), gentamycin (GM) (n=11, 7.80%), and imipenem (IPM) (n=6, 4.25%) (Table 1).

Similar patterns of resistance where reported in previous studies in Ecuador, demonstrating DEC resistance to: ampicillin (85%), ciprofloxacin (63%), trimethoprim/sulfamethoxazole (70%), amoxicillin-clavulanic acid (46%), cefotaxime (40%) (Montero *et al.,* 2016), that way studies in Esmeraldas showed a high percentage of resistance for: cephalothin (48.80%), ampicillin (76.19%), trimethoprim/sulfamethoxazole (73.80%) (Paéz *et al.,* 2016).

Additionally, multiple resistance reached 78.87% in this study, considered from resistance for at least three differents antibiotics. This data supports the existent of high resistance rates in DEC, reported by Canizalez and others (2016) up to 91% of *E. coli* intestine pathogen resistance for at least one antimicrobial. Similarly, multiple resistance to antibiotics of DEC in Peru for: ampicillin (85%), cotrimoxazole (79%), tetracycline (65%) and nalidixic acid (28%) (Ochoa *et al.,* 2011).

These resistance patterns indicate: DEC with higher frequency in B-lactam antibiotics: penicillin (ampicillin), cephalosporin (cephalothine and cefotaxime), therefore: 61.97% presented resistance to ampicillin and cephalothine (first generation); 8.5% resistance to ampicillin, cephalothine and cefotaxime (third generation); 1.41% with resistance to more that penicillins and cephalosporines to carbapenems (imipenem). Furthermore amoxicillin-clavulanic acid would be an alternative to B-lactam resistances. However, this study presented resistance in 45.77% of all DEC analyzed and in 3.52% amoxicillin-clavulanic acid and imipenem resistance was detected.

Comparing the antibiotics resistances between pathotypes, no statistically significant difference could be found. That way the resistances were registered in a uniform manner in all evaluated pathotypes (table 3). Furthermore multiple resistance was determined for cases as well as controls. In cases, up to 10 resistances were observed, whereas in controls

up to 8 resistances of all 11 antibiotics tested were registered. No significant association of resistance to any specific antibiotic to cases was determined. This proves to be true looking at studies in Esmeraldas which revealed antibiotic resistance to a ampicillin and sulfamethoxazole-trimethroprim in cases and controls (Eisenberg et al., 2012) and studies in Quito which found no statistically significant association between any specific pathotype with any specific antibiotic resistance (Montero et al., 20016).

Studies in Peru reported molecular mechanisms of resistance in antibiotic families: quinolones, beta-lactams, chloramphenicols and tetracyclines, therefore mutations in gyrA could be verified in 64% and parC in 5.8% of DEC cases with resistance to quinolones (nalidixic acid) (Pons *et al.,* 2014), presence of resistance genes like: *blaTEM* (resistance to B-lactams) present in 35%, *cat* (resistance to chloramphenicol) present in 87%, and *tetA* (resistance to tetracycline) in 31% of DEC (Mercado *et al.,* 2011). Nevertheless, in this study no antibiotic resistance genes were evaluated but these resistance mechanisms in pathotypes of evaluated *E. coli* were registered.

These data support the DEC resistance rates reported by Canizalez *et al.* (2016); up to 91% of *E. coli* intestine pathogen resistance for at least one antimicrobial. In the same way, multiple resistance to antibiotics for DEC in Peru have been reported: ampicillin (85%), cotrimoxazole (79%), tetracycline (65%) and nalidixic acid (28%) (Ochoa *et al.,* 2011). Ochoa *et al.* (2009) and studies in Latin America (Bessone *et al.,* 2017; Canizalez *et al.,* 2016; Paniagua *et al.,* 2007), where ETEC strains presented multiple resistance principally to: cephalothin, ampicillin, trimethoprim/sulfamethoxazole, cephotaxime, chloramphenicole, among other antibiotics.

A major frequency of antibiotic resistance was observed in cases (isolates from diarrheal cases), resistance to ampicilina, cefotaxime, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole streptomycin and imipenem but none of these differences were statistically significant (Table 2). However, when separating the analysis by pathotypes, we found difference statistically significant (OR with IC95% 7.99 (1.24-51.5); p-value= 0.029) for amoxicillin-clavulanic acid in controls against cases for EIEC pathothype (Table 3). These results disagree with other studies shows greater antibiotic resistance in cases (Ochoa *et al.,* 2009).

