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A Study to Identify the Etiological Agents Causing Diarrhea in Two Low-Income Ecuadorian Communities

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"A study to identify the Etiological Agents Causing Diarrhea in two Low- Income Ecuadorian Communities"

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DEDICATORIA

A las personas que accedieron a participar en el estudio de Casos y Controles en las ciudades de Quito y de Borbón.

A las mujeres de mi familia.

RESUMEN

Introducción- Las estrategias para disminuir la mortalidad y morbilidad por las enfermedades diarreicas requieren un enfoque sitio-específico, de ahí la importancia del entendimiento de los patógenos que pueden causar diarrea.

Métodos- Se realizó un estudio de casos y controles donde se determinó la presencia de quince agentes etiológicos de la diarrea aguda en muestras fecales.

Resultados- De las muestras analizadas 51% de los casos y 27% de los controles de una comunidad urbana, y 62% de los casos y 18% de los controles de una comunidad rural de Ecuador, resultaron positivos para la presencia de uno o más patógenos. Rotavirus y Shigellae se asociaron a la presencia de diarrea en la comunidad urbana, las co-infecciones resultaron en un incremento del riesgo de diarrea que las infecciones por un solo patógeno. *Campylobacter* y *Entamoeba histolytica* fueron frecuentemente halladas en personas asintomáticas, mientras que *Escherichia coli* enteropatogénica y *Salmonella* no-typhi no fueron detectados en ninguna muestra analizada.

Conclusiones- Consistentemente con el estudio Global Enteric Mulcicenter Study desarrollado en África-Subsahariana y el Sur de Asia, hemos encontrado que un pequeño grupo de microorganismos patógenos es el causante de la mayoría de los casos de diarrea en las comunidades incluidas en este estudio.

ABSTRACT

Background-Continued success in decreasing diarrheal disease burden requires targeted interventions. To develop such interventions, it's crucial to understand which pathogens cause diarrhea.

Methods- Using a case-control design we tested stool samples, collected in both rural and urban Ecuador, for fifteen pathogenic microorganisms.

Results-Pathogens were present in 51% of case and 27% of control samples from the urban community, and 62% of case and 18% of control samples collected from the rural community. Rotavirus and Shigellae were associated with diarrhea in the urban community; co-infections were more pathogenic than single infection; *Campylobacter* and *Entamoeba histolytica* were found in large numbers in cases and controls; and non-typhi *Salmonella* and enteropathogenic *E. coli* were not found in any samples.

Conclusions-Consistent with the Global Enteric Multicenter Study, focused in south Asia and sub-Saharan Africa, we found that in Ecuador a small group of pathogens accounted for a significant amount of the diarrheal disease burden.

CONTENT

FIGURES AND TABLES

INTRODUCTION

DIARRHEAL DISEASE: A PUBLIC HEALTH PROBLEM WORLDWIDE

From centuries there have been reports of diarrheal diseases worldwide, showing devastating results over human health and survival.[1] At present, Epidemiology, Microbiology, Public Health, and other sciences contributed to reduce the burden of the disease by the detection of factors, etiological agents or behaviors related to the rates of mortality and morbidity due to the disease.

Preventable strategies such as maternal weaning, improving sanitary conditions, and safe water access, among others, helped to reduce the transmission of the disease. Also, the identification of the etiological agents had facilitated to relate environmental and behavioral factors with the microbiological presence. Nowadays, diagnosis and treatment of the disease has been improved in terms of access and efficacy. However, diarrhea is one of the leading causes of death in young children worldwide in undeveloped countries were a lack of epidemiological decisions had perpetuate the disease. Moreover, epidemiological and microbiological research can help to understand the disease and to decision-make around the problem.

DIARRHEIC DISEASES IN ECUADOR

In the year 2011 the Instituto Nacional de Estadísticas y Censos (INEC) of the Ecuadorian Republic declared the infectious diarrheal disease (CIE-10 code

A09) as the thirteenth cause of death among infants showing a rate of 0,21 per 1000 inhabitants.[2] Even thought, Ecuadorian number of deaths for diarrheal disease WHO data since 1979 to 2009 shows a progressive diminution among different groups of ages (Table 1), the number of cases for diarrheal diseases has a significantly increase (Table 2). These data may reflect improved strategies in the diagnoses and treatment of the disease, but at the same time, possibly revealing the persistence of etiological agents and transmission factors of the disease in Ecuador. For that reason, it is important to fight against the disease by knowing the etiological agents of the diarrheal disease to infer the environment or behavioral factors associated with the persistence of the infectious etiological agent in the way to reduce not only mortality but also morbidity due to the disease.

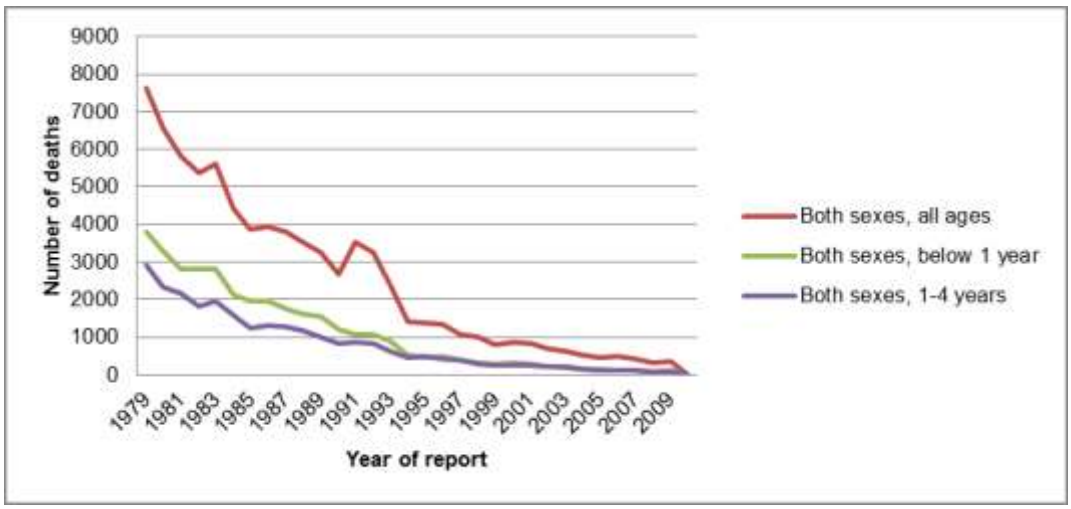

Figure 1

Ecuadorian number of deaths intestinal infectious diseases. WHO database data.

