Campylobacter jejuni infection and virulence-associated genes in children with moderate to severe diarrhoea admitted to emergency rooms in northeastern Brazil

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Campylobacter is an important cause of foodborne gastroenteritis. We determined the occurrence of *Campylobacter jejuni* and *Campylobacter coli*, using culture-based methods and PCRs targeting virulence-associated genes (VAGs) among children aged ≤ 14 years who were treated for diarrhoea at emergency rooms in northeastern Brazil. Genomic DNA was extracted directly from stool samples collected from 366 children. A questionnaire was also applied to qualify the clinical conditions presented by each child at the time of admission. *C. jejuni* and *C. coli* were detected in 16.4 % (60/366) and 1.4 % (5/366) of the diarrhoeal samples, respectively, by PCR, a much higher proportion than that detected by conventional methods. *C. jejuni* VAGs were detected in the following proportions of *hipO*-positive samples: *ciaB*, 95 % (57/60); *dnaJ*, 86.7 % (52/60); *racR*, 98.3 % (59/60); *flaA*, 80 % (48/60); *pldA*, 45 % (27/60); *cdtABC*, 95 % (57/60); and pVir 0 % (0/60). Particular symptoms, such as blood in faeces, vomiting, fever, and/or abdominal pain, were not associated with detection of *C. jejuni* nor were they associated with any particular VAG or combination of VAGs ($P > 0.05$). *C. jejuni* and its VAGs were detected in a substantial proportion of the children admitted. Further efforts shall be directed towards elucidating whether these genetic factors or their expressed proteins play a role in *Campylobacter* pathogenesis.

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INTRODUCTION

The genus *Campylobacter*, a group of thermotolerant, microaerophilic, Gram-negative bacteria, includes a number of pathogens that primarily cause gastrointestinal disease in humans, particularly *Campylobacter jejuni* and *Campylobacter coli*. *Campylobacter*-associated gastroenteritis is thought to occur through zoonotic transmission, being acquired from exposure to tainted food and/or contaminated drinking water (Sherman *et al.*, 2010). *Campylobacter* infection frequently presents as self-limiting acute enteritis with diarrhoea, malaise, fever and abdominal pain, sometimes with vomiting and the presence of blood in faeces

(Allos, 2001); disruption of epithelial cells and inflammation of the intestinal mucosa are hallmark features of severe cases (Beltinger *et al.*, 2008). *C. jejuni* and *C. coli* cause significant morbidity worldwide, especially in children (Amieva, 2005; Tam *et al.*, 2003; Wang *et al.*, 2008; Fernández *et al.*, 2008).

Adherence and colonization are crucial steps in the pathogenesis of *C. jejuni*. Flagella have a major role in invasion; markedly reduced internalization *in vitro* has been reported with *flaA*[−] *C. jejuni* mutants (Wassenaar, 1997). The genes *racR* and *dnaJ* are determinants for *C. jejuni* colonization and are presumably expressed in response to conditions encountered in the intestinal microenvironment, such as differences in temperature between environmental reservoirs and human intestines (Konkel *et al.*, 1998; Brás *et al.*, 1999). The *ciaB* gene encodes *Campylobacter* invasion

Abbreviations: CDT, cytolethal distending toxin; MicD, microbiological diagnosis; VAGs virulence-associated genes.

antigen B protein, which confers invasive properties, as shown by *C. jejuni* *ciaB* null mutants which display a significant reduction in internalization (Konkel *et al.*, 1999). Also, CiaB and other secreted proteins require functional flagella export apparatus for their secretion (Konkel *et al.*, 2004; Dasti *et al.*, 2010). The *pldA* gene encodes a protein involved in the synthesis of an outer membrane phospholipase that has also been related to cellular invasion (Ziprin *et al.*, 2001). The plasmid pVir is associated with the ability of *C. jejuni* to adhere to and invade cells *in vitro* and has been shown to increase virulence *in vivo* (Bacon *et al.*, 2000). Tracz *et al.*, 2005 showed that in very invasive infections, with a dysenteric phenotype, pVir could be detected. Last but not least, cytolethal distending toxin (CDT), a very efficient bacterial toxin, causes cell distension and death due to blocking of cell division. This toxin is produced by a variety of Gram-negative bacteria including *Campylobacter* species, *Shigella* species, *Escherichia coli* and *Haemophilus ducreyi* (Young & Schauer, 2000; Yamasaki *et al.*, 2006). The CdtA and CdtC subunits appear to be responsible for binding to the membrane receptors of susceptible cells, while the CdtB subunit is responsible for the toxic effect at the nucleus level, causing dsDNA cleavage as a result of its DNase I (EC 3.1.21.1) activity (Lara-Tejero & Galán, 2001).