Unexpectedly, *E. coli* pathotypes from Borbon, had a significantly higher proportion of antibiotic resistances in comparison with Quito and Esmeraldas (rural): ampicillin (p=

0.033), amoxicillin-clavulanic acid (p= 0.000), cephalothin (p= 0.003), ciprofloxacin (p= 0.048), streptomycin (p= 0.000) and tetracycline (p= 0.016) (Table 4). The most important determinant of the emergence of bacterial antibiotic resistance, both in individuals and populations, is antibiotic use (Komolafe, 2003). The presence of higher rates in rural areas can be explained by poor management in the health system, and informal healthcare providers, in such a way that a number of studies document the effect of social and behavioural factors on antibiotic resistance (Yagupsky, 2006). Furthermore, it has been noted elsewhere that sub-therapeutic dosing may be common in low- and middle-income countries (Sacristán *et al.,* 2014). There is a marked difference higher between bacterial resistance in urban sites and rural sites (Martinez, 2009), in this study this difference is also recorded.

Antibiotic resistance genes in both pathogenic and non-pathogenic bacteria can be transmitted from animals to humans through the consumption of food, or through direct contact with animals or their waste in the environment ambient. (Chantziaras *et al.,* 2014), suggesting that bacterial resistances in animals play an important role in the bacterial resistance of the community.

The large variations in proportions of resistant bacteria in the rural and urban sities, suggest differences in veterinary practice in Ecuador, which has been registered in other contries (Garcia-Migura *et al.,* 2014; da Costa *et al.,* 2013) futhermore, in rural sity exist more direct contact with production animals and companion animals, which non-therapeutic use of antibiotics in leadsing to increased antibiotic resistance in the community (da Costa *et al.,* 2013).

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TABLES

Table 1. Antibiotic resistance

Antibiotics	Pathotypes							
	EPEC[a] (N=45)	ETEC(N=26)	EHEC (N=3)	EIEC(N=8)	DAEC (N=30)	EAEC (N=23)	two genes[b] (N=6)	TOTAL (N=141)
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
ampicillin (AM)	29 (64.4)	17 (65.38)	1 (33.33)	7 (87.50)	28 (93.33)	20 (86.96)	4 (66.67)	106 (75.18)
amoxicillin-clavulanic acid (AMC)	19 (42.22)	9 (34.61)	0 (0.00)	1 (12.50)	21 (70.00)	12 (52.17)	3 (50.00)	65 (46.10)
cefotaxime (CTX)	1 (2.22)	1 (3.84)	0 (0.00)	0 (0.00)	8 (26.67)	2 (8.70)	0 (0.00)	12 (8.51)
cephalothin (CF)	33 (73.33)	20 (76.92)	2 (66.67)	5 (62.50)	28 (93.33)	18 (78.26)	4 (66.67)	111 (78.72)
chloramphenicol ©	13 (28.89)	1 (3.84)	0 (0.00)	1 (12.50)	8 (26.67)	7 (30.43)	2 (33.33)	32 (22.69)
ciprofloxacin (CIP)	5 (11.11)	1 (3.84)	1 (33.33)	1 (12.50)	3 (10.00)	0 (0.00)	0 (0.00)	11 (7.80)
trimethoprim/sulfamethoxazole (SXT)	23 (51.11)	16 (61.54)	2 (66.67)	7 (87.50)	28 (93.33)	17 (73.91)	2 (33.33)	95 (67.38)
gentamicin (GM)	3 (6.67)	0 (0.00)	0 (0.00)	1 (12.50)	6 (20.00)	1 (4.35)	0 (0.00)	11 (7.80)
streptomycin (S)	26 (57.78)	14 (53.85)	0 (0.00)	8 (100.00)	28 (93.33)	20 (86.96)	4 (66.67)	100 (70.92)
tetracycline (TE)	28 (62.22	8 (30.77)	0 (0.00)	4 (50.00)	25 (83.33)	18 (78.26)	2 (33.33)	85 (60.28)
imipenem (IPM)	3 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)	1 (3.33)	2 (8.70)	0 (0.00)	6 (4.25)

[b]two genes of pathotypes: strain contain 2 different genes.