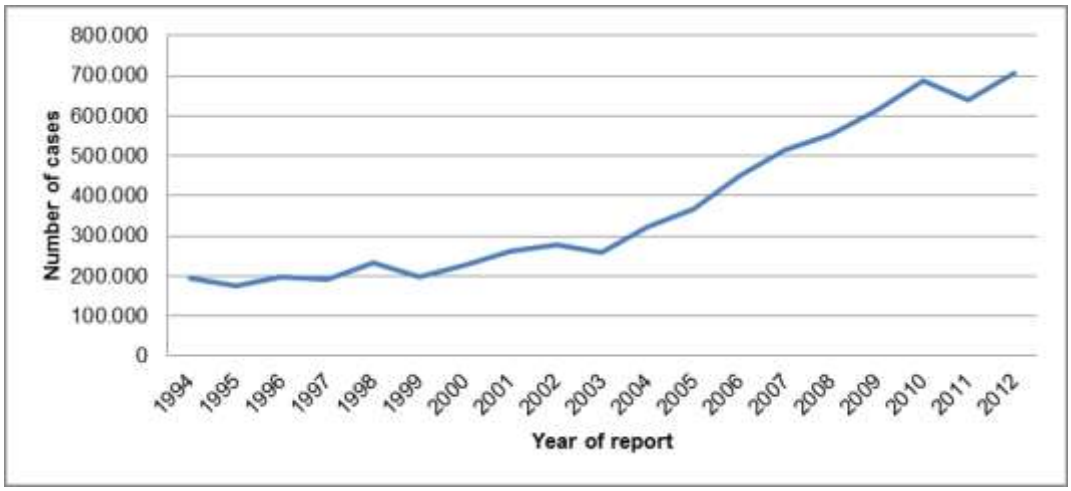

Figure 2

Ecuadorian number of intestinal infectious disease cases reported by year. SIVE-ALERTA data 2014.

COMMUNITY ACQUIRED PATHOGENS RELATED TO ACUTE DIARRHEAL DISEASE

Some community acquired pathogens related to acute diarrheal episodes are reviewed here.

Campylobacter. The first description of a *Campylobacter* species dates from more than a century ago, when *C. fetus* was associated to fetal and reproductive tract animal infections which can lead to abortions and could cause septicemic infections in humans. In the 1970 *Campylobacter* species such as *C. jejuni*, *C. coli*, *C. upsaliensis* and *C. lari* were isolated from diarrheal samples in humans. Biologically, *Campylobacter* is a genus of small spirillum Gram negative bacteria that possess a polar flagellum. The laboratory growth condition include microaerophilic (O_2 3-15% and, CO_2 3-5%) and 42ºC incubation, which inhibits

other bacteria but allows *Campylobacter* growth environments for its cultivation. *Campylobacter* diarrheagenic species transmission mainly is due to the consumption of contaminated food, other ways that have been describe include untreated water or milk, or working with animals (specially for *C. coli* transmission).[3,4]

Enteropathogenic *Escherichia coli* (EPEC). EPEC first report outbreak was recorded at 1940s in infants (United Kingdom); the outbreak was attributed to *E. coli* serotype O:111. Nowadays, there are 12 O serogroups which are included in this pathotype group. EPEC attaching and effacing (A/E) lesions in intestinal microvilli on enterocites are the more remarkable feature of this bacterium. The chromosomal pathogenicity island LEE (locus enterocyte effacement) encodes an intimin *eae*, an intimin receptor Tir, and a type III secretion system *esc* and *sep* which translocate the Tir receptor to the host enterocyte. EPEC strains also possess a virulence plasmid EAF that encode a bundle forming pili *bfp* with adherence properties. Typical EPEC possess the LEE island (*eae*$^{+}$) and the EAF plasmid (*bfp*$^{+}$). While atypical EPEC strains only possess the LEE island (*eae*$^{+}$, *bfp*$^{-}$). EPEC strains (*eae*$^{+}$) possessing Shiga toxin genes (*stx-1* and *stx-2*) are called enterohaemorragic *E. coli* (EHEC). Typical and atypical EPEC isolation in the present days is often a usual finding among developing countries, for example among developing Peruvian children under 2 years with and without diarrheal disease, or in Uruguayan and Brazilian cases.[5]

Enterohaemorrhagic *Escherichia coli* (EHEC) are agents whose infection can lead to a diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. EHEC possess Shiga-toxin (*stx*) virulence factor, but, also possess a LEE pathogenicity

island. It has been described two different LEE acquisition in ancestral EPEC that raised EHEC1 and EHEC2 clone linages.[6] EHEC causes hemolytic uremic syndrome (HUS) due to the effect of *stx* in the endothelial cells of the renal capillary. These bacteria are transmitted from domestic animals such as cattle and sheep.[7,8] Even though there are high rates of EPEC infection in developing countries, low rates or no cases EHEC are seen in the same locations. Immune responses against components of the EPEC LEE may protect the individuals from EHEC infections. Moreover, a mouse model demonstrated that EPEC infection may have a protective effect against EHEC.[9]

