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***Campylobacter jejuni* and *Campylobacter coli* in children from communities in Northeastern Brazil: molecular detection and relation to nutritional status**

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INTRODUCTION

Campylobacter is a leading bacterial cause of food-born disease worldwide (Center for Disease Control – CDC/USA, 2009; Vaillant *et al*, 2005), representing a huge health challenge. This mainly zoonotic genus includes at least 18 species (Humphrey *et al*, 2007; On, 2001) of which *Campylobacter jejuni* and *Campylobacter coli* are the most common pathogens and account for the majority of diagnosed human *Campylobacter* infections (Tam *et al*, 2003; World Health Organization – WHO, 2001). Young children remain most susceptible both in the developing and the developed world (Amieva, 2005; Coker *et al*, 2002; Huilan *et al*, 1991; Podewils *et al*, 2004). Rarely, some infections due to specific *C. jejuni* serotypes result in serious post-infectious complications like bacteremia, arthritis, Reiter's syndrome and Guillain-Barré syndrome (GBS) (Hannu *et al*, 2002; Kaira *et al*, 2009; Nielsen *et al*, 2010). *Campylobacteriosis* is also the commonest risk factor for post-infectious irritable bowel disease (IBD), which occurs in approximately 20–30% patients following infection (Marshall *et al*, 2006).

C. jejuni can disrupt the integrity of the intestinal barrier by targeting epithelial tight junctions (MacCallum *et al*, 2005; Chen *et al*, 2006). Recently, it was demonstrated that *Campylobacter* infection promotes translocation of non-invasive bacteria via a lipid raft-mediated transcellular process, an event that may be associated with intestinal inflammation and even the onset of IBD in susceptible individuals (Kalischuk *et al*, 2009). *Campylobacter* can also present as an opportunistic infection, perhaps related to immunosuppression seen with malnutrition (Kakai *et al*, 1995; Fernández *et al*, 2008). Other virulence factors can also be involved in the pathogenesis of *Campylobacter* infections. (Al-Mahmeed *et al*, 2006, Zilbauer

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et al, 2008). However, the association of *Campylobacter* infection with malnutrition remains unclear and is the focus of this report.

Routine detection of *Campylobacter* species in clinical laboratories is based on culture of stool specimen on selective media followed by phenotypic identification. *Campylobacter* species appear to possess uniquely fastidious growth requirements and culture methods are also biased toward the detection of *C. jejuni* and *C. coli* (Moore et al, 2005). Molecular methods based on Polymerase Chain Reaction (PCR) amplification have several advantages over classical bacteriology (concerning to detection limits, species identification level, speed and automation possibility) and may provide an alternative to culture methods for the detection of *Campylobacter*, particularly for epidemiological studies, where many samples are examined and diagnosis can be expensive and laborious (Abubakar et al, 2007; Ajjampur et al, 2008; Amar et al, 2004). The application of PCR-based assays to the detection of *Campylobacter* species in clinical and food samples has been previously reported by several research groups since 1992 (Kulkarni et al, 2002; Lawson et al, 1999; Linton et al, 1997; Lübeck et al, 2003; Oyofe et al, 1992; Samie et al, 2007; Waegel and Nachamkin, 1996; Vanniasinkam et al, 1999).

In the present study we determined the prevalence of *C. jejuni* and *C. coli* using molecular biology probes and its association with nutritional status in children from urban communities in Northeastern Brazil. In addition, we explored the occurrence and variability of cytolethal distending toxin (CDT) encoding genes from *C. jejuni* positive samples, evaluated here as a virulence factor.

MATERIAL AND METHODS

Ethical clearance, study site and population

This study was approved by the local and national Ethical Committee in Brazil and by the University of Virginia Institutional Review Board. A consent form was read and signed by the parents or guardians of each child. The study site was at urban communities located close by to the Institute of Biomedicine for Brazilian Semi-Arid & Clinical Research Unit/Center for Global Health (IBSAB&UPC/CGH), Federal University of Ceara, in the city of Fortaleza, state of Ceara, Brazil.

A survey was done from March to July 2007, during the rainy season, to screen for enteric pathogens in children 2-36 months old. A total of 325 children were screening and they were divided in two groups, diarrhea (83 children) and non-diarrhea, the controls (242 children). Diarrhea was defined as three or more unformed or watery stools, in a 24 hour period, as noted by the mother or caretaker. The diarrhea group was defined as children with history of diarrhea in the last two weeks as reported by their parents or guardian. Age matched controls were randomly selected using a list of sequential random numbers in ascending order supplied by a computer program. In total, 166 children (2-36 months old; 83 cases and 83 controls) were enrolled in this study.