[a]Isolates of Typical EPEC and atypical EPEC together, due the small amount of Typical EPEC.

Table 2. Antibiotic resistance of diarrheagenic *E. coli* in cases and controls.

Antibiotics	Control (N=60)	Case (N=81)	P-value*
	N (%)	N (%)	
ampicillin (AM)	43 (71.67)	63 (77.8)	0.337
amoxicillin-clavulanic acid (AMC)	31 (51.67)	34 (41.98)	0.312
cefotaxime (CTX)	3 (5.00)	9 (11.11)	0.234
cephalothin (CF)	50 (83.33)	61 (75.31)	0.229
chloramphenicol ©	13 (21.67)	19 (23.46)	0.841
ciprofloxacin (CIP)	3 (5.00)	8 (9.88)	0.352
trimethoprim/sulfamethoxazole (SXT)	39 (65.00)	56 (69.14)	0.590
gentamicin (GM)	5 (8.33)	6 (7.41)	1
streptomycin (S)	40 (66.67)	60 (74.07)	0.353
tetracycline (TE)	38 (63.33)	47 (58.02)	0.730
imipenem (IPM)	2 (3.33)	4 (4.94)	0.700

* statistically significant: P-value ≤ 0.05

Table 3. Clinical antibiotic resistance among the different diarrheagenic *E. coli* in isolates from cases and controls

	EPEC[a]		ETEC		EHEC		EIEC		DAEC		EAEC		two pathotypes[a]	
	Control	Case	Control	Case	Control	Case	Control	Case	Control	Caso	Control	Case	Control	Case
	n= 24 (%)	n= 31 (%)	n= 7 (%)	n= 19 (%)	n= 2 (%)	n=1 (%)	n= 2 (%)	n= 6 (%)	n= 12 (%)	n=18 (%)	n=12 (%)	n=11 (%)	n= 1 (%)	n=5 (%)
AM	14 (58.3)	15 (71.4)	4 (57.14)	13 (68.42)	1 (50.00)	0 (0.00)	2 (100.00)	5 (83.33)	11 (91.67)	17 (94.44)	11 (91.67)	9 (81.82)	0 (0.00)	4 (80.00)
AMC	11 (45.83)	8 (38.10)	2 (28.57)	7 (36.84)	0 (0.00)	0 (0.00)	0 (0.00)	1 (16.67)	9 (75.00)	12 (66.67)	9 (75.00)[c]	3 (27.27)[c]	0 (0.00)	3 (60.00)
CTX	0 (0.00)	0 (0.00)	0 (0.00)	1 (5.26)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (25.00)	5 (27.78)	0 (0.00)	2 (18.18)	0 (0.00)	0 (0.00)
CF	20 (83.33)	14 (66.67)	5 (71.43)	14 (73.68)	1 (50.00)	1 (100.00)	1 (50.00)	4 (67.67)	12 (100.00)	16 (88.89)	11 (91.67)	7 (63.64)	0 (0.00)	4 (100.00)
C	7 (29.17)	6 (28.57)	0 (0.00)	1 (5.26)	0 (0.00)	0 (0.00)	0 (0.00)	1 (16.67)	3 (25.00)	5 (27.78)	3 (25.00)	4 (36.36)	0 (0.00)	2 (40.00)
CIP	1 (4.17)	4 (19.5)	0 (0.00)	1 (5.26)	1 (50.00)	0 (0.00)	0 (0.00)	1 (16.67)	1 (8.33)	2 (11.11)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
SXT	12 (50.00)	11 (52.38)	3 (42.86)	13 (68.42)	2 (100.00)	0 (0.00)	2 (100.00)	5 (83.33)	11 (91.67)	17 (94.44)	9 (75.00)	8 (72.73)	0 (0.00)	2 (66.67)
GM	3 (12.50)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (16.67)	2 (16.67)	4 (22.22)	0 (0.00)	1 (9.09)	0 (0.00)	0 (0.00)
S	13 (54.17)	13 (61.90)	3 (42.86)	11 (57.89)	0 (0.00)	0 (0.00)	2 (100.00)	6 (100.00)	11 (91.67)	17 (94.44)	11 (91.67)	9 (81.82)	0 (0.00)	4 (80.00)
TE	16 (66.67)	12 (57.14)	2 (28.57)	6 (31.58)	0 (0.00)	0 (0.00)	1 (50.00)	3 (50.00)	9 (75.00)	16 (88.89)	10 (83.33)	8 (72.73)	0 (0.00)	2 (40.00)
IPM	1 (4.17)	2 (9.52)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (5.56)	1 (8.33)	1 (9.09)	0 (0.00)	0 (0.00)