Previous data from our group, obtained from a case-control study of diarrhoea acquired in the community, demonstrated the presence of *C. jejuni* and *C. coli*. The prevalence rates for *C. jejuni* reached 9.6% for the diarrhoea group and 7.2% for the non-diarrhoea group (da Silva Quetz *et al.*, 2010). These data prompted us to assess the detection rate of *Campylobacter* in children from the same geographical area with community-acquired diarrhoea who were treated in local hospitals. Thus, in the present work we focused our efforts on describing the importance of *C. jejuni* as an enteropathogen among children who were treated for diarrhoea at emergency rooms in Fortaleza, Ceara, an impoverished area in Brazil. We also characterized *C. jejuni* virulence-associated genes (VAGs) that may play a role in *C. jejuni* pathogenicity. We performed these analyses on DNA extracted directly from faecal material. Moreover, we examined the data for correlations between selected clinical parameters and the molecular profile of each sample.

METHODS

Ethical clearance, study site and population. This study was part of a project entitled 'Diarrhea Enteric Card (DEC)' aiming to develop a point-of-care microfluidic PCR-based multiplex assay for four common bacterial enteric pathogens (Yager *et al.*, 2006; Weigl *et al.*, 2006). The project was approved by the local and national ethical committees in Brazil (HIAS 80/06 and CONEP 13523/2007, respectively). A consent form was read and signed by the guardians of each child whom received medical care due to a diarrhoeal event, after being admitted to emergency rooms of two paediatric hospitals in Fortaleza, Ceará, Brasil: the Albert Sabin Children Hospital (Hospital Infantil Albert Sabin, HIAS) and the Center for Child Assistance Lúcia de Fátima R.G. Sá (CAC). A questionnaire was used

to qualify the clinical symptoms presented by each child at the time of admission, for example, presence of blood in stools, vomiting, fever, and abdominal pain. Exclusion criteria included antibiotic treatment or breastfeeding prior to admission.

Stool sample collection and microbiological tests. From May 2008 to April 2009, stool samples were collected from 366 children (0–14 years of age) with diarrhoea, who were treated in emergency rooms in Fortaleza, Ceara, Brazil (HIAS and CROA). Fresh stool specimens (one per subject) were obtained in collection cups and all specimens were processed within 4 h of collection. The stool specimens were tested for the presence of *Campylobacter*, diarrhoeagenic *E. coli*, *Salmonella* and *Shigella*, following standard microbiological procedures. The remaining faecal material was aliquoted and stored at -80°C until required for DNA extraction directly from faecal samples.

Phenotypic identification of *Campylobacter* species was performed by analysing colony morphology and growth characteristics. *Campylobacter* species typically produce non-haemolytic, translucent, watery colonies, some producing discrete, colourless colonies, and cells typically present cytochrome oxidase (EC 1.9.3.1) activity and appear as Gram-negative 'gull's wings'-shaped bacilli under light microscopy using Gram stain. The microbiological diagnosis (MicD) was designated as being 'suggestive' or 'not suggestive' of *Campylobacter*.

Genomic DNA. Genomic DNA was extracted from 366 stool samples using a QIAamp DNA stool Mini kit (Qiagen). The purity of DNA extracted was determined by measuring A_{260}/A_{280} . In order to certify the quality of our extraction procedures, we performed an internal control PCR for all DNA extracted directly from faecal aliquots just before the PCR diagnosis procedures. This internal control PCR amplified the *rrs* (16S rRNA) gene (Wang *et al.*, 2002; Brandal *et al.*, 2007). Positive control DNA was extracted from *C. jejuni* ATCC 33291 and *C. coli* INCQS 00263 (kindly provided by Oswaldo Cruz Foundation, Rio de Janeiro, Brazil).

Oligonucleotides, PCR and multiplex-PCR amplifications. PCR primers for detection of *C. jejuni* and *C. coli*, as well as the *cdtABC* operon, were used as previously described (da Silva Quetz *et al.*, 2010). Primers used for detection of VAGs in *C. jejuni* and the *rrs* internal control PCR along with the optimal cycling conditions are listed in Table 1. All primers were synthesized by Invitrogen.