Demographic data and anthropometric measures

An experienced field team, composed of one nurse coordinator and three healthcare workers, participated in the field activities of this study. The demographic and clinical data were accessed in a case report form applied by the healthcare workers, followed and revised by the field nurse coordinator.

Anthropometric measures, including weight, height, arm circumference and skin fold thickness were collected. The arm mid-upper circumference was obtained using a tape made from a flexible material for pediatric use. The measurement of skin fold thickness was measured from

the midpoint of the posterior non-dominant arm, using a caliper. Also, each child wore light clothing to measure the weight. The weight of each child was measured using a calibrated weighing scale with 100 grams (g) precision (Tanita Solar Scale, Tanita Corporation of American Inc., Arlington, IL, USA). The length or height was measured in the supine position for children under 24 months old and standing for children aged 24 months or older. An anthropometric rod was used for this measurement with intervals of 0.1 centimeter (cm). We also calculated the body mass index (BMI) and z-scores for height-for-age (HAZ), weight-for-age (WAZ) and weight-for-height (WHZ).

Stool sample collection and transport

Stool samples were collected from all children participating in this study. The samples were collected at each household and transported in iced boxes within 4 hours to the laboratory at UPC/IBIMED. All the samples were further aliquoted without dilution. These aliquots were kept in the freezer at -80°C until all the molecular tests were performed.

Bacterial culture and maintenance

We used as controls *C. jejuni* ATCC 33291 and *C. coli* INCQS/FIOCRUZ 00263. *C. coli* control was provided by the Reference Microorganisms Laboratory of the National Institute of Quality Control in Health – Oswaldo Cruz Foundation (INCQS/FIOCRUZ, Rio de Janeiro, RJ, Brazil). Both agents were incubated separately, in a microaerobic atmosphere at 37°C using culture medium *Difco*™ *Columbia Blood Agar* base (Difco BD® 279240, DIFCO-BD - Sparks, MD, USA) with addition of 5% of sheep blood, *Campylobacter Growth Supplement* (Oxoid SR0232E) and *Campylobacter Blaser-Wang Selective Supplement* (Oxoid SR0098E, OXOID - Cambridge, GB), according to manufacturer's instructions. Briefly, the lyophilized agents were reconstituted with sterile saline solution and an aliquot of solution was added to the culture plates. After the period of growth (48 hours), the microorganisms were analyzed for their morphological characteristics using Gram staining and cytochrome oxidase test. *Campylobacter* sp. is a Gram-negative rod with a typical morphology in stained smears in the form of “S” gull wings or in large spirals.

Genomic DNA

QIAamp DNA stool Mini kit (QIAGEN, Valencia, USA) was performed for genomic DNA extraction from stool samples in accordance with the manufacturer's instructions. We used an incubation of the specimen in a lysis buffer at 95 °C instead of 70 °C, option given for enhancing pathogens DNA extraction. DNA extraction from cultured *C. jejuni* was made with boiling water method. Briefly, colonies of *C. jejuni* ATCC 33291 were suspended in 100 µL of sterile deionized water and boiled for 10 minutes. After centrifugation (956 × g for 10 minutes), the supernatant was used as template for PCR.

DNA from cultured *C. coli* was extracted with a modified protocol using *QIAamp DNA stool Mini kit*, since boiling water is not appropriate for *C. coli*'s DNA extraction (Mohan *et al*, 1998). The modified procedure involved an incubation of 100µL of a water suspension of the cultured *C. coli* INCQS/FIOCRUZ 00263 with the same volume of a lysis buffer for 5 minutes at 95 °C instead of incubation with Inhibitex resin provided by the kit.

All genomic DNA extracted were controlled using the 230, 260, 280 and 320 nm wave lengths in a spectrophotometer (Eppendorf BioPhotometer Plus, Hamburg, Germany). The 260nm absorbance reading provides the total amount of nucleic acid yield, and the 320nm absorbance reading reduces the background influence on total amount of nucleic acid yield. The 260/280 nm and 260/230 nm were used to ensure DNA quality. Samples with ration between 1.8 and 2.0 were presumed to be free of contamination, such as ribonucleic acid, protein, and reagents (Sambrook and Russel, 2001).