[b]two pathotypes: strain contain 2 different genes.

[a]Isolates of Typical EPEC and atypical EPEC together, due the small amount of Typical EPEC.

[c]Statistically significant (OR with IC95% 7.99 (1.24-51.5); *p-value*= **0.029**).

Table 3.1. Multiresistences in *E.coli* pathotypes

	Pathotypes							
	EPEC[a] (N=45)	**ETEC (**N=26)	**EHEC** (N=3)	**EIEC** (N=8)	**DAEC** (N=30)	**EAEC** (N=23)	**two pathopypes**[b] (N=6)	**TOTAL** (N=141)
MULTIRESISTENCE	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
≥ 2	40 (88.89)	22 (84.61)	2 (66.67)	8 (100.00)	30 (100.00)	22 (95.65)	3 (50.00)	127 (90.07)
≥ 3	30 (66.67)	18 (69.23)	1 (33.33)	8 (100.00)	30 (100.00)	22 (95.65)	3 (50.00)	112 (79.43)
≥ 4	27 (60.00)	13 (50.00)	-	5 (62.50)	29 (96.67)	21 (91.30)	3 (50.00)	98 (69.50)
≥ 5	19 (42.22)	6 (23.08)	-	3 (37.50)	28 (93.33)	17 (73.91)	2 (33.33)	75 (53.19)

[b]two pathotypes: strain contain 2 different genes.

[a]Isolates of Typical EPEC and atypical EPEC together, due the small amount of Typical EPEC 5 L- M

Table 4. Clinical antibiotic resistance among the different diarrheagenic *E. coli* in isolates from Borbon, Esmeraldas and Quito.

	BORBON (2018)	**ESMERALDAS (2016)**	**QUITO (2014)**	
	Controls and cases (N=141)	Controls and cases (N=84)	Controls and cases (N=107)	**P-value***
ANTIBIOTICS	N (%)	N (%)	N (%)	
ampicillin (AM)	106 (75.18)	64 (76.19)	66 (61.68)	**0.033**
amoxicillin-clavulanic acid (AMC)	65 (46.10)	8 (9.52)	4 (3.74)	**0.000**
cefotaxime (CTX)	12 (8.51)	6 (7.14)	5 (4.67)	0.497
cephalothin (CF)	111 (78.72)	41 (48.81)	35 (32.71)	**0.000**
chloramphenicol ©	32 (22.69)	15 (17.86)	7 (6.54)	**0.003**
ciprofloxacin (CIP)	11 (7.80)	5 (5.95)	1 (0.93)	**0.048**
trimethoprim/sulfamethoxazole (SXT)	95 (67.38)	62 (73.81)	71 (66.36)	0.495
gentamicin (GM)	11 (7.80)	7 (8.33)	9 (8.41)	0.982
streptomycin (S)	100 (70.92)	52 (61.90)	3 (2.80)	**0.000**
tetracycline (TE)	85 (60.28)	52 (61.90)	53 (49.53)	0.144
imipenem (IPM)	6 (4.26)	0 (0.00)	0 (0.00)	**0.016**
Multiresistance	112 (79.43)	65 (77.38)	76 (71.03)	0.293

* statistically significant: P-value ≤ 0.05

SUPPLEMENTARY INFORMATION

Table 5. Strain with two pathotypes genes.

