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Zoonotic Enteropathogens in a semi-rural community close to Quito

María Soledad Sarzosa Moreta

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

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María Soledad Sarzosa Moreta

Firmas

Gabriel Trueba, Ph.D.
Director de la Maestría en Microbiología

Director del Trabajo de Titulación

Jay Graham, Ph.D.

Miembro del Comité de Tesis

Sonia Zapata, Ph.D.

Miembro del Comité de Tesis

Hugo Burgos, Ph.D.
Decano del Colegio de Posgrados

Quito, 15 de diciembre 2016

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Firma del estudiante: _____

Nombre: María Soledad Sarzosa Moreta

Código de estudiante: 00123453

C. I.: 1722169305

Lugar, Fecha Quito, 15 de Diciembre de 2016

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RESUMEN

La diarrea, actualmente sigue siendo una causa importante de morbilidad y mortalidad en niños (0-5 años); aproximadamente 760.000 millones de casos se presentan por año, y se encuentran mayormente concentrados en países en vías de desarrollo. En este estudio se investigó la presencia de siete enteropatógenos zoonosicos: *Campylobacter* (*C. jejuni, C. coli* y *Campylobacter* spp.), *Salmonella* spp., *Yersinia enterocolitica*, *Giardia duodenalis, Cryptosporidium parvum*, *E. coli* enteropatógenica atípica (aEPEC) y *E. coli* productora de Shiga toxinas (STEC), en muestras fecales de niños y animales domésticos, y su posible transmisión zoonótica en una parroquia semi-rural. Esta tesis está compuesta de dos trabajos científicos: en el primero, es un reporte de siete enteropatógenos zoonóticos en dos barrios; uno con alta presencia de animales domésticos y el otro con baja presencia de animales domésticos. El segundo artículo, describió la transmisión zoonótica de *Giardia duodenalis* mediante genotipificación molecular. En el primer artículo, se encontró que: Chinangachi poseía mayor variedad de patógenos que el Centro; *Giardia duonelalis,* aEPEC, and *Cryptosporidium parvum* fueron los patógenos más abundantes en ambas comunidades. Sin embargo no se encontró diferencias significativas en la prevalencia de enteropatógenos zoonóticos entre los hogares con animales y sin animales en ambas comunidades. La genotipificación de *Giardia duodenalis*, permitió la identificación de 3 ensamblajes (ensamblajes AII, BI y C en niños y ensamblajes AV, BIII, BI y C en animales de granja), pero la transmisión zoonótica entre animales y niños aparentemente no fue muy común.

Palabras clave: Yaruqui, Quito, diarrea, animales, enteropatógenos zoonóticos, transmisión, *Giardia duodenalis,* ensamblajes.

ABSTRACT

Diarrhea is a significant cause of morbidity and mortality in children (0-5 years old); approximately 760.000 million cases per year occur and they are mostly concentrated in low and middle income countries (LMICS). We investigated the presence of seven enteropathogens: *Campylobacte*r species (*C. jejuni, C. coli* and *Campylobacter* spp.), *Salmonella* spp., *Yersinia enterocolitica, Giardia duodenalis*, *Cryptosporidium parvum*, enteropathogenic *E. coli* (aEPEC) and Shiga Toxin-producing *E. coli* (STEC), in fecal samples from children and domestic animals and their possible zoonotic transmission in a semi-rural parish. This thesis contains two scientific papers: first paper, describes a study of seven zoonotic enteropathogens in two neighborhoods; one with large numbers and the other with low numbers of domestic animals. The second paper, describes zoonotic transmission of *Giardia duodenalis* through molecular genotyping. In the first paper, we found: that Chinangachi presented more variety of pathogens than Centro; *Giardia duonelalis,* aEPEC, and *Cryptosporidium parvum* were the more prevalent pathogens in both communities; statistical analyses had demonstrated that there was no significant difference in prevalence of zoonotic enteropathogens between households with animals and without animals in both communities. Genotyping of *Giardia duodenalis*, identify the presence of 3 assemblages (assemblages AII, BI and C in children and assemblages AV, BIII, BI and C in livestock) but, zoonotic transmission between livestock and children did not appear to be common.

Key words: Yaruqui, Quito, diarrhea, animals, zoonotic enteropathogens, transmission, *Giardia duodenalis,* assemblages.

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PART I

FIRST SCIENTIFIC PAPER: CHARACTERIZATION OF ZOONOTIC ENETEROPATHOGENS IN HUMANS AND DOMESTIC ANIMALS FOUND IN A SEMI-RURAL COMMUNITY IN QUITO

Abstract

Zoonotic enteropathogens are an important cause of acute diarrhea in children under the age of five years old around the world. Animals play an important role as source of transmission especially in low- and middle income countries (LMICs). In this study, we investigated the presence of seven zoonotic enteropathogens: *Campylobacte*r species (*C. jejuni, C. coli* and *Campylobacter* spp.), *Salmonella* spp., *Escherichia coli* pathotypes (atypical enteropathogenic *E. coli* (aEPEC) and Shiga Toxin-producing *E. coli* (STEC)), *Yersinia enterocolitica, Giardia duodenalis*, and *Cryptosporidium parvum* in two communities of the parish of Yaruqui (with low and high livestock). We found that: we found: that Chinangachi presented more variety of pathogens than Centro; *Giardia duodenalis,* aEPEC and *Cryptosporidium parvum* were the more prevalent pathogens in both communities and statistical analyses determine that there was no significant difference in the prevalence of zoonotic enteropathogens ($X^2(1) = 0.341$, $p > 0.05$) between neighborhoods.

Key words: zoonotic enteropathogens, diarrhea, Yaruqui, animals, prevalence.

Introduction

Diarrhea is still a significant cause of morbidity and mortality in children 5 years old or younger; approximately 123.6 million diarrheal episodes are presented per year (Umesh D. Parashar, 2003) and 760.000 million children are affected and concentrated in low and middle income countries (Fischer Walker C, 2013) (OMS, 2013). Poverty, limited access to clean water, limited sanitary infrastructure, contaminated food and exposure to farm animals are important factors (Teka T, 1996) (Black RE, 2004).