Oligonucleotides and PCR amplifications

PCR primers are listed in Table 1 and they were synthesized by Invitrogen Life Technologies (INVITROGEN, Sao Paulo, SP, Brazil).

Two sets of PCR reactions were performed separately; one for *hipO* and another set for *ask* detection using a final volume of 25 µl. Each reaction contained 12.5 µL of *GoTaq Green Master Mix 2X* (PROMEGA, Madison, WI, USA) which contains *Taq* polymerase, deoxynucleotide triphosphates, and MgCl₂; 200 nM of forward and reverse primers (*hipO* primer pair for *C. jejuni* detection, and *ask* primer pair for *C. coli* detection), 2.5 µl of template DNA, and made up to a final volume of 25 µl with nuclease-free water (PROMEGA, Madison, USA). Thermocycling in a *MyCycler*TM (BIO-RAD, San Diego, USA) consisted of 1 cycle of initial denaturation at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 45 s; followed by one cycle for a final extension at 72 °C for 10 min. Samples were held at 4°C until analysis. A water-template negative control and a positive control of purified *Campylobacter* DNA - *C. jejuni* ATCC 33291 and *C. coli* INCQS/FIOCRUZ 00263 - were included in each PCR run.

Multiplex PCR amplification

Multiplex PCR reactions were performed for detection of *cdtABC* operon in a final volume of 25 µl. Each reaction contained 12.5µL of *Multiplex Master Mix 2X* (QIAGEN, Valencia, USA) which contains *HotStarTaq* polymerase, deoxynucleotide triphosphates, and MgCl₂; 200 nM of forward and reverse primers, all together (*cdtA*, *cdtB* and *cdtC* pair primers), 2.5 µl of template DNA, and made up to a final volume of 25 µl with nuclease-free water (PROMEGA, Madison, USA). Thermocycling in a *MyCycler*TM (BIO-RAD, San Diego, USA) consisted of 1 cycle of initial denaturation and enzyme activation at 95 °C for 15 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 56° C for 30 s, and extension at 72° C for 45 s; followed by one cycle for final extension at 72° C for 10 min. Samples were held at 4° C until analysis. A water-template negative control and a positive control of purified *C. jejuni* ATCC 33291 DNA were included in each PCR run. PCR and Multiplex PCR setup were performed using dedicated pipettes and aerosol barrier tips to minimize the risk of PCR contamination. A total of 10 µl of each PCR or Multiplex PCR product was run on a 1.2% agarose gel in Tris acetate-EDTA buffer. Gels were stained with ethidium bromide and photographed using an UV transilluminator *ChemiDoc XRS*TM (BIO-RAD, San Diego, USA).

ELISA for *Campylobacter* sp.

Stool specimens were evaluated for the presence of *Campylobacter* with an ELISA assay, the *Campylobacter ProSpect Microplate Assay* from REMELTM (Lenexa, KS, USA), according to manufacturer's guidelines. The plate readings were done spectrophotometrically at 450 nm wavelength (ELISA *Microplate Reader 230S* - ORGANON TEKNIKA®, Durham, NC, USA). The results were interpreted according to the instruction's guide after validation of each test, based on expected absorbance readings in positive and negative control wells.

Statistical analysis

Data were entered twice by two independent persons and validated using Excel and Access softwares (Microsoft Corp., Cupertino, CA) and analyzed using SPSS version 11.0 (SPSS IncTM, Chicago, IL, USA). The graphics were performed using the GraphPad Prism version 4.0 (GraphPadTM Software, San Diego, CA, USA). We used the EpiInfo software version 6.0 (Center for Disease Control, Atlanta, GA, USA) for the calculation of z-scores. Shapiro-Wilk test was used to verify data normality and Levene's test to check the variances equality. Student's *t*, Chi-Square, Fisher's Exact or Anova tests were used depending on data requirements. The differences were considered significant when the P value was less than 0.05.

RESULTS

Age, sex and nutritional parameters

The diarrhea and non-diarrhea groups were similar regarding age, gender, height, weight, arm circumference, and BMI ($p > 0.05$, *Student's t* test). Skinfold thickness was significantly less in the children with diarrhea (mean $\pm$ sd; 7.072 cm $\pm$ 1.849, 7.763 cm $\pm$ 2.065; $p = 0.0374$, *Student's t* test). Children with diarrhea also had lower WAZ (-0.296 $\pm$ 1.343) and WHZ (0.306 $\pm$ 1.300) compared to non-diarrhea group (0.156 $\pm$ 1.318 for WAZ and 0.769 $\pm$ 1.093 for WHZ; $p = 0.044$ and $p = 0.022$ for WAZ and WHZ, respectively; *Student's t* test). HAZ was similar in both groups ($p > 0.05$).