Enterotoxigenic *Escherichia coli* (ETEC) has been found in Colombian communities (Cartagena) as a main diarrheal pathogen ($p<0.03$, OR 2.51), among Peruvian children older than 6 months (OR 4.56 $p=0.026$), and into Ecuadorian communities.[10,11]

Escherichia coli Shigellae. Humans are the only known host for these bacteria, consequently transmission occurs via fecal-oral route, fomites, or contaminated water consumption. Some *E.coli* Shigellae have Shiga toxin and may cause dysentery and even can cause HUS.[12] Breastfeeding plays a role as a protective factor against the disease caused by this pathogen.[13]

Rotavirus. As other reovirus, rotavirus comprises 11 double strain RNA segments and three layers of envelope proteins: a core, an inner capsid and an outer capside. Rotaviral A group genotypes are named by the combination of two antigenic outer capsid proteins VP7 (a glycoprotein named type G) and, VP4 (a protease named type P). 23 alelic variants had been described for VP7 and 32 for VP4 ones. These viruses are subject of genetic shift caused by reassortment of

the RNA segments resulting in a large variety of possible genotypes. Additionally it is common to find mixed rotaviral infections. It has been reported higher assortment rates in rotavirus from domestic animals than in human counterparts. The virus is spread for fecal-oral transmission.[14]

Cryptosporidium. *Cryptosporidium* are oocysts-forming apicomplexa protozoan having 21 confirmed species that parasite a broad range of vertebrate hosts. *Cryptosporidium parvum* and *C. hominis* are the two more frequent species associated to human diarrheal disease, however *C. felis*, *C. meleagridis*, *C. canis*, *C. suis*, *C. muris* and *C. baileyi* (infecting cats, birds and humans, dogs, pigs, rodents and birds primary hosts respectively) have been reported in human cases. *Cryptosporidium* virulence is multifactorial, and comprises host factors such as age, sex, the status of the immune system, but also parasite genotypic characteristics.[15] The infection is presented often as outbreaks due to contaminated water and few by contaminated food consumption. Water contamination by animal hosts seems to be the most important mechanism. Its live cycles begins when after oocyst ingestion and exystation, sporozoites are released and invade intestinal epithelial cells. In these cells sporozoites passes through a sexual and an asexual cycle of division. The sexual cycle produce microgametes and macrogametes which are fertilized by the first ones, then the oocyst encyst and sporulates. After this process two types of cyst are produced: the thin walled which auto-infect other host cells and the thick walled which are excreted with the feces.[16]

Aeromona species *hydrophila*, *veronii*, *sobria* and *cavia* are considered emerging pathogens and potential risk for the developing of diarrhea in humans.

This genus lives in aquatic environments, and had been isolated even in biofilms of chlorinated water containers. However, routes of transmission and pathogenicity of these bacteria has not been well understood.[17]

Giardia lamblia, also called *G. intestinalis* or *G. duodenalis* is a protozoan parasite which infects the upper intestinal tract in humans. The infection cause a not well understood state of asymptomatic carriage, while in some other cases it has been associated to sporadic diarrheal episodes, or even acute diarrhea. Genotypes of *G. lamblia* A and B are the most frequently related to the symptomatic infection state. Even when studies in developing countries had failed to prove the burden of *G. lamblia* as an important intestinal pathogen,[18] the parasite infection seems to be a causative of diarrhea among people in developed countries and in fewer than three months aged infants in developing countries.[19,20]

ASYMPTOMATIC CARRIERS

Important approaches employ case-control and cohort designs that able the investigators to calculate statistical indicators such as the odds ratio or the attributable fraction to inform about the burden of one pathogen for the presence of the diarrheal disease.

Some factors had been reported as main contributors for asymptomatic carriage of intestinal pathogens. Firstly, some pathogen characteristics such as, the occurrence of long periods of excretion (*Campylobacter*, *Salmonella* and norovirus) after the presence of the diarrheal episodes, the presence of different strains or genotypes some of them more or less pathogenic than the other ones,

the need of interaction with other pathogens or even, the latent phase of an infection. Second, host factors including intestinal microbiota that work as an important protective barriers by producing inhibitory growth compounds (produced by the metabolism of fatty chains, bacteriocines, colicines) of some pathogenic bacteria. Also, a theory called environmental enteropathy that is state of chronic inflammatory bowel disease present in children who live under poor hygienic conditions may change the course of some intestinal infections.[21] Third, some environmental factors such as the inoculum needed to cause an asymptomatic infection, or the sensitivity of the diagnostic test to estimate the presence of a given microorganism.[22] As an illustration, *Campylobacter* species carriers have been reported among people living under poor hygienic conditions.[23] In South-American countries these data seems to be high, for example, a Brazilian study showed isolation percentages among people without diarrhea of 4,9% for *C. jejuni* (among diarrheal samples of 5,8%?), and 2 for *C. coli* (diarrheal isolation frequencies were 2,2);[24] Chilean isolation percentages of asymptomatic carriers were 4 (diarrheal 9 to 14);[25,26] Also, African rates reflects 9,5% on non-diarrheal isolations (and 9,5% among cases).[27]

SOME DIARRHEAL PATHOGENS PRESENT WITH SEASONALITY PATTERNS

A review that included studies developed among tropical countries that reported rotaviral disease presence and seasonality data showed that its presence is improved by drier and colder climates among the year.[28] *Cryptosporidium* transmission could be affected by seasonal rainfall peaks, or by changes in farm

animal waste release.[16] European countries data report case distribution peaks of intestinal disease due to *Cryptosporidium*, *Campylobacter*, STEC, *Salmonella enterica* serovar Enteritidis and *Escherichia coli* Shigellae in the summer and autumn seasons.[29]