Two sets of PCRs or multiplex-PCRs were performed in duplicate and the results were coincident. PCRs contained 12.5 μl GoTaq Green Master Mix 2 $\times$ (Promega); 200 nM each forward and reverse primers and 2.5 μl template DNA, made up to a final volume of 25 μl with nuclease-free water (Promega). Multiplex-PCRs contained 15 μl Qiagen Multiplex Master Mix Solution 2 $\times$ and Q-solution enhancer (Qiagen); 200 nM each forward and reverse primers and 2.5 μl template DNA, made up to a final volume of 25 μl with nuclease-free water. Each reaction product was run on a 3% agarose gel in Tris/acetate-EDTA buffer. Gels were stained with ethidium bromide and photographed using a ChemiDoc XRS UV transilluminator (Bio-Rad).

ELISA for detection of *Campylobacter* antigen. In order to provide more support to the molecular assay, we performed a *Campylobacter* ProSpecT Microplate Assay (Remel) on 74 samples, following the manufacturer's guidelines. The plates were read spectrophotometrically at 450/620 nm using an ELISA Microplate Reader (Bio-Rad) and the results were interpreted according to the manufacturer's instructions. The analysis was done in order to assess the degree of concordance between the results of PCR and ELISA.

Statistical analysis. Data were entered twice by two independent persons, validated using Excel software (Microsoft) and analysed using SPSS version 13.0. The analysis of associations was performed in contingency tables, using Fisher's exact test. To compare means,

Table 1. PCR primers, amplification conditions, and expected PCR products for *C. jejuni* VAGs

For all PCR conditions, an initial denaturation step (5 min for single PCR and 15 min for multiplex PCR at 95 °C) and a final extension step (10 min at 72 °C) were performed in a MyCycler thermocycler (Bio-Rad). F, Forward; R, reverse.

Target gene	Primer sequence (5'→3')	GenBank accession no.	Amplicon size (bp)	PCR conditions (35 cycles)	Reference(s)
Internal control					
<i>rrs</i> *† <i>E. coli</i> 16SrRNA	F: CCCCTGGACGAAGACTGAC R: ACCGCTGGCAACAAAGGATA	JN547259.1	401	20 sec at 95 °C, 20 sec at 63 °C, 45 sec at 72 °C	Wang <i>et al.</i> (2002), Brandal <i>et al.</i> (2007)
<i>C. jejuni</i> VAGs					
<i>ciaB</i> s*‡ <i>Campylobacter</i> invasion antigen B	F: TCATGCGGTGGCATTAGAAATGGG R: AGGTCTAACTTCATCAACCCCTTTGCCA	AF114831.1	658	30 sec at 95 °C, 30 sec at 64 °C, 45 sec at 72 °C	Konkel <i>et al.</i> (1999)
<i>dnaJ</i> *‡ chaperone protein DnaJ	F: AGGCTTTGGCTCATCACGTCG R: GGTGCTTCACCGCGTATGG	AF053962.1	574		Konkel <i>et al.</i> (1998)
<i>racR</i> *‡ DNA-binding response regulator	F: TGGGGCTTCAAATCGGTGCTGA R: GCGACCGATGATAACATCAAGGCT	GQ491062.1	326		Hamidian <i>et al.</i> (2011)
<i>flaA</i> *‡ flagellin	F: AGCTGCTTCGCAACTTTCTACGGT R: TGCACCTCTCGGCTGCAAAGTCT	AF369585.1	325	30 sec at 95 °C, 30 sec at 59 °C, 45 sec at 72 °C	Fitzgerald <i>et al.</i> (2001)
<i>pldA</i> *‡ phospholipase A	F: AAGAGTGAGGCGAAATTCCA R: GCAAGATGGCAGGATTATCA	CP000025.1	385		Fouts <i>et al.</i> (2005)
pVir† virulence plasmid	F: GAACAGGAAGTGGAAAACTAGC R: TTCCGCATTGGGCTATATG	AF226280.2	708	30 sec at 95 °C, 30 sec at 55 °C, 1 min at 72 °C	Bacon <i>et al.</i> (2002)

*Chromosomal gene.

†Single PCR.

‡Multiplex PCR.

the normality of the data distribution was first checked. When normal distribution of the data was confirmed, the comparison of the two means was performed using Student's *t*-test; an ANOVA test was used to compare three or more means. Sub-hypotheses were analysed by using the Bonferroni test. When normality was not accepted, the comparison of two means was performed by using the Mann-Whitney test. The significance level was set at $P < 0.05$. The Kappa index was used to determine the degree of concordance between the results of PCR and ELISA tests.