The role of zoonosis in diarrheal disease in industrialized nations is typically associated with food contamination and is considered very important (Cleaveland S. C., 2001) (Woolhouse M. E., 2005). Approximately 9.4 million foodborne illness are caused by zoonotic enteropathogens causing 1.351 deaths in the United States (Elaine Scallan, 2011).The impact of these pathogens in LMICS is estimated in 1.5 billion diarrhea cases annually (Fischer Walker C, 2013), where diarrhea is responsible of 526.000 deaths (UNICEF, 2016).

Some case-control studies have demonstrated the potential of some zoonotic pathogens to cause acute diarrhea in children around the world. *E. coli* pathotypes: atypical enteropathogenic *E. coli* (aEPEC) and Shiga Toxin-producing *E. coli* (STEC) were reported in children from Brazil (Rebecca R. Seigel, 1996), Peru (Ochoa TJ1, 2009) and India (Ghosh AR, 1991); *Campylobacter* spp. was found in Peru (Ochoa TJ1, 2009); *Salmonella* spp. was detected in Mexican and Bangladesh children (Paniagua GL, 2007) (Albert MJ, 1999); while *Y. enterocolitica* has been mentioned as a relevant cause of diarrhea in Poland (Krzysztof Fiedoruk, 2015), Iraq (Kanan TA, 2009) and China (Wang

X, 2015); *Giardia duodenalis* and *Crypstosporidium parvum* are common parasites in LMICS (Stephanie M. Fletcher, 2013), like Ethiopia (de Lucio A, 2016), Mexico (Larrosa-Haro A, 2010) and Libya (Ghenghesh KS, 2016).

A previous report suggested potential transmission of 2 zoonotic enteropathogens (*Campylobacter jejuni* and atypical enteropathogenic *E. coli*) from domestic animals to humans in Ecuador in a region with large livestock and poultry populations (Vasco K., 2016); the present study was carried out in a neighboring location during the same period of time. In the present report, we compared the prevalence of pathogens in households with different levels of contact with livestock.

Material and Methods

Study location.

The study was conducted in 2 communities (Chinangachi (semi-rural) and Centro (semi-urban)) in the Yaruqui parish, located in northeast Quito, the capital of Ecuador.

Ethical considerations.

The study protocol was approved by the Institute for Animal Care and Use Committee at the George Washington University (IACUC#A296), as well as the Bioethics Committee at the Universidad San Francisco de Quito (#2014-135M) and the George Washington University Committee on Human Research.

Specimen collection, transportation and conservation.

Fecal samples were collected from children 0 to 5 years old and from all domestic animals in the same household from February to August of 2015 in two communities.
All the information concerning socio-demographic characteristics of the individuals involved in the study like: gender, age, antibiotic use and contact with animals were obtained the day before sample collection. Fresh fecal samples were collected from children after obtaining informed consent from their parents. We collected the animal fecal samples from the floor, taking care not to contaminate them with other residues. Then samples were transported at 4°C to the laboratory of the Microbiology Institute at Universidad San Francisco de Quito. All samples were cultured as soon as they arrived; two 1.5 ml aliquots of the sample were stored frozen (at -80°C) to run an ELISA

test for *Cryptosporidum parvum* and *Giardia duodenalis* detection; and the other tube was used for DNA extraction. The rest of the sample was conserved with 10% formalin at 4°C for identification of helminths.

Identification of Zoonotic Enterophatogens.

Fecal samples were analyzed for identification of seven zoonotic enteropathogens: atypical enteropathogenic *E. coli* (aEPEC), Shiga Toxin-producing *E. coli* (STEC), *Campylobacter* species (*C. jejuni, C. coli* and *Campylobacter* spp.), *Salmonella spp*, *Yersinia spp, Cryptosporidium parvum* and *Giardia duodenalis*,

Microbiological and Immunological Identification.

Pathotypes of E. coli.

All samples were cultured in MacConkey Lactose Agar (Difco, Saparks, Maryland) at 37°C for 18h, five lactose-positive isolates were cultured in Nutrient Agar (Difco, Sparks, Maryland) at 37°C for 18h, colonies from these five isolates were pooled using a toothpick, then suspended in 300µL of sterile distilled water, and boiled for 10 minutes to release DNA. The five isolates were tested for ß-D glucoronidase activity in Chromocult® Coliform agar (Merck KGaA, Darmstadt, Germany). PCR positive colonies were grown and cryopreserved in Brain and Hearth Infusion medium with 20% glycerol (Difco, Sparks, Maryland) at -80°C.

Yersinia spp.

For *Yersina* spp., all samples were pre-enriched. Samples were first inoculated in PBS 1X by 21 days at 4°C, then cultured in Cefsulodin Irgasan Novobiocin agar (Oxoid Ltd, Basingstoke, Hampshire, England) at 28°C for 24 and 48h. Suspicious colonies were subjected to oxidase test (Bactident Oxidase, Merck) and if they were negative we

confirmed with RapiD-20E (bio Merieux, Marcy, l'Etolie, France).

Salmonella spp.

For *Salmonella* spp., samples were pre-enriched. All samples were first inoculated in Selenite Broth (Merck KGaA, Darmstadt, Germany) at 37°C for 18h, then streaked on Xylose-lysine-deoxycholate agar (Difco, Sparks, Maryland) at 37°C for 18h. Colonies unable to ferment lactose were tested for urease activity (Christenson urea agar, Difco, Sparks, Maryland) and sulfide, indole, motility activity (SIM Medium, Difco, Sparks, Maryland), then suspicious colonies were tested by Rapid-20E (bio Merieux, Marcy, l'Etolie, France).

Campylobacter species.

Samples were cultured on Campylobacter Agar with 5% lysed horse blood and modified Preston Campylobacter Selective Supplement (Oxoid Ltd, Basingstoke, Hampshire, England) (at 42°C for 48h) in microaerobic conditions using CampyGen CO_2 (Oxoid Ltd, Basingstoke, Hampshire, England). All colonies were Gram-stained and tested for oxidase (Bactident Oxidase, Merck).

Giardia duodenalis and Cryptosporidium parvum.

Both parasites were detected using Enzyme-Linked Inmunosorbent Assay (Ridascreen®Giardia, r-Biopharm, Darmstadt, Germany). Also all positive samples for *Giardia duodenalis* ELISA were confirmed by microscopy.