CO-INFECTIONS

Unicomb (1996) identified rotavirus associated diarrheal cases that were in a mixed infection with other pathogens such as *Vibrio cholera*, enteropathogenic *Escherichia coli*, and *E. coli* Shigellae in stool samples of 5810 subjects under 2 years old, in Bangladesh, India from 1987 to 1988. The study scored the severity of the disease by using indicators such as dehydration, number of stool bowel movements, fever, vomiting, and duration of diarrhea. Rotavirus/*E. coli* co-infection was not associated with a worst outcome than rotavirus or *E. coli* alone. While, co-infections with rotavirus/*V. cholera* and rotavirus/*E. coli* Shigellae were more severe infections ($p<0.01$ and $p<0.02$ respectively) than the rotavirus, *V. cholera* and *E. coli* Shigellae alone.[30]

Ochoa (2009) described co-infection more often among cases than in controls in a study of Peruvian children under 2 years old: 13.3 versus 5 percent among cases and controls respectively ($p<0,001$). The most common cases were rotavirus/EAEC and rotavirus/*Campylobacter*.[31]

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TITLE

Identifying etiological agents causing diarrhea in low income Ecuadorian communities

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INTRODUCTION

Despite the decline in diarrhea-associated child mortality over the past four decades, diarrheal disease is still the second leading cause of death among children under 5 in developing countries.[1,2] To continue to reduce diarrhea-associated mortality, targeted interventions are needed. Informing the development of these interventions require an understanding of the social, environmental, and biological drivers of diarrheal disease. To this end, the Global Enteric Multicenter Study (GEMS) was conducted to ascertain the etiological agents causing the diarrhea in children under five.[3] The GEMS study sites were located in sub-Saharan Africa and south Asia, where diarrheal disease burden is highest in the world.[1,2] The GEMS study, unfortunately, did not include study sites within Latin America, where improved water and sanitation facilities are lacking and diarrheal disease is a large concern. To complement the GEMS study, we conducted a case control study in a rural and urban community within Ecuador.

Clinical investigations of diarrheagenic microbial agents are time consuming, expensive, difficult to achieve in the clinical setting, and the results seldom benefit the patient,[4,5] nevertheless, understanding which agents cause disease can provide useful epidemiological information for designing intervention strategies.[6,7] For example, *Vibrio* spp. infection has been associated with raw or undercooked shellfish[6] and non-typhi *Salmonella* spp. is often linked to food-borne outbreaks.[8] In addition to pathogen-specific transmission modes, diarrhea transmission also varies by social and environmental context. For example, it has

been observed that the rate of salmonellosis decreases when the level of development increases in a country.[9]

In spite of the importance of understanding the epidemiology of diarrheal disease, little is known about the etiological agents causing diarrhea in Latin American. For example, in Ecuador the reported incidence of diarrheal disease increased from 17 per 1,000 inhabitants in 1994 to 46 per 1,000 inhabitants in 2012,[10] yet, there is limited researching describing the pathogen diversity of these diarrheal cases. Of the twelve studies published about diarrheic disease in Ecuador during the past 30 years (Table 1), seven studies presented prevalence data that contained scant etiologic information, while five studies analyzed data from case-control studies that highlighted etiological relationship between disease and pathogens.[11,12] Each study focused on at least one pathogen, with some studies identifying on viral causes of diarrhea (e.g., rotavirus and norovirus) and others describing bacterial and parasitic causes. Furthermore, each study was limited to either a rural or urban setting.

A few older studies provide relevant etiological data. In the early 1980s a prospective study in rural northeastern Brazil found that rotavirus, ETEC, STEC, Shigellae and *Giardia lamblia* were important causes of the disease; rotavirus was more prevalent during dry seasons and ETEC during rainy seasons.[13] In the mid 1980's a case control study in urban Brazil revealed that EPEC, rotavirus, *Salmonella*, Shigellae, and ETEC and, co-infections with more than one pathogen were associated with disease.[14] More recently, a passive surveillance in Lima Peru showed that the most common agents associated to diarrhea in under 1 year old

infants were: EAEC, EPEC, *Campylobacter* and rotavirus; EAEC, EPEC and *Campylobacter* were frequently found in asymptomatic individuals.[15,16]

Here we present data from a comparative case-control study describing the etiology of diarrheal disease in an urban (low-income neighborhood in Quito) and a rural (low-income community Northwestern Ecuador) setting. Within each community, stool samples were collected from study participants and tested for 15 important viral, bacterial, and protozoan pathogens.

MATERIALS AND METHODS

Human subjects. We conducted a case -control study recruiting participants in two locations: Guamaní, a low-income urban neighborhood in the Ecuadorian capital city of Quito, and Borbón, a low–income rural community in the Northwestern Ecuador. Guamaní is located in the province of Pichincha at an altitude of 2,700 meters above sea level (m.a.s.l.). The population consists of 65,065 individuals; almost all (98%) households in this area have access to a drinking water source that is considered improved by the World Health Organization (WHO) criteria. Borbón is a small town in Esmeraldas province of northwestern Ecuador, at an approximate altitude of 15 m.a.s.l. Borbón has a population of 7,696 people. In contrast to Guamaní, less than 3 in 5 households in Borbón (58%) have access to an improved drinking water source.[17]