	PATHOTYPE								GENES						
CODE	**EPEC (eaeA)**	**ETEC (*lt*)**	**ETEC (*sta*)**	**EIEC (*ipaH*)**	**DAEC (*afa*)**	**EAEC (*aggR*)**	**EHEC (*stx1*)**	**EHEC (*stx2*)**	***eaeA***	***lt***	***sta***	***ipaH***	***afa***	***aggR***	***stx1,stx2***
B75.5 L- M+	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	B75.5 -/+				B75.5 -/+		
C14	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	C14.2 -/+				C14.2 -/+		
B201 L- M+	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG	B201.5 -/+					B201.5 -/+	
B244	NEG	POS	POS	NEG	NEG	NEG	-	-		B244.3	B244 .3				
B295	NEG	POS	POS	NEG	NEG	NEG	-	-		B295.2	B295.2				
R66	NEG	POS	POS	NEG	NEG	NEG	-	-		R66.5	R66.5				

L=Lactose; M: 4-metilumberiferil-β-D-glucorónico (Mug).

POS= positive; NEG=negative

Table 4. 1. Clinical antibiotic resistance among the different diarrheagenic *E. coli* in isolates from Borbon, Esmeraldas and Quito in cases.

	BORBON (2018)	**ESMERALDAS (2016)**	**QUITO (2014)**	
	Case (N=81)	Case (N=52)	Case (N=61)	**P-value***
	N (%)	N (%)	N (%)	
ampicillin (AM)	63 (77.78)	38 (78.08)	45 (73.77)	0.786
amoxicillin-clavulanic acid (AMC)	34 (41.98)	5 (9.62)	4 (6.56)	**0.000**
cefotaxime (CTX)	9 (11.11)	2 (3.85)	3 (4.92)	0.202
cephalothin (CF)	61 (75.31)	24 (46.15)	21 (34.43)	**0.000**
chloramphenicol ©	19 (23.46)	11 (21.15)	3 (4.92)	**0.009**
ciprofloxacin (CIP)	8 (9.88)	3 (5.77)	1 (1.64)	0.129
trimethoprim/sulfamethoxazole (SXT)	56 (69.14)	38 (73.08)	50 (81.97)	0.218
gentamicin (GM)	6 (7.41)	4 (7.69)	9 (14.75)	0.289
streptomycin (S)	60 (74.07)	33 (63.46)	3 (4.92)	**0.000**
tetracycline (TE)	47 (58.02)	31 (59.62)	32 (52.46)	0.710
imipenem (IPM)	4 (4.94)	0 (0.00)	0 (0.00)	**0.058**
Multiresistance	66 (81.48)	39 (75.00)	51 (83.61)	0.491

* statistically significant: P-value ≤ 0.05

Table 4. 2. Clinical antibiotic resistance among the different diarrheagenic *E. coli* in isolates from Borbon, Esmeraldas and Quito in controls.

	BORBON (2018)	**ESMERALDAS (2016)**	**QUITO (2014)**	
	Control (N=60)	Control (N=32)	Control (N=46)	**P-value***
	N (%)	N (%)	N (%)	
ampicillin (AM)	43 (71.67)	26 (81.25)	21 (45.65)	**0.002**
amoxicillin-clavulanic acid (AMC)	31 (51.67)	3 (9.38)	0 (0.00)	**0.000**
cefotaxime (CTX)	3 (5.00)	4 (12.5)	2 (4.35)	0.292
cephalothin (CF)	50 (83.33)	17 (53.13)	14 (30.43)	**0.000**
chloramphenicol ©	13 (21.67)	4 (12.5)	4 (8.70)	0.163
ciprofloxacin (CIP)	3 (5.00)	2 (6.25)	0 (0.00)	0.261
trimethoprim/sulfamethoxazole (SXT)	39 (65.00)	24 (75.00)	21 (45.65)	0.023
gentamicin (GM)	5 (8.33)	3 (9.38)	0 (0.00)	0.117
streptomycin (S)	40 (66.67)	19 (59.38)	0 (0.00)	**0.000**
tetracycline (TE)	38 (63.33)	21 (65.63)	21 (45.65)	0.114
imipenem (IPM)	2 (3.33)	0 (0.00)	0 (0.00)	0.267
Multiresistance	46 (76.67)	26 (81.25)	25 (54.35)	**0.014**

* statistically significant: P-value ≤ 0.05

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