Molecular Determination.

Pathotypes of E. coli.

DNA was used for polymerase chain reaction (PCR) to identify *eae* (Karns J, 2007), *bfpA* (Tornieporth NG, 1995), *stx-1* and *stx-2* (Pollard D, 1990). Colonies of all positive pools were tested individually by PCR.

Salmonella spp.

Serovars were identified by the use of 10 pair of primers in a multiplex PCR in two different reactions (STM and STY), both together allow the identification of 30 different serotypes that represent the most common clinical isolates of *S. enterica subsp. enterica* (Kim S, 2006); reactions were performed whit specifications and conditions described by (Vasco K., 2016). Amplified products were separated by electrophoresis on 2.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator.

Campylobacter spp.

Campylobacter jejuni/coli were confirmed by PCR reaction developed by (Persson S, 2005).

Giardia duodenalis.

Nested PCR was performed for the *tpi* gene (Irshad M. Sulaiman, 2003): the first PCR reaction was conducted in 30 µl, containing 1X PCR Buffer (5X Green GoTaq® Reaction Buffer Promega, Madison, WI, USA), 2,5mM $MgCl_2$ ($MgCl_2$ Promega, Madison, WI, USA), 150µM of each dNTP (dNTP mix Promega, Madison, WI, USA), 0,2µM of each primer, 5 units of Go Taq polymerase (GoTaq® DNA Polymerase Promega, Madison,

WI, USA), 2X BSA (Bovine Serum Albumine Acetylated, Promega, Madison, WI, USA) and 6µl of DNA. For nested reaction, the same concentrations were used except for 1,5mM $MgCl_2$ and 6µl of the primary PCR product was used as template. The same conditions from (Irshad M. Sulaiman, 2003) were used except for the annealing temperatures, for first reaction 60°C, and 50°C for secondary reaction in a (T100 Thermal Cycler *BIO-RAD*, Berkley, CA, USA). The amplified products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator, all positive products for the second reaction were sequenced in (Functional-Biosciences Inc, Madison, WI, USA) and analyzed with MEGA 6.0.

Cryptosporidium parvum.

Positive samples for *Cryptosporidium parvum* ELISA were tested by PCR (N. Jothikumar, 2008), with some modifications. The reaction was conducted in 30 µl, containing 1X PCR Buffer (5X Green GoTaq® Reaction Buffer Promega, Madison, WI, USA), 2mM $MgCl_2$ ($MgCl_2$ Promega, Madison, WI, USA), 200µM of each dNTP (dNTP mix Promega, Madison, WI, USA), 0.25 µM of each primer, 2 units of Go Taq polymerase (GoTaq® DNA Polymerase Promega, Madison, WI, USA) and 6µl of DNA. PCR comprised an initial denaturation step of 2 min at 95°C, then 44 cycles of: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by final extension of infinite at 12°C. The amplified products were separated by electrophoresis on 2.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator.

Identification of other intestinal parasites.

All fecal samples from children were tested by microscopy to detect helminths and protozoa.

Data Analysis.

Chi square test was performed using Microsoft Office Excel 2013 (Redmond, WA, USA) and Graph Pad Software, Inc. (La Jolla, CA, USA).

Results

Participants.

A total of 135 households were studied in both communities. Seventy-four households in Centro: 52 (70%) without animals and 22 (30%) with animals. Sixty-one households in Chinangachi: 19 (31%) without animals and 42 (69%) with animals. In Centro, 143 samples were taken: 89 (62%) human samples, 54 (38%) animal samples; dogs (17%), chickens (7%) and guinea pigs (4%). In Chinangachi, 229 samples were taken: 71 (31%) human samples and 158 (69%) animal samples: dogs (17%), chickens (14%), pigs (10%) and guinea pigs (10%) (Table 1).

Prevalence and distribution of zoonotic enteropathogens in children and livestock from Centro and Chinangachi.

Children in the Centro community had *Giardia duodenalis* (9%, most abundant) followed by aEPEC (13%) and *Salmonella* spp. (2%). Children in Chinangachi presented *Giardia duodenalis* (32%, most abundant), followed by aEPEC and *Crypstosporidium parvum* (6%), *Salmonella* spp, STEC, *C. jejuni* and *Campylobacter* spp had (1%) (Table 2).

Domestic animals in Centro had *Giardia duodenalis* (20%, most abundant), followed by aEPEC (6%) and *Cryptosporidium parvum* (4%). Livestock in Chinangachi had *Giardia duodenalis* (20%), followed by aEPEC and *Crypstosporidium parvum* (8%), *C. coli* (4%), STEC (2%), *Salmonella* spp., *C. jejuni* and *Campylobacter* spp. (1%) (Table 2).

Campylobacter species, STEC and *Yersinia* spp. were absent in Centro, while *Yersinia* spp. was the only pathogen missed in Chinangachi. The source of the pathogens for both communities are shown in Appendix 1 and 2.

Prevalence of other intestinal parasites in children.

Ascaris spp. and *Entomoeba histolytica/dispar* were the only two pathogenic parasites found in both communities, with the following prevalence: *Ascaris* spp. (12%) in Centro and (7%) in Chinangachi; and *Entomoeba histolytica/dispar* (20%) in Centro and (14%) in Chinangachi (Table 3).

Prevalence of zoonotic enteropathogens in children from houses with animals and without animals.

In Centro: *G. duodenalis, aEPEC* (14% for both pathogens) and *Salmonella* spp. (5%) were more prevalent in houses with animals, while aEPEC (15%) was more found in households without animals. In Chinangachi: *G. duodenalis* (37%), *C. parvum* (16%) and aEPEC (11%) were more prevalent in families without animals, while *C. jejuni*, *Campylobacter* spp., STEC and *Salmonella* spp. ((2%) for the four pathogens) were just found in households with animals (Table 4).

Co-infections in children.

Co-infections, defined as the presence of two or more pathogens in one stool sample, were found in both communities. Children from families with animals had more co-infections (Centro (23%); Chinangachi (19%)). The more frequent co-infections were: (*G. duodenalis + Entomoeba histolytica/dispar)* in both communities; *(G.duodenalis + C. parvum)* in Chinangachi and (*aEPEC+ Entomoeba histolytica/dispar*) in Centro (Table 5).