Guamaní study: A case was defined as anyone who came to the local health center suffering from diarrhea (3 loose stools in 24 hours) and had not taken antibiotic or similar medication in the two weeks prior to enrollment. Controls were defined as those who came to the health center for another reason, and did not have diarrheal symptoms during the past two weeks and had not taken antibiotics in the two weeks prior to enrollment. Each enrolled case was matched by age (within one month for those less than a year, within six months for those between 1 and 10 years, and within one year for those older than 10 years) and gender to an appropriate control. The recruitment took place between March and May 2012 during the rainy season (median rainfall is 145.8, 372 and 55 millimeters during March, April and May respectively; mean temperature is 13.9, 13.9 and 15.5 degrees Celsius during March, April and May respectively).[18,19,20] Borbón study: Using a cohort of 400 households that were being followed in a multiyear observational study, each household was visited daily for a period of two weeks. During each visit, a household respondent was asked if any household member had diarrhea (3 loose stools in 24 hours). If more than one household member were case candidates, only one randomly selected case was included in the study. For every case identified three randomly selected controls (individuals without diarrheal symptoms during the past week) were selected among the 400 households in the cohort. Stool samples were collected between July and August 2012, which corresponds to the dry season (median rainfall is 18.2 and 25.6 millimeters during July and August respectively, mean temperature is 25.7 and 25.6 degrees Celsius during July and August respectively).[21,22] All the participants declared that they did not take antibiotics in the past week. Prior to enrollment in the Guamaní and Borbón studies, all individuals signed a consent document

outlining the study procedures, which were approved by the Ethics Committees of University Michigan and Universidad San Francisco de Quito, with local and national government approval by Ecuadorian Public Health Ministry.

Laboratory procedures. All participants provided stool samples to the study team within one hour of the bowel movement; samples were placed in a cooler if the transport to the laboratory took over an hour. All bacteria culturing and sample preservation began less than 8 hours after collection. Identification of Bacteria: To identify diarrheagenic enterobacteria, stool samples were cultured in four agar media: MacConkey Lactose agar (Difco, Sparks, Maryland); MacConkey Sorbitol agar (Difco, Sparks, Maryland); *Salmonella-Shigella* agar (Difco, Sparks,Maryland); and Xylose Lysine Deoxycholate agar (Difco Sparks, Maryland). To investigate *Campylobacter* sp., samples were culture in *Campylobacter* Oxoid agar(Oxoid Ltd, Basingstoke, Hampshire, England) using CampyGen CO_2 (Oxoid Ltd,Basingstoke, Hampshire, England). *Campylobacter jejuni-coli* suspicious colonies were Gram-stained, tested for oxidase and confirmed by PCR (hippuricase and aspartokinase) genes.[23,24] To classify *Vibrio* spp., samples were cultured in TCBS agar (Difco, Sparks, Maryland) sucrose positive and sucrose negative colonies were subjected to API-20E (bio Merieux, Marcy l'Etolie, France) tests. Lactose fermenting and ß-D-glucuronidase positive colonies (using Chromocult agar from Difco, Sparks, Maryland) were subjected to polymerase chain reaction (PCR) for the presence of the *ipaH*, *estA*, *eltA*, *bfpA*, *stx*-1, *stx*-2 genes to detect *E. coli* pathotypes enteroinvasive (EIEC), enterotoxigenic (ETEC), enteropathogenic (EPEC) and shiga-toxigenic (STEC) respectively.[25,26,27] The ß-D-glucuronidase negative *E. coli* were also tested for the

presence of *stx*-1 and *stx*-2 genes. Lactose negative and xylose negative colonies were tested them using API-20E tests to determine whether colonies were *Shigellae* and *Salmonella* spp. Suspect *Shigellae* were confirmed by the presence of *ipaH* gene. Identification of Viruses: Immunochromatographic tests were used for detection of norovirus (Rida®Quick Norovirus, r-Biopharm, Darmstadt, Germany) and rotavirus (Rida®Quick Rotavirus, r-Biopharm, Darmstadt, Germany). Identification of Parasites: Enzyme-Linked Immunosorbent Assay was used to detect *Giardia lamblia* (Ridascreen®*Giardia*, r-Biopharm, Darmstadt, Germany) and *Cryptosporidium parvum* (Ridascreen®*Cryptosporidium*, r-Biopharm, Darmstadt, Germany). Identification of Amoeba: PCR from stool samples was performed in order to detect *Entamoeba histolytica.*[28] We also used microscopy to detect protozoa (including Kinyoun's acid-fast stain).

Statistical analyses. Statistical analyses were performed using Microsoft Office Excel 2010, and CDC Epi Info 7.1.0.6. We calculated odds ratio (OR) to compare pathogens present in case and control samples. A Matched Pair Odds Ratio and the exact confidence intervals were calculated for the Guamaní analyses. For the Borbón analyses, a weighted stratified Mantel Haenszel Odds Ratio was calculated by using three age groups: 0 to 4.9 years, 5 to 13 years and, older than 13 years.

RESULTS

Participants. In Guamaní, the sample size was 200 (100 cases and 100 controls). The median age was 14 years for both the cases and controls (Standard Deviation (SD) = 15.6 and 16.2 for the cases and controls respectively). Children under 5 years represented 49 cases and 49 controls and males represented 54 cases and 54 controls. In Borbón, the sample size was 151 (39 cases and 112 controls). The median age was 13 (SD = 19) and, 24 (SD = 19) years among cases and controls respectively. Males represented 23 cases and 57 controls.

Pathogens. Pathogens were isolated from case samples more often in Borbón (62%) than in Guamaní (51%). Moreover, among case samples, co-infections were more prevalent in Borbón (23%) compared with Guamaní (16%) (Table 2). The odds of pathogen-associated diarrhea, including both single infections and co-infections, were also higher in the rural than in the urban settings (Borbón OR 7.36, 95% CI 3.2-16 and Guamaní OR 4.4, 95% CI 1.9-12). The presence of each specific microorganism, as an agent of a single infection and a co-infection, isolated from both urban and rural locations is shown in Table 3. The pathogens associated with diarrhea were *Shigellae* and rotavirus in Guamaní, and rotavirus and *Giardia lamblia* in Borbón. *Salmonella* spp. and EPEC were not found in any stool sample collected from either location; whereas, *Cryptosporidium* was found in 4 case samples in Borbón and 2 case samples in Guamaní, and was not found in any control samples. All *Cryptosporidium* positive cases were found in

children under 5 years old (4 of 21 in Borbón and 2 of 49 in Guanamí). Higher rates of *Campylobacter* and *Entamoeba histolytica* were found in samples from the urban site in both cases and controls, in comparison to the rural site.