C. jejuni/coli detection by PCR and relation to nutritional status

We performed PCR amplification for *hipO* and *ask* genes in duplicate for each sample in all 166 samples (83 cases and 83 controls). The results are summarized in Table 2. Figure 1 (A and B) shows the PCR products on agarose gel for *C. jejuni* and *C. coli*. There were no significant differences between case and control groups regarding to *C. jejuni/coli* prevalences. In the diarrhea group, 7.2% (6/83) of samples had amplification for *hipO* gene and 3.6% (3/83) of samples were positive for *C. coli*. Co-infection (*C. jejuni* and *C. coli* detection) was found in 2.4% (2/83) of cases. In the control group, 6.0% of samples were positive for *C. jejuni*. Only one sample from control group presented *ask* gene amplification and represented also a co-infected sample (1.2% of controls, 1/83).

Stool samples with *hipO* positive gene (positive molecular detection of *C. jejuni*), showed a correlation with malnutrition in children as shown in Figure 2. Children with *C. jejuni* (*hipO*⁺) positive stool samples had significantly lower mean of WAZ (mean $\pm$ sd, -1.039 $\pm$ 1.293, $n=14$) than children with *hipO*⁻ stool samples (0.037 $\pm$ 1.313; $n=130$, $p=0.0073$, Anova test). The mean of WHZ for children in *C. jejuni* positive samples (mean $\pm$ sd, -0.476 $\pm$ 1.246, $n=14$) was also significantly lower than that in children with *C. jejuni* negative samples (0.651 $\pm$ 1.167, $n=130$; $p = 0.0022$, Anova test). These observed differences were not related to occurrence of diarrhea, suggesting that the infection/carriage of *C. jejuni* may be associated with “subclinical” injury resulting in a significant negative impact on nutritional status. In fact, diarrhea cases with *hipO*⁺ had lower WHZ than to diarrhea cases without *C. jejuni* (ie *hipO*⁻; $p < 0.05$).

The *cdtABC* Operon

We performed Multiplex PCR amplification for *cdtABC* operon for all samples which showed positive or indeterminate results for ELISA *ProSpect* (20 cases and 31 controls), but only the *hipO*⁺ samples presented *cdtABC* amplification. For this reason, the results were presented as percentage of *hipO*⁺ samples (Table 2). In diarrhea group, 50.0% (4/8) of samples had amplification of *cdtABC* operon. In the non-diarrhea group, 66.7% (4/6) of *hipO*⁺ samples presented *cdtABC* operon amplification. There was no significant difference between groups. The multiplex PCR products for the *cdtABC* genes are shown in Figure 1C.

DISCUSSION

This was a case-control study where both groups had similar age and sex. However, the diarrhea group showed significantly lower WAZ and WHZ compared to the control group without diarrhea. Several reports including this group of research have shown before that diarrheal diseases were cause of malnutrition as well as malnutrition contributed to increase the risk for more diarrheal diseases (Black *et al*, 1984; Checkley *et al*, 2008; Guerrant *et al*, 2008). The extensive etiology study of diarrhea, from this same area, has been done before, including association or risk factor for malnutrition (Lima *et al*, 2000; Moore *et al*, 2000). Although we

cannot distinguish cause from effect of infection on malnutrition in this study, the results are consistent with the literature, recognizing association and, furthermore, it adds *C. jejuni* to this list of specific pathogens associated with malnutrition.

The innovation of this study is the molecular detection of *C. jejuni/coli* direct from frozen fecal samples and therefore samples unavailable for detection by standard microbiological methods. The traditional methods for identification of *Campylobacter* genus involves the use of proper culture media followed by biochemical tests, which require approximately 72-96 hours to complete phenotypic identification. Molecular detection of *Campylobacter* has been described as credible and reliable tool (Kulkarni *et al*, 2002; Lawson *et al*, 1999; Linton *et al*, 1997; Lübeck *et al*, 2003; Oyofe *et al*, 1992; Samie *et al*, 2007; Waegel and Nachamkin, 1996; Vanniasinkam *et al*, 1999).