Serovars of *Salmonella* spp.

Trought PCR we could identify 3 servoras; one children, two dogs and one chicken presented serovar Infantis; one children and three dogs had serovar Typhimurium; one children carried serovar Poona (Table 6).

Discussion

The prevalence of zoonotic enteropathogens was greater in Chinangachi than Centro but it was not statistically significant (X^2=0.30, p>0.05). Also Chinangachi showed more diversity of zoonotic enteropathogens than Centro: *Giardia duodenalis*, aEPEC, *Crypstosporidium parvum,* non typhi *Salmonella*, *C. coli,* STEC, *C. jejuni* and *Campylobacter* spp.; while in Centro we found only four enteropathogens: *G. duodenalis,* aEPEC *Salmonella* spp and *Cryptosporidium* parvum (Table 2).

The more prevalent pathogen for both communities was *Giardia duodenalis,* followed by aEPEC and *Cryptosporidium parvum* (Table 2). There was only significant difference in the prevalence of *Giardia duodenalis* (X^2=1.72 p<0.05), (in humans and livestock) between both communities. Respect to co-infections, prevalence of pathogens (X^2=0.34, p>0.05) was not statistically different in families with animals and without animals, in both communities.

Three *Salmonella enterica* serovars were identified in our study in children and livestock: Poona, Infantis and Typhimurium were found in children; while serovar Infantis and Typhimurium were just isolated from livestock (Table 6). Serovars Infantis and Typhimurium are associated with chickens (Basler C F. T., 2014) and dogs (Leopard, 2015), serovar Poona is a rare serotype associated with pet reptiles (Basler C, 2014).

The statistical analyses, determine that neighborhood and presence of animals apparently is not influencing the prevalence of zoonotic enteropathogens in children.

Differences in the sanitary infrastructure between both communities, may be influencing in the prevalence of zoonotic enteropathogens, but further statistical analyses should be performed.

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PART II

SECOND SCIENTIFIC PAPER: CHARACTERIZATION OF *GIARDIA DUODENALIS* INFECTING HUMANS AND DOMESTIC ANIMALS IN A SEMI-RURAL PARISH OF QUITO, ECUADOR

Abstract

We studied the prevalence of *Giardia duodenalis* in children (0-5 years old) and livestock within 5 semi-rural communities in the parish of Yaruqui. We conducted Genotyping, (using *tpi* gene) among samples found positive for *G. duodenalis* by ELISA and microscopy. We observed the presence of 3 assemblages in humans and livestock. Assemblages AII, BI and C were found in children and assemblages AV, BIII, BI and C were present in livestock. In this study, transmission of *G. duodenalis* between domestic animals and children was not common, however two *G. duodenalis* assemblages/sub-assemblages (C and BI), were found in both children and domestic animals.

Key words: Giardia duodenalis, children, livestock, Yaruqui, Genotyping, assemblages.

Introduction

Giardia duodenalis (also known as *G. lamblia and G. intestinales*) is a binucleated flagellate parasite member of diplomonads, and part of Excavata supergroup (Simpson, 2003). The parasite cycle has two important steps; from ingestion of cyst to formation of trophozoite (excystation), and from motile trophozoite to infective cyst (encystation) (Johan Ankarklev, 2010). The cysts can be found in the environment for prolonged periods. The infective dose is low (10 cysts) (Ulloa-Stanojlović FM, 2016) and the parasite is generally acquired by consumption of contaminated water and food (Johan Ankarklev, 2010).

This parasite causes one of most common parasitic infections worldwide and contributes to an estimated of 280 million symptomatic human infections (called giardiasis) (WHO, 2015). The infection is characterized by watery diarrhea, epigastric pain, nausea, vomiting and weight loss. Symptoms are severe in children and chronic infections are common but about a half of them are asymptomatic during epidemics (Cotton JA, 2011) (Buret, 2007). In developing countries *Giardia* is not associated with acute diarrhea in children but some data suggest that in their first trimester of life, infants could present it (M., 2012).

Genetic studies of *G. duodenalis* through the technique of Multilocus genotyping, based on the use of four markers *bg, gdh, tpi* and *SSuRNA*, shows 8 genetic groups within giardia complex known as assemblages (Xiao Y. F., 2011). Assemblages A and B are found in humans and animals. Assemblages (C to H), are found in animals: assemblage C and D have been identified in dogs, wolves, coyotes and cats;

assemblage E have been found in pigs, goats and water buffaloes; assemblage F have been found in cats; assemblage G have been found in rats and assemblage H have been identified in seals (Xiao Y. F., 2011). In some occasions these assemblages have been found in humans (Johan Ankarklev, 2010). This is why some authors consider *G. duodenalis* a zoonotic pathogen, but this fact is still issue of discussion because some sub-assemblages (like AII) have demonstrated to cause diarrhea only in humans but not in animals (Xiao Y. F., 2011).

Previous studies made in Ecuador reveal that giardiasis has a prevalence of 20 to 24% in humans on rural and urban communities (Rinne S, 2005) (Atherton R, 2013) (Vasco G, 2014), few reports of prevalence in animals are found; prevalence of 20%, 25% was found in dogs in Cuenca and Loja (Calle, 2015) (Castillo, 2011), studies in other animal species were not found. Just one study of genotyping is present in Ecuador and was made in human adult samples from rural communities of province of Esmeraldas, with use of *gdh* marker (Atherton R, 2013). We explored the possibility of zoonotic transmission of *Giardia duodenalis* in children (0-5) years old in 5 semi-urban communities in Ecuador.

Material and Methods

Study Area.

The study was conducted between June 2014 to July 2016 in 5 semirural communities Oton de Velez (Community 1), Chinangachi (Community 2), Centro (Community 3), Tejar (Community 4), San Vicente (Community 5,) on parish Yaruqui, located on North East of Quito, capital of Ecuador. Yaruqui has highest of 2.527 m. a. s. l., temperature between 12 to 18 °C, and area of 3.116.28 Km^2.

Ethical considerations.