Co-infections. Co-infections, defined as the presence of two or more pathogens in one stool sample, were found in both the urban and rural communities. The presence of more than one pathogen increased the odds of having diarrhea in Borbón for any infection OR 7.36, 95% CI 3.2-16, an infection with only one pathogen OR 3.5, 95% CI 1.5-8, and an infection with 2 pathogens OR 16,95% CI 3.2-86 (Table 2). We found that rotavirus had higher odds for causing diarrhea in both the urban sites (OR 12, CI 95% 1.23-358 and OR 14.8, CI 95% 1.7-132 respectively) if the sample was co-infected. This was also true for *Giardia lamblia* samples from the rural site (OR 8, CI 95% 2-32). Within Borbón co-infections of rotavirus/*G. lamblia* were associated with diarrhea (OR 24, CI 95% 1.9-302). Table 4 presents a complete list of co-infections identified in the stool samples from the studied populations.

DISCUSSION

A vast number of pathogens that can cause diarrheal disease; however, most studies suggest that only a few pathogens account for a majority of the disease burden.[3,6,29] By in large, these same etiological agents have been consistently associated to disease over time, although there are a few important emerging pathogens such as norovirus and *Cryptosporidium parvum*. Our study in

rural and urban Ecuador complements the information provided by the Global Enteric Multicenter Study (GEMS) carried out in sub-Saharan Africa and Asia.

Consistent with GEMS, rotavirus was associated with diarrheal disease in both our urban and rural low-income community, whereas *Shigellae* was only important in our urban setting. *Giardia* was found in a large number of samples in both our urban and rural settings and in cases and controls. The higher prevalence of *Giardia* in Borbón is likely due to the study design differences between the sites. The Guamaní samples came from individuals attending a health center; whereas samples from Borbón came from an active surveillance within the community. Moreover, a single infection with *Giardia* was not associated with diarrhea in either setting, which is consistent with findings from a recently published systematic review.[30] Unlike single infections, a co-infection with *Giardia* and rotavirus was associated with diarrhea in our rural setting. This is in agreement with two prior studies one performed in our study region[31] and the other in Israel.[32] *Giardia* infections also occur in a high proportion of individuals with subclinical or mild symptoms.

Campylobacter coli and *C. jejuni* were isolated in higher numbers in the urban community in both cases and controls, and therefore were not associated with diarrhea. Neither ETEC nor *Shigellae* and EPEC were significantly associated with diarrhea in our rural setting. Data collected from the past 10 years in the same rural site demonstrated consistent association of these three pathogens with diarrhea.[33] Environmental factors, such as climate[33,34] or reduced virulence of pathovars circulating at the time of the study, could also provide an explanation for

the data's presentation. The greater role of *Shigellae* in Guamaní may due to the high population density in the urban setting.

Interestingly, we did not find any non-typhi *Salmonella*. Although this is consistent with the GEMS study, our low sample size does not preclude the fact that non-typhi *Salmonella* occurs at lower levels. In fact, similar studies in Brazil found non-typhi *Salmonella* spp in diarrheagenic stool samples.[35,36,37,38] These discrepancies may result from differences in animal husbandry of livestock, or from other environmental characteristics that may affect transmission of this intestinal pathogen.

Although we only isolated *C. parvum* in 2 samples in Guamaní and 4 samples in Borbón, it is of interest that all 6 samples were from cases of children under 5 years. So in Borbón, 4 of the 21 cases were positive for *C. parvum*. In comparison, 3 of the 21 cases were positive for rotavirus, suggesting that *C. parvum* is an important pathogen in this rural area of Ecuador. This is in general consistent with the GEMS study, which focused on children under five years old. The importance of *C. parvum* as a cause of diarrhea dropped off significantly after the age of 2 in the GEMS data, showing that this pathogen is less likely to cause disease in older children. An important distinction between the GEMS study and ours is that we did not use a severity scale in isolating cases; however, we assume that the Guamaní (urban) clinical cases were generally more severe than the Borbón (rural) community cases.

High rates of asymptomatic carriage of intestinal pathogens were observed in the two locations (27% in Guamaní and 18% in Borbón). Asymptomatic carriage may be due to several factors, including strain pathogenicity, host

immunity against pathogenic factors, intestinal microbiota and herd immunity.[5,39,40] Asymptomatic *Campylobacter* spp. carriage among people living in Latin-America countries has been documented since the late 1980's;[41] specifically, *C. coli* carriage has been documented among people living or handling with pigs, sheep or chickens.[42,43] These findings support the hypothesis that *Campylobacter* spp. is present in environments where people live in close proximity with animals and under poor sanitation conditions.[41]

Some of the discrepancies between rural and urban settings could be explained by ecologic differences. Guamaní is located in the Andes at an elevation of 2,700 meters, has low humidity and samples were collected during the rainy season. Borbón is located near the coast, (low elevation and high humidity) and samples were obtained during the dry season.

Our pathogen detection rate among cases in this study, 51% in the urban and 62% in the rural settings, is consistent with similar studies in South America (13.1% to 62.2%)[36,44] and Ecuador (27% to 65%, Table 1). There are a variety of reasons for not being able to explain 100% of the diarrhea cases. First, not all cases were infectious in origin. Second, we used diagnostic procedures that had sensitivity limitations. Our results differed from GEMS´, possibly because our procedures and reagents (such as EIA kits) were different and our sample sizes were significantly smaller. Additionally, we did not investigate all potential pathogens included in the GEMS study such as adenovirus, *Yersinia enterocolitica*, *Clostridium perfringens* infections, or enterotoxins (*Staphylococcus aureus* or *Bacillus cereus*).