In pilot studies (data not published) we tested sensitivity and specificity of our PCR assays, and the efficiency of our DNA extraction technique. Comparing to the microbiologic diagnostic, our PCR tests have 88% of sensibility and 95% of specificity. The samples used were tested at similar conditions as the ones used in this presented work.

We also tested our DNA extraction and pre-treatment methods, which supposedly could improve the extraction and decrease the interference of PCR inhibitory substances present in fecal material. The commercial kit used for DNA extraction directly from stool (*QIAmp* DNA Stool Mini kit - Qiagen, USA) contains a resin capable of adsorbing the DNA-damaging substances and PCR inhibitors present in fecal material. Also, in our tests, extractions without prior treatment showed better results in PCR reactions performance. Those preliminary tests let us to conclude that the direct use of untreated fecal samples (as target for pathogen DNA extraction) is efficient and reduces time and labor work.

In order to provide more support to these molecular probes used for diagnosis, we conducted an enzyme-immunosorbent assay (ELISA *ProSpect*® *Campylobacter* Microplate Assay; called *ProSpect* ELISA). This immune-enzymatic tool provided some clue to these biology molecular probes, since all positive samples for PCR (*hipO*⁺ and/or *ask*⁺ samples) were also positive for *ProSpect* ELISA. Comparing to the microbiologic diagnostic, we tested *ProSpect* ELISA's sensitivity and specificity, and we found 92% of sensitivity and 95% of specificity.

Although molecular tools do not replace conventional methods, these techniques can be useful in assessing fastidious pathogens that can be difficult to culture in some settings (Foxman and Riley, 2001). The advantages of methods based on nucleic acids molecular detection include: a) speed and specificity; b) possibility of directly detection without culture; and c) greater sensitivity for pathogens that may cause subclinical but important infections. The disadvantages are: a) cost; b) difficulty in achieving standardization of molecular methods (Abubakar *et al*, 2007; WHO, 2001); c) inability to obtain an isolate for further phenotypic investigations (Wright and Wynford-Thomas, 1990). Nevertheless, genetic studies are extremely helpful in the elucidation of pathogens and their virulence genes.

The prevalence rate found in this study for *C. jejuni* (9.6% for diarrhea group and 7.2% for non-diarrhea group) are quite similar to the prevalence rates seen in previous Brazilian studies using traditional method of microbiologic culture and biochemical tests: (i) 7.5% *Campylobacter* isolation rate from a prospective (over 30 months) study of diarrhea diseases in a village closeby to Fortaleza in Northeastern Brazil (Guerrant *et al*, 1983), and (ii), in a case-control study in southeastern Brazil, *Campylobacter* sp. was isolated in 11.2% of cases of diarrhea and 6.6% of controls (Mendes *et al*, 1987). In a recent African study, however, *Campylobacter* was not found in control group (Samuel *et al*, 2006). Two earlier African studies also encountered 6% prevalence rates of *C. jejuni/coli* in control groups (Aboderin *et al*, 2002; Bhadra *et al*, 1992).

C. coli detection showed a trend toward increased numbers of *ask* gene detection in children with diarrhea compared to those without diarrhea, but the difference did not reach statistical significance. Since the prevalence found in this study was low, we will need a better sample size to clarify if *C. coli* is a significant enteropathogen associated or not with diarrheal diseases.

The isolation of *Campylobacter* from children without diarrhea is well-documented in developing countries (Coker *et al*, 2002; Hassan *et al*, 2006; Huilan *et al*, 1991; Molback *et al*, 1994) and this common occurrence of asymptomatic *Campylobacter* carriers provides evidence of development of immune protection against clinical disease but not against colonization (Havelaar *et al*, 2009).

We have detected a low prevalence rate of co-infections, *C. jejuni* and *C. coli*, in only 1.8% and this finding was also seen in another pediatric campylobacteriosis study reported from India (Bhadra *et al*, 1992).

The present study showed an association of *C. jejuni* molecular detection with worsened nutritional parameters in children. Similar findings have also been reported in African and South American studies. In Africa, two studies have demonstrated that the isolation rate of *Campylobacter* is higher among malnourished children with diarrhea (Kakai *et al*, 1995; Lloyde-Evans *et al*, 1983). In Chile, it was determined that the occurrence of *Campylobacter* species is significantly most frequent among healthy malnourished children than among healthy well-nourished children (Fernández *et al*, 2008).