The study protocol was approved by the Institute for Animal Care and Use Committee at the George Washington University (IACUC#A296), as well as the Bioethics Committee at the Universidad San Francisco de Quito (#2014-135M) and the George Washington University Committee on Human Research Institutional**.**

Sampling Techniques.

Fecal samples were collected from children between ages of 0 to 5 years and all the animals that lived with the children, between June 2014 to July 2016 in 5 communities. A total of 253 samples from children and 592 samples from domestic animals were collected.

Specimen collection, transportation and conservation.

All the information concerning to their socio-demographic characteristics of the individuals of the study: like sex, age, use of antibiotics and contact with animals were taken the day before sample collection. Fresh fecal samples were collected from children with the previous concern of them parents, using a clean fecal container and spoon, also the samples from animals were collected from the floor, taking care not to contaminate them with the residues of the floor and labeled correctly, then samples were transported at 4°C, as soon as possible to the lab. On the lab a cryo tube was filled to practice ELISA and other for DNA extraction, both were saved at -80°C. Residue sample were conserved with10% formalin at 4°C, for microscopy.

Identification of *G. duodenalis* and DNA extraction.

All samples were tested for detection of *G. duodenalis* using Enzyme-Linked Inmunosorbent Assay (Ridascreen®Giardia, r-Biopharm, Darmstadt, Germany). Positive samples were confirmed by microscopy to detect cysts and trophozoites of *G. duodenalis* and other intestinal parasites using saline solution (0.9% sodium chloride solution) and Lugol's iodine staining at 10X and 40X magnifications. DNA extraction was done using (PowerFecal® DNA Isolation Kit MO BIO Laboratories IncCarlsbad, CA, USA), and DNA was preserved at -20°C until PCR.

Nested PCR.

All extracted DNA from fecal samples that were positive for ELISA and Microscopy for *G. duodenalis,* were tested using nested PCR amplification of the: *SSU-rRNA* (Richard M. Hopkins, 1997), *bg* (Corrado Minetti, 2015), *gdh* (Jianbin Ye, 2014) and *tpi* (Irshad

M. Sulaiman, 2003) with the correspond primers. But, PCR protocols for *SSU-rRNA, bg* and *gdh* were tested with original protocols and some modifications without success.

Just PCR for *tpi* gene was successfully performed with some modifications from the original, obtaining a product of 605pb for first and 530pb for secondary reactions. The first PCR reaction was conducted in 30 µl, containing 1X PCR Buffer (5X Green GoTaq® Reaction Buffer Promega, Madison, WI, USA), 2,5mM $MgCl_2$ ($MgCl_2$ Promega, Madison, WI, USA), 150µM of each dNTP (dNTP mix Promega, Madison, WI, USA), 0,2µM of each primer, 5 units of Go Taq polymerase (GoTaq® DNA Polymerase Promega, Madison, WI, USA), 2X BSA (Bovine Serum Albumine Acetylated, Promega, Madison, WI, USA) and 6µl of DNA. For nested reaction the same concentrations were used except for 1,5mM $MgCl_2$ and 6µl of the primary PCR product was used as template; PCR water was used as negative control for both reactions. The same conditions from (Irshad M. Sulaiman, 2003) were used except for the annealing temperatures, for first reaction 60°C, and 50°C for secondary reaction in a (T100 Thermal Cycler *BIO-RAD*, Berkley, CA, USA). The amplified products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV trans-illuminator. The PCR was conducted in Microbiology Institute San Francisco de Quito University.

DNA sequencing and Phylogenetic Analysis.

All samples positive for *tpi* nested PCR, were sent to be sequenced in (Functional-Biosciences Inc, Madison, WI, USA) Nucleotide sequences were analyzed and compared to those in the Gene Bank, (Appendix 3). Nucleotide alignments were carried with Clustal and the phylogenetic tree was generated with MEGA 6.0 using Neighbor Joining method.

Statistical Analysis.

Chi square test was performed with Graph Pad Software, Inc. (La Jolla, CA, USA).

Results

Prevalence of *Giardia duodenalis* in Yaruqui.

From a total of 845 fecal samples collected in the both years, 131 samples were positive for *Giardia duodenalis* by ELISA and microscopy; 63 (48.0%) were from children, 39 (29.8%) from dogs, 13 (9.9%) from pigs, 6 (4.6%) from rabbits, 3 (2.3%) from chickens, 3 (2.3%) from guinea pigs, 2 (1.5%) from cats, 1 (0.76%) from quail and 1 (0.76%) from sheep. Community 3 (Chinangachi) had the highest prevalence of *Giardia* 41.2% followed by Community 1 (Oton) (26.7%), Community 2 (Centro) (14.5%), Community 5 (9.1%) and Community 4 (San Vicente) (8.4%) (Table 7).

DNA sequencing and Phylogenetic Analysis.

We were able to amplify the *tpi* gene from 10 human and 5 animal samples (out of 131 *G. duodenalis* positive samples); sequences of 7 (46.7%) amplicons belonged to assemblage A (6 amplicons from children were sub-assemblage AII and 1 amplicon from dog sub-assemblage AV). Four amplicons (26.7%) belonged to assemblage B (1 amplicon from dog was sub-assemblage BIII, 2 amplicons from children and 1 from rabbit were sub-assemblage BI). Four amplicons belonged to assemblage C (26.7%), (2 were from children, 1 from pig and 1 from a dog). A summary of the origin of the DNA sequences and their accession numbers for Gen Bank are found in Table 8. The phylogenetic tree, which indicates similarity between sequences was generated with MEGA 6.0 through Neighbor Joining method (Figure 1).

Association between assemblages and Diarrhea.

From 10 human fecal samples that amplified the *tpi* gene, 2 were fluid (1 assembly AII and one assembly C). The other 8 samples presented a normal consistency.

Discussion

Most human samples (n = 6) contained amplicon sequences belonging to sub-assemblage AII, which indicate human origin (Thompson, 2008; Xiao L. a., 2008; Caccio, 2005) and did not overlap with sequences found in animals. The sub-assemblage AV has been previously reported in dogs, similar to what we observed (Xiao Y. F., 2011).

Two human samples produced amplicons with sequences showing high nucleotide similarity to assembly C, similar sequences were found in a dog and a pig; this assemblage has been found in animals, mostly dogs and others such as: foxes, coyotes and seals (Covacin C, 2011) (Reboredo-Fernández A, 2015); in some occasions it has been reported in humans too. (Traub, 2004).