This study confirms that in Ecuador, a small and consistent subset of pathogens-including rotavirus and pathogenic *E. coli*-, are the major causes of diarrhea. The specific set of important etiologic agents, however, varies depending on the urban/rural setting, socioeconomic level, and other important environmental drivers. Thus, site-specific etiological studies can help inform targeted interventions and control strategies.

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Author's contributions: Gabriela Vasco coordinated the collection, survey and recruitment in Guamaní study, conducted microbiological tests of stool samples from Guamaní and Borbón, conducted the statistical analyses, and wrote the first manuscript. Gabriel Trueba supervised the survey and reviewed the manuscript. Richard Atherton and Manuel Calvopiña tested the samples for protozoan pathogens. William Cevallos directed the survey and recruitment in Borbón.Thamara Andrade tested the samples for *Entamoeba histolytica.* Martha Eguiguren wrote the survey for the Guamaní study. Joseph Eisenberg developed the overall study design, revised the manuscript and, advised on the statistical analyses.

Table 1

Summary of published data on the presence of enteric pathogenes in Ecuador. Studies that reported % positive cases used a cross sectional design, and studies that reported an Odds ratio or Risk ratio used a case-control design.

	Year of publication	Number of tested samples	Age range	% positive cases	Measure of Association	Setting	Reference
Campylobacter	1987	100	0 to 24 months	23	-	Urban	45
Campylobacter	1994	177	Under 5 years	3,37	-	Urban	46
Cryptosporidium	1986	169	Under 5 years	11.24	-	Urban	47
Shigellae	1987	100	0 to 24 months	12	-	Urban	45
Shigellae	1994	177	Under 5 years	3.7	-	Urban	46
Shigellae	2007	915	All ages	0.9	0.6 (0.06, 2.7)ǂ	Rural	33
Shigellae	2012	2936	All ages	-	1.6 (1.1–2.4)*	Rural	48
Shigellae and EIEC	2012	3314	All ages	17.1	3.6 (2.4, 5.0)*	Rural	31
Entamoeba histolytica	1987	100	0 to 24 months	1	-	Urban	45
Enteroinvasive *E. coli*	2007	915	All ages	8.9	3.1 (1.6, 6.0)ǂ	Rural	33
Enteropathogenic *E. coli*	2007	915	All ages	1.7	1.9 (0.4, 8.2)ǂ	Rural	33
Enterotoxigenic *E. coli*	2007	915	All ages	7.6	6.9 (2.8, 18.6)	Rural	33
Giardia lamblia	1987	100	0 to 24 months	5	-	Urban	45
Giardia lamblia	1994	177	Under 5 years	63	-	Urban	46
Giardia lamblia	2012	244	Under 5 years	18.3	-	Urban	49
Giardia lamblia	2012	3314	All ages	31.5	2.6 (2.1, 3.2)*	Rural	31
Giardia lamblia	2012	2936	All ages	-	1.5 (1.0, 2.2)*	Rural	48
Norovirus	2012	244	Under 5 years	29.5	-	Urban	49
Rotavirus	1981	702	Under 3 years	21.1	-	Urban	50
Rotavirus	1986	1722	Under 3 years	21.8	-	Urban	51
Rotavirus	1987	100	0 to 24 months	21	-	Urban	45
Rotavirus	1994	177	Under 5 years	7.5	-	Urban	46
Rotavirus	2007	1656	All ages	23.35	9.2 (6.1, 13.9)ǂ	Rural	52
Rotavirus	2008	728	Under 5 years	37	-	Urban	53
Rotavirus	2009	3300	All ages	22.3	10.6 (7.9, 14.3)ǂ	Rural	54
Rotavirus	2012	3314	All ages	22.2	10.7 (7.9, 15.1)*	Rural	31
Rotavirus	2012	2936	All ages	-	1.7 (1.1, 2.5)*	Rural	48
Salmonella spp.	1987	100	0 to 24 months	3	-	Urban	45

ǂOdds ratio (95% CI), OR compared the odds of having diarrhea given infection with the odds of not having diarrhea given infection *Crude Risk ratio (95% CI)

Table 2

Number of infections by case/control status and odds ratios for diarrhea relative to case status in Guamaní and Borbón, Ecuador. Estimates in bold are statistically significant (p<0.05).

	GUAMANÍ (100 cases and 100 controls)				BORBÓN (39 cases and 112 controls)			
	Total (%)	Cases (%)	Controls (%)	Pair-Matched Odds Ratio (95% CI)	Total (%)	Cases (%)	Controls (%)	Mantel-Haenszel Odds Ratio (95% CI)
Zero Pathogens	122 (61)	49 (49)	73 (73)	**0.74 (0.5-1.04)**	107 (71)	15 (38)	92 (82)	**0.22 (0.8-0.61)**
Pathogens (including co-infections)	78 (39)	51 (51)	27 (27)	**4.4 (1.9-12)**	44 (29)	24 (62)	20 (18)	**7.36 (3.2-16)**
1 pathogen	61 (30.5)	35 (35)	26 (26)	**3.14 (1.3-8.7)**	32 (21)	15 (38)	17 (15)	**3.5 (1.5-8)**
Co-infection	17 (8.5)	16 (16)	1 (1)	**19 (1.1-326)**	12 (8)	9 (23)	3 (2.6)	**11 (2.7-43)**
2 pathogens	9 (4.5)	8 (8)	1 (1)	13 (0.7 - 230)	9 (6)	7 (18)	2 (1.8)	**16 (3.2-86)**
3 pathogens	8 (4)	8 (8)	0 (0)	7 (0.3- 135)	3 (2)	2 (5)	1 (0.9)	6 (0.5-68)

Table 3

Pathogen distribution and odds ratio for diarrhea reported as a crude estimate as well as stratified by single infection and co-infection (two or more infections) in Guamaní and in Borbón. Estimates in bold are statistically significant (p<0.05).