The mechanisms by which *Campylobacter* induces disease are not clearly understood (Poly and Guerry, 2008). However, two mechanisms for gastrointestinal illness are postulated based on clinical syndromes found in patients: (i) intestinal adherence and toxin production which may impair the fluid absorption by the intestine, thus causing secretory diarrhea (Wassenaar, 1997); and (ii) bacterial invasion and proliferation within the intestinal mucosa resulting in inflammatory or bloody diarrhea (Janssen *et al*, 2008). *C. jejuni* produces CDT (Guerrant *et al*, 1987; Johnsson and Lior, 1988) that causes cytodistention of affected epithelial cells and arrest of cell cycle resulting in apoptosis. CDT comprised three subunits - *cdtA*, *cdtB*, and *cdtC* - which are all necessary for cytolethal distending toxin activity (Lara-Tejero and Gálan, 2001). Although the genes encoding this protein seem to be universally present in *C. jejuni*, the efficiency of strains invading cultured human cells varies greatly, implying that different strains may have important genetic differences (Al-Mahmeed *et al*, 2006).

In order to explore this possibility, we performed a multiplex PCR assay to identify the presence of these CDT encoding genes in stool samples positive for *C. jejuni*. We found *cdtABC* operon genes in 57.1% of all *hipO*⁺ samples, with no significant differences between diarrhea and non-diarrhea groups. These data did not answer why a child presented diarrhea or not, but gave us a clue about the existence of genetic diversity of the circulating strains of *C. jejuni* in this study site.

In conclusion, these findings suggest that PCR is a potential diagnostic tool for detecting *C. jejuni/coli* direct from frozen stool samples. The influence of malnutrition as a risk for harboring *Campylobacter* must still be determined. This report suggests that further studies are warranted to evaluate the prevalence, heterogeneity and combinations of virulence-related genes in *Campylobacter* infections in children as well as their association with malnutrition.

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Figure 1.

Photos of the PCR results for (A): *C. jejuni*'s *hipO* gene amplification, 176bp; (B): *C. coli*'s *ask* gene amplification, 502bp; and (C): Multiplex PCR result for *cdtA* (175pb), *cdtB* (418pb) and *cdtC* (270bp) genes amplification. All three figures: Columns M = molecular marker, 100bp. (A): Columns 1,2,3,4 e 9: negative samples; Columns 5-8 and 10: positive samples; Column 11: sterile deionized H₂O (negative control); Column 12: positive control (DNA extracted from *C. jejuni* ATCC® 33291 - boiling water extraction). (B): Columns 1-3, 5, 8, 9, 11 and 12: negative samples; Columns 4, 6, 7 and 10: positive samples; Column 13: sterile deionized H₂O (negative control); Column 14: positive control (DNA extracted from *C. coli* INCQS/FIOCRUZ 00263 – *QIAamp* Stool Mini Kit extraction, modified protocol). (C): Columns 1-4, 9 and 10: negative samples; Columns 5-8 and 11: positive samples; Column 12: sterile deionized H₂O (negative control); Column 13: positive control (DNA extracted from *C. jejuni* ATCC 33291 - boiling water extraction). **NOTE:** PCR = Polymerase Chain Reaction; *hipO* = hippurate hydrolase encoding gene of *C. jejuni*; *ask* = aspartate kinase encoding gene of *C. coli*; *cdt* = cytolethal distending toxin encoding genes.

Figure 2.

Graphic representation of the correlation between the mean values of WAZ and WHZ z-scores for *hipO*⁺ positive (PCR *C. jejuni* detection) and *hipO*⁻ samples. The WAZ and WHZ z-scores were obtained from calculations performed with the height, weight and age data. Children with *hipO*⁺ samples presented poorer mean WAZ (-1.033 ± 1.29 , n=14) and WHZ (-0.476 ± 1.25 , n=14) values than children with *hipO*⁻ samples (0.037 ± 1.31 , n=130 for WAZ and 0.65 ± 1.17 , n=130 for WHZ) as noted with Anova test (p=0.0073 and p= 0.0022 for WAZ and WHZ mean values, respectively). **NOTE:** Z-scores = Nutritional parameters for assessment of malnutrition; WAZ= weight-for-age z-score; WHZ= weight-for-height z-score; *hipO*⁺= positive samples for molecular detection of *C. jejuni*; *hipO*⁻= negative samples for molecular detection of *C. jejuni*; *hipO*= *C. jejuni*'s hippurate hydrolase encoding gene, PCR= Polymerase Chain Reaction; *= statistically significant difference.