Two other sequences from humans were sub-assemblage BI which occasionally infect humans but is common in animals (S.M. Caccio, 2008). One amplicon from a rabbit belonged to sub-assemblage BI and it was also found in a child in the same household as the rabbit. Another amplicon from a dog was sub-assemblage BIII which according to the literature, is common in humans (Tawin Inpankaew, 2014).

Communities that had more animals (Communities 1 and 3) had the highest prevalence of *G. duodenalis* (26.7%, 41.2% respectively). In contrast, people from more urban communities (Communities 2, 5, 4) had lower prevalence (14.5%, 9.1% and 8.4% respectively) however this difference was not significant (X^2 =0.23, p>0.05).

Most *G. duodenalis* human positive samples (80%) had normal consistency, which may indicate that infections were asymptomatic. Asymptomatic carriage of *G. duodenalis* has been described in Ecuador by (Vasco G, 2014), (Atherton R, 2013) and other reports also indicate that *G duodenalis* does not cause acute pediatric diarrhea among infants and children in developing countries (Levine, 2012).

For the genotyping procedure we were able to amplify only *tpi* gene, instead of the four markers *SSuRNA, gdh, bg* and *tpi* which are recommended to identify sub-assemblages (Xiao Y. F., 2011)(Caccio S. M., 2008) (Traub, 2004) However in this report the nucleotide sequence of *tpi* contained polymorphisms which were easily assigned to different sub-assemblages.

We were able to amplify the *tpi* from only 15 samples 11.5% (15 out of 131 samples). Studies from distinct authors (Schuurman T, 2007)(Christen Rune Stensvold, 2012) (Dorien Van den Bossche, 2015), had demonstrated that: microscopy, immunoassay and PCR had different specificity ranges. Genotyping using PCR could be hampered affected by some factors including the hardness of the cysts walls (Surl CG, 2011), the presence of PCR inhibitors such as bile salts, carbohydrates and heme (Oikarinen S, 2009).

In conclusion, we found evidence of zoonotic transmission in four children (two children amplicons belonged to sub -assemblage BI and two to assemblage C). The Chi square test, demonstrates that there was no association between the prevalence of *G. duodenalis* and presence of animals in Yaruqui.

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PART III

TABLES AND FIGURES

Neighborhood	**El Centro (urban)**	**Chinangachi (semi-rural)**
Total households visited	**n = 70 (%)**	**n = 61 (%)**
HHs with animals	22 (30)	42 (69)
HHs without animals	52 (70)	19 (31)
Child fecal samples collected	**n = 89**	**n = 71**
Animal fecal samples collected	**n = 54**	**n = 158**
Chickens	10 (7)	32 (14)
Guinea pigs	6 (4)	22 (10)
Pigs	2 (1)	23 (10)
Dogs	25 (17)	37 (16)
Cattle	0	10 (4)
Rabbits	2 (1)	7 (3)
Ducks	1 (1)	7 (3)
Sheep	1 (1)	4(2)
Cats	5 (3)	7 (3)
Quail	0	1(1)
Geese	0	2 (1)
Goat	1 (1)	3 (1)
Turkey	0	3 (1)
Parakeet	1 (1)	0

Table 1. Descriptive analysis of study households and source of fecal samples collected in Yaruqui, Ecuador.

	Centro				**Chinangachi**			
	Human		**Livestock**		**Human**		**Livestock**	
Pathogens	**+/Total**	**P**	**+/Total**	**P**	**+/Total**	**P**	**+/Total**	**P**
C. jejuni	0/89	0,00	0/54	0,00	1/71	0,01	2/158	0,01
C. coli	0/89	0,00	0/54	0,00	0/71	0,00	7/158	0,04
aEPEC	12/89	0,13	3/54	0,06	4/71	0,06	13/158	0,08
*Campylobacte*r spp.	0/89	0,00	0/54	0,00	1/71	0,01	2/158	0,01
STEC	0/89	0,00	0/54	0,00	1/71	0,01	3/158	0,02
Salmonella spp.	2/89	0,02	0/54	0,00	1/71	0,01	6/158	0,04
Giardia duodenalis	8/89	0,09	11/54	0,20	23/71	0,32	31/158	0,20
Cryptosporidium parvum	0/89	0,00	2/54	0,04	4/71	0,06	8/158	0,05
Yesinia spp.	0/89	0,00	0/54	0,00	0/71	0,00	0/158	0,00
Total	**20/89**	**0,25**	**15/54**	**0,30**	**35/71**	**0,49**	**72/158**	**0,46**

Table 2. Prevalence of pathogens in children and livestock in the two Communities of Yaruqui.

Parasites	Centro		Chinangachi	
	+/Total	Prevalence	+/Total	Prevalence
Ascaris spp.	11/89	0,12	5/71	0,07
Entomoeba histolytica/dispar	18/89	0,20	10/71	0,14
Total	**29/89**	**0,33**	**15/71**	**0,21**

Table 3. Other intestinal parasites found in children in Yaruqui

Pathogen	Centro		Chinangachi	
HHWA	**+/Total**	**Prevalence**	**+/Total**	**Prevalence**
C. jejuni	0/22	0,00	1/42	0,02
Campylobacter spp.	0/22	0,00	1/42	0,02
aEPEC	3/22	0,14	3/42	0,07
STEC	0/22	0,00	1/42	0,02
Salmonella spp.	1/22	0,05	1/42	0,02
G. duodenalis	3/22	0,14	13/42	0,31
C. parvum	0/22	0,00	3/42	0,07
HHWOA	**+/Total**	**Prevalence**	**+/Total**	**Prevalence**
C. jejuni	0/52	0,00	0/19	0,00
Campylobacter spp.	0/52	0,00	0/19	0,00
aEPEC	8/52	0,15	2/19	0,11
STEC	0/52	0,00	0/19	0,00
Salmonella spp.	1/52	0,02	0/19	0,00
G. duodenalis	6/52	0,12	7/19	0,37
C. parvum	0/52	0,00	3/19	0,16

Table 4. Prevalence of pathogens in children from households with animals (HHWA) and households wihitout animals (HHWAO) for both communities in Yaruqui.