		Single infections		Co-infections		All Infections	Single Infections	Co-Infections
	Total	Case	Controls	Case	Controls	Odds Ratio (Exact 95% CI)	Odds Ratio (95% CI)	Odds Ratio (95% CI)
GUAMANÍ						Matched OR		
Rotavirus	10	0	0	10	0	**21 (1.23-358.4)**	1 (0.01-50)	**12 (1.23-358)**
Norovirus	5	2	1	2	0	4 (0.4-197)	2 (0.1-117)	5 (0.24-104)
Shigellae	11	3	0	8	0	**23 (1.35-390)**	7 (0.36-135)	17 (0.98-294)
Campylobacter jejuni	11	7	4	0	0	2 (0.5-8)	2 (0.5-8)	1 (0.02-50)
Campylobacter coli	5	3	2	0	0	1.5 (0.25-9)	7 (0.36-136)	1 (0.02-50)
EIEC	8	3	2	3	0	5 (0.55-236)	2 (0.18-22)	7 (0.36-136)
ETEC (*elt*A)	3	0	1	1	1	0.5 (0.008-9.6)	0.33 (0.0002-15)	1 (0.1-78)
ETEC (*est*A)	3	2	0	1	0	7 (0.36-135)	5 (0.24-104)	3(0.1-74)
STEC (*stx*-1)	3	1	1	1	0	3 (0.12-73)	1 (0.02-50)	1(0.02-50)
Vibrio parahaemolyticus	1	1	0	0	0	3 (0.12-73)	3 (0.12-74)	1(0.02-50)
Giardia lamblia	15	2	7	6	0	1.25 (0.27-6.3)	0.16 (0.004-1.37)	13(0.73-231)
Cryptosporidium parvum	2	2	0	0	0	8 (0.24-104)	5 (0.24-104)	1 (0.02-50)
Entamoeba histolytica	26	9	8	8	1	2.1 (0.82-6.2)	1.1 (0.36-3.7)	15 (0.85-262)
BORBÓN*						Mantel-Haenszel OR		
Rotavirus	9	2	1	5	1	**12 (2.4-60)**	6 (0.52-68)	**14.8 (1.7-132)**
Norovirus	1	0	1	0	0	NA	NA	NA
E. coli Shigellae	2	0	0	1	1	2.92 (0.18-48)	NA	2.92 (0.18-48)
Campylobacter jejuni	1	0	1	0	0	NA	NA	NA
Campylobacter coli	1	0	0	1	0	NA	NA	NA
EIEC	6	1	2	1	2	1.46 (0.25-8.3)	NA	1.44 (0.13-16)
ETEC (*eltA*)	2	2	0	0	0	NA	NA	NA
ETEC (*estA*)	3	1	1	1	0	6 (0.53-68)	2.9 (0.18-48)	NA
Giardia lamblia	25	6	9	7	3	**4.16 (1.7-10.2)**	2 (0.7-6.3)	**8 (2-32)**
Cryptosporidium parvum	4	2	0	2	0	NA	NA	NA
Entamoeba histolytica	5	1	2	2	0	4.6 (0.74-28)	1.4 (0.12-16)	NA

*Two samples from Borbón were not tested for *Entamoeba histolytica* NA: Not enough data for analyses

Table 4

Co-infections found in Guamaní and in Borbón. Estimates in bold are statistically significant (p<0.05).

	TOTAL (%)	Number Cases	Number Controls	OR (95% CI)
Guamaní				
Shigellae /Rotavirus/EIEC	2	2	0	5 (0.24-104)
Shigellae /Rotavirus/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
Shigellae/EIEC	1	1	0	3 (0.12-74)
Shigellae/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
ETEC *elt* /*Entamoeba histolytica*	1	0	1	0.3 (0.01-8)
ETEC *est*/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
Giardia lamblia/*Shigellae* /Rotavirus	3	3	0	7 (0.36-136)
Giardia lamblia/ETEC *elt*/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
Giardia lamblia/Rotavirus	2	2	0	5 (0.24-104)
Norovirus/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
Norovirus/STEC/*Entamoeba histolytica*	1	1	0	3 (0.12-74)
Rotavirus/*Entamoeba histolytica*	2	2	0	5 (0.24-104)
Total	17	16	1	**19 (1.1-326)**
Aggregated[1] *Shigellae* /Rotavirus	6	6	0	13 (0.73-231)
Aggregated *Giardia lamblia*/Rotavirus	5	5	0	11 (0.6-199)
Borbón				
Shigellae /EIEC	1	1	0	NA
Giardia lamblia/*Campylobacter coli*	1	1	0	NA
Giardia lamblia/*Cryptosporidium*	1	1	0	NA
Giardia lamblia/*Shigellae*/ EIEC	1	0	1	NA
Giardia lamblia/EIEC	1	0	1	NA
Giardia lamblia/ETEC *est*	1	1	0	NA
Giardia lamblia/Rotavirus	3	2	1	6 (0.5-68)
Giardia lamblia/Rotavirus/*Entamoeba histolytica*	2	2	0	NA
Rotavirus/*Cryptosporidium*	1	1	0	NA
Total	12	9	3	**17 (3.2-88)**
Aggregated *Giardia lamblia*/Rotavirus	5	4	1	**24.13 (1.9-302)**
Aggregated *Shigellae* /EIEC	2	1	1	5.12 (0.33-79)

NA: Insufficient data for analysis [1]Includes all co-infections that contain both pathogens

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