Table 1

Oligonucleotide sequence of primers^a used in PCR and Multiplex PCR assays.

Oligo	Primer name	Oligonucleotide Sequence (5' - 3')	Nucleotide Position	Target gene	GenBank accession no.	PCR conditions (35 cycles) ^g	Product Lengths (bp)
hipO ^b	hipO-F	ATGATGGCTTCTTCGGATAG	685-704	hipO	FJ655194.1	30" at 95°C, 30" at 51°C, 45" at 72°C	176
	hipO-R	GCTCCTATGCTTACAACTGC	548-529				
ask ^c	ask-F	GGTATGATTCTACAAAGCGAG	18-38	ask	AF017758.1		502
	ask-R	ATAAAAGACTATCGTCGCGTG	519-499				
cdtA ^d	cdtA-F	TTGGCGATGCTAGAGTTTGG	443-462	cdtA			175
	cdtA-R	ACCGCTGTATTGCTCATAGGG	617-597				
cdtB ^e	cdtB-F	CTCGCGTTGATGTAGGAGCTA	1111-1131	cdtB	AY445094.1	30" at 95°C, 30" at 56°C, 45" at 72°C	418
	cdtB-R	GCAGCTAAAAGCGGTGGAGTA	1528-1508				
cdtC ^f	cdtC-F	AGCCTTTGCAACTCCTACTGG	1653-1673	cdtC			270
	cdtC-R	GCTCCAAAGGTTCCCATCTTC	1922-1903				

NOTES:

d, e, f primers pairs for detection of *C. jejuni*'s cytolethal distending toxin (*cdtA*, *B* and *C*) encoding operon genes, chromosomal genes, multiplex PCR detection.

^a All primers were designed by our group.

^b hipO: primer pair for detection of *C. jejuni*'s hippuricase hydrolase (*hipO*) encoding gene, chromosomal gene, single PCR detection.

^c ask: primer pair for detection of *C. coli*'s aspartate kinase (*ask*) encoding gene; chromosomal gene, single PCR detection.

^g For all PCR conditions, an initial denaturation step (10 minutes for single PCR and 15 minutes for multiplex PCR at 95°C) and a final extension (10 minutes at 72°C) were performed. F = Forward primer; R = Reverse primer.

Table 2Prevalence of *Campylobacter* sp., *C. jejuni*, *C. coli*, and co-infection in the population studied.

Assay	Results	Groups		Total % of positive samples (positive/n)
		Diarrhea % of positive samples (positive/n)	Non-diarrhea % of positive samples (positive/n)	
ELISA	ELISA <i>ProSpect</i> (<i>Campylobacter</i> sp.)	23.7 (19/80)	31.7 (26/82)	27.8 (45/162)
PCR	<i>hipO</i> ⁺ (<i>C. jejuni</i>)	7.2 (6/83)	6.0 (5/83)	6.63 (11/166)
	<i>ask</i> ⁺ (<i>C. coli</i>)	3.6 (3/83)	0.0 (0/83)	1.81 (3/166)
	<i>hipO</i> ⁺ / <i>ask</i> ⁺ (<i>C. jejuni</i> and <i>C. coli</i>)	2.4 (2/83)	1.2 (1/83)	1.81 (3/166)
	<i>hipO</i> ⁺ and/or <i>ask</i> ⁺ (<i>C. jejuni</i> and/or <i>C. coli</i>)	13.25 (11/83)	7.2 (6/83)	10.24 (17/166)
	<i>cdtABC</i> ⁺ (<i>C. jejuni</i>)	50.0 (4/8)	66.7 (4/6)	57.1 (8/14)

NOTE: ELISA: ELISA *ProSpect Campylobacter* Microplate Assay (Remel, KS, USA); *hipO*⁺: *C. jejuni*'s hippuricase hydrolase encoding gene amplified; *ask*⁺: *C. coli*'s aspartate kinase encoding gene amplified; *cdtABC*⁺: *C. jejuni*'s cytolethal distending toxin encoding operon genes amplified. Non significant differences related to *Campylobacter* detection (ELISA or PCR) were found between diarrhea and non-diarrhea groups (*Student's T*, *Chi-Square* or *Fisher's Exact* tests).