Co infection	Centro				Chinangachi			
	HHWA		HHWOA		HHWA		HHWOA	
	+/Total	P	+/Total	P	+/Total	P	+/Total	P
G. duodenalis + Entomoeba histolytica/dispar	1/22	0,05	1/52	0,02	1/42	0,02	1/19	0,05
G. duodenalis + Campylobacter spp.	0/22	0,00	0/52	0,00	1/42	0,02	0/19	0,00
G. duodenalis + aEPEC	0/22	0,00	0/52	0,00	2/42	0,05	0/19	0,00
G.duodenalis + C. parvum	0/22	0,00	0/52	0,00	2/42	0,05	2/19	0,11
G. duodenalis + Ascaris spp	0/22	0,00	0/52	0,00	1/42	0,02	0/19	0,00
aEPEC+ Entomoeba histolytica/dispar	1/22	0,05	1/52	0,02	0/42	0,00	0/19	0,00
aEPEC+ Ascaris spp.	1/22	0,05	0/52	0,00	0/42	0,00	0/19	0,00
Ascaris spp. + Entomoeba histolytica/dispar	1/22	0,05	0/52	0,00	0/42	0,00	0/19	0,00
G.dudodenalis + aEPEC+ Entomoeba histolytica/dispar	1/22	0,05	0/52	0,00	0/42	0,00	0/19	0,00
G. duodenalis + STEC + C. parvum	0/22	0,00	0/52	0,00	1/42	0,02	0/19	0,00
Total	**5/22**	**0,23**	**2/52**	**0,04**	**8/42**	**0,19**	**3/19**	**0,16**

Table 5. Co-infections in children from households with animals (HHWA) and without animals (HHWAO) for both communities in Yaruqui.

Specie	Serovar		
	Poona	Infantis	Typhimurium
Children	1	1	1
Chiken		1	
Dog		2	3
Total	**1**	**5**	**4**

Table 6. Serovars of *Salmonella enterica subsp enterica* in children and animals in Yaruqui.

	Communities						
Source	**C1 Oton**	**C2 Centro**	**C3 Chinangachi**	**C4 San Vicente**	**C5 El Tejar**	**Total (number of samples)**	**Total Prevalence in animal species**
Children	22	8	23	4	6	63	**48,0**
Chicken	0	0	3	0	0	3	**2,3**
Guinea pigs	1	0	2	0	0	3	**2,3**
Pig	2	0	10	1	0	13	**9,9**
Dogs	5	9	14	5	6	39	**29,8**
Rabbit	4	0	1	1	0	6	**4,6**
Sheep	1	0	0	0	0	1	**0,76**
Cat	0	2	0	0	0	2	**1,5**
Quail	0	0	1	0	0	1	**0,76**
Total (n)	35	19	54	11	12	131	**100**
Prevalence in Communities	**26,7**	**14,5**	**41,2**	**8,4**	**9,1**	**100**	

Table 7. Prevalence of *Giardia duodenalis* in Yaruqui, by neighborhood and animal species.

Infected host	Community	Assemblages and sub-assemblages	Accesion number Gen Bank
Dog	Comunity 2	AV	KX830814
Dog	Comunity 4	C	KX830811
Human	Comunity 2	AII	KX830802
Human	Comunity 3	BI	KX830806
Human	Comunity 3	AII	KX830804
Human	Comunity 3	C	KX830808
Human	Comuntity 3	AII	KX830805
Human	Comunity 3	AII	KX830815
Human	Comunity 3	AII	KX830803
Human	Comunity 4	BI	KX830809
Human	Comunity 5	AII	KX830813
Rabbit	Comunity 4	BI	KX830810
Swine	Comunity 3	C	KX830807
Dog	Comunity 5	BIII	KX830812
Human	Comunity 5	C	KX830816

Table 8. Assemblages and sub-assemblages of Giardia duodenalis with use of tpi gene in Yaruqui with their Gen Bank accession numbers.

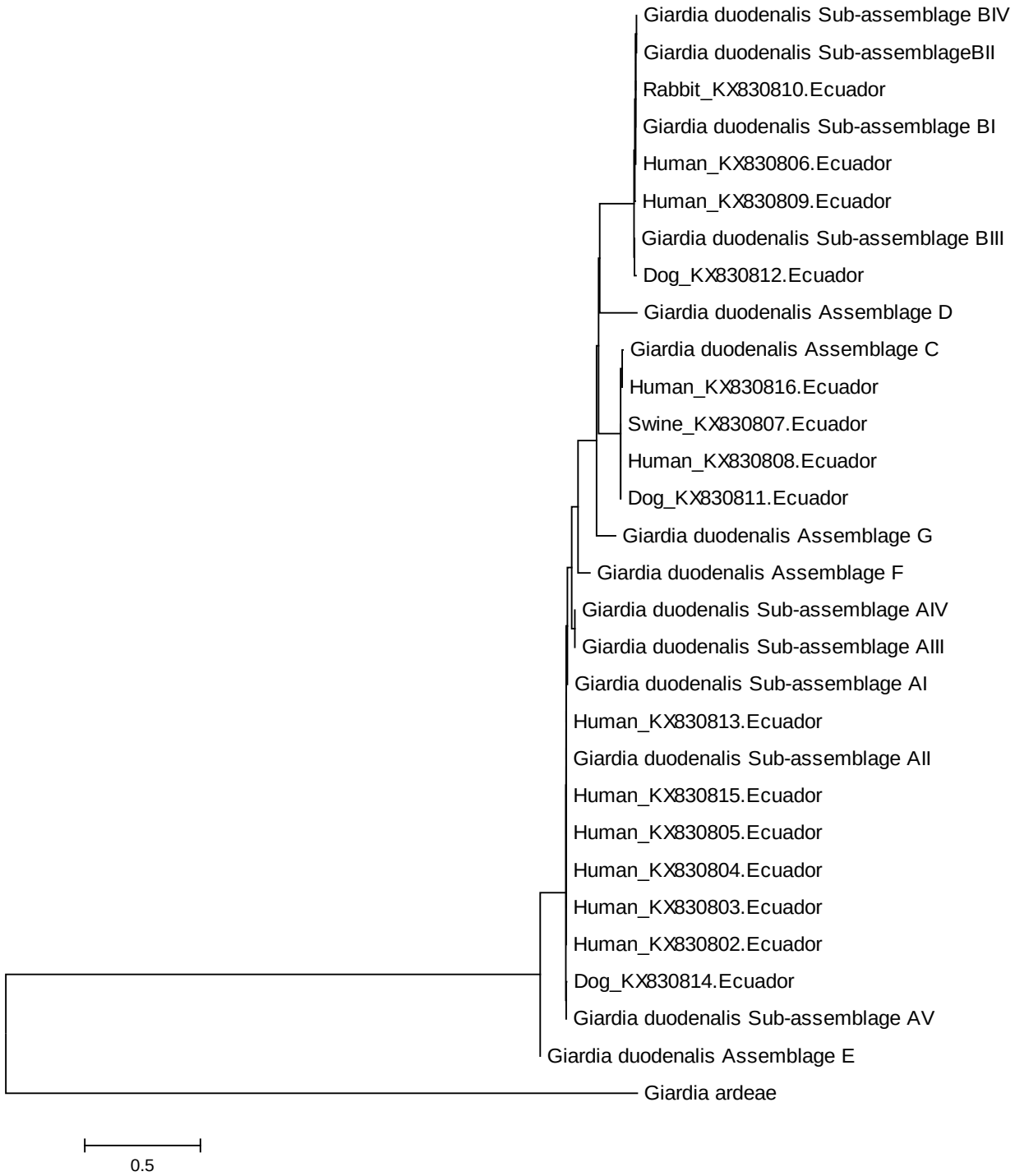

Figure1. Phylogenetic tree of *tpi* gene sequences from Yaruqui using Neighbor Joining method. All accession numbers for the sequences from Ecuador are shown in the tree and accession numbers from sequences from Gen Bank are shown in Appendix 3.

PART IV

ANNEXES

Source	# Samples	(%)	*C. jejuni*	(%)	*C. coli*	(%)	*aEPEC*	(%)	*Campylobacter sp*	(%)	*STEC*	(%)	*Salmonella sp*	(%)	*Yersinia*	(%)	*Giardia duodenalis*	(%)	*Cryptosporidium parvum*	(%)
Children	89,0	62,2	0,0	0,0	0,0	0,0	12,0	8,4	0,0	0,0	0,0	0,0	2,0	1,4	0,0	0,0	8,0	5,6	0,0	0,0
Chickens	10,0	7,0	0,0	0,0	0,0	0,0	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Guinea pigs	6,0	4,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Pigs	2,0	1,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Dogs	25,0	17,5	0,0	0,0	0,0	0,0	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	9,0	6,3	0,0	0,0
Cattle	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Rabbits	2,0	1,4	0,0	0,0	0,0	0,0	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Ducks	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Sheep	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Cats	5,0	3,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	2,0	1,4	2,0	1,4
Quail	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Geese	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Goat	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Horse	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Parakeet	1,0	0,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Total	**143,0**	**100,0**	**0,0**	**0,0**	**0,0**	**0,0**	**15,0**	**10,5**	**0,0**	**0,0**	**0,0**	**0,0**	**2,0**	**1,4**	**0,0**	**0,0**	**19,0**	**13,3**	**2,0**	**1,4**

Appendix 1. Source of enteropathogens in Centro.

Source	# Samples	(%)	*C. jejuni*	(%)	*C. coli*	(%)	*aEPEC*	(%)	*Campylobacter sp*	(%)	*STEC*	(%)	*Salmonella sp*	(%)	*Yersinia*	(%)	*Giardia duodenalis*	(%)	*Cryptosporidium parvum*	(%)
Children	71,0	31,0	1,0	0,4	0,0	0,0	4,0	1,7	1,0	0,4	1,0	0,4	1,0	0,4	0,0	0,0	23,0	10,0	4,0	1,7
Chickens	32,0	14,0	1,0	0,4	2,0	0,9	4,0	1,7	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0	3,0	1,3	1,0	0,4
Guinea pigs	22,0	9,6	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	4,0	1,7	1,0	0,4
Pigs	23,0	10,0	0,0	0,0	2,0	0,9	2,0	0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	8,0	3,5	1,0	0,4
Dogs	37,0	16,2	0,0	0,0	0,0	0,0	6,0	2,6	1,0	0,4	1,0	0,4	5,0	2,2	0,0	0,0	14,0	6,1	2,0	0,9
Cattle	10,0	4,4	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Rabbits	7,0	3,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0
Ducks	7,0	3,1	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Sheep	4,0	1,7	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4
Cats	7,0	3,1	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Quail	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4	1,0	0,4
Geese	2,0	0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Goat	3,0	1,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Turkey	3,0	1,3	1,0	0,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,0	0,4
Horse	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Total	**229,0**	**100,0**	**3,0**	**1,3**	**7,0**	**3,1**	**17,0**	**7,4**	**3,0**	**1,3**	**4,0**	**1,7**	**7,0**	**3,1**	**0,0**	**0,0**	**54,0**	**23,6**	**12,0**	**5,2**

Appendix 2. Source of enteropathogens in Chinangachi.

Accesion Number	Especie/Assemblage and subassemblage
AF069556	*Giardia duodenalis* Subassemblage AI
AF069557	*Giardia duodenalis* Subassemblage AII
EU637582	*Giardia duodenalis* Subassemblage AIII
JQ928710	*Giardia duodenalis* Subassemblage AIV
EF688030	*Giardia duodenalis* Subassemblage AV
EF688030	*Giardia duodenalis* Subassemblage BI
BAH34c8B	*Giardia duodenalis* Subassemblage BII
AF069561	*Giardia duodenalis* Assemblage BIII
AF069560	*Giardia duodenalis* Assemblage BIV
AY228641	*Giardia duodenalis* Assemblage C
DQ246216	*Giardia duodenalis* Assemblage D
AY655705	*Giardia duodenalis* Assemblage E
AF069558	*Giardia duodenalis* Assemblage F
EU781013	*Giardia duodenalis* Assemblage G
AF069564	*Giardia ardeae*

Appendix 3. Accession numbers for the sequences obtained from Gene Bank for the *tpi* gene