RESULTS AND DISCUSSION

Population description

The details of the population studied, including age, gender and family income are summarized in Table 2. The majority of children treated were 2 years old or younger (214/366, 58.5%), which is consistent with the age considered most

susceptible to diarrhoeal diseases worldwide (Podewils *et al.*, 2004; Vu Nguyen *et al.*, 2006). The group treated were considered to be similar with respect to their age ($P = 0.787$, Student's *t*-test). The female/male ratio was 0.58, with boys accounting for the majority of the patients ($P = 0.024$). Approximately 75 % of all the children admitted lived in homes where the monthly income was less than US\$ 250. Nearly 90 % of the patients' households had piped water. About 9.5 % (35/366) of the patients' mothers were illiterate or had less than 3 years of schooling, 47.8 % (175/366) had between 4 and 11 years of schooling, and 24.0 % (88/366) had more than 12 years of schooling.

Assessment of clinical parameters

The diarrhoeal illnesses of the children enrolled in this hospital-based study were moderate to severe based on the

Table 2. Details of the studied population and parental socioeconomic information from 366 children treated for diarrhoea at emergency rooms in Fortaleza, Brazil, during the period May 2008–April 2009

Characteristic	No. (%)	Median	Range
Male patients	212 (57.9)		
Age (years)			
≤2	214 (58.5)	0.85	
2–5	86 (23.5)	2.85	
≥5	66 (18.0)	7.57	
Total	366 (100)	1.52	0.02–13.92
Monthly income (US\$*)			
<200	77 (21.9)	125.00	
200–250	188 (53.4)	207.50	
>250	87 (24.7)	400.00	
Total	352 (96.1)	207.50	0–1500

*1 US\$=2 Real – mean exchange rate of during the time of study.

symptoms reported and considering the fact that the guardians took their child to the appropriate health personnel for immediate attention.

According to the parental responses to the questionnaires, >95 % of the children were experiencing acute diarrhoea less than 13 days prior to date of attendance, as opposed to the 3.1 % with persistent diarrhoea, experiencing diarrhoea for 14–30 days prior to date of attendance. Other data regarding clinical parameters are as follows: 22.6 % (83/366) of children presented blood in the stool, 72.9 % (267/366) presented abdominal pain and vomiting, and fever was reported in 74.6 % (273/366) of cases.

Microbiological detection vs molecular detection

Using traditional diagnostic techniques, a bacterial pathogen was detected in 30.3 % (111/366) of the stool samples examined. *Campylobacter* species were found in 6.0 % (22/366) of cases. The overall distribution of pathogens found is summarized in Table 3. Although there is a paucity of population-based data about *Campylobacter* infections in Brazil, previous case-control studies have reported that the prevalence of *Campylobacter* in stool samples from diarrhoeic children is between 1.7 % (Moreno *et al.*, 2010) and 11.2 % (Mendes *et al.*, 1987). The prevalence found with our culture-based methods (6.0 %) is consistent with these findings.

After the internal control for DNA extraction was confirmed as positive (Fig. 1a), the PCR assays for *Campylobacter* diagnosis were performed. *C. jejuni* and *C. coli* were detected in 16.4 % (60/366) and 1.4 % (5/366) of DNA samples, respectively (Table 4). Our PCR-based methods detected a higher rate of *Campylobacter* infections, with overall infection rate of 17.8 %. Assuming a prevalence of 6.0 % of *Campylobacter* infection in Brazilian sites, the specificity, positive and negative predictive values

Table 3. Bacterial enteropathogens identified by traditional microbiological diagnostic techniques in the 366 stool specimens.

Micro-organism	No. positive samples (%)
<i>Campylobacter</i> spp.	22 (6.0)
<i>E. coli</i> non-O157:H7	21 (5.7)
<i>Salmonella</i> spp.	8 (2.2)
<i>Shigella</i> spp.	60 (16.4)
No enteric pathogen detected	255 (69.7)

(PPV, NPV) and sensitivity were, 90.1, 34.6, 98.7 and 81.8 %, respectively. The sensitivity of our PCR protocol, however, must still be confirmed through further experiments using spiked stool samples.

Previous studies have shown improvements in the rate of detection of *Campylobacter*. These studies suggest that culture-based methods underestimate the prevalence of *Campylobacter* infection and that PCR-based methods are more efficient for detection of *Campylobacter* from faecal samples declared negative by culture-based methods (Maher *et al.*, 2003; Singh *et al.*, 2011). Using molecular approaches, Inglis *et al.* (2011) revealed that 12.2 % and 4.3 % of *C. jejuni* and *C. coli* infections, respectively, have been missed by culture-based methods of identification. In India, Sinha *et al.* (2004) reported that the rate of *Campylobacter* detection using molecular tools reached 19 %, as opposed to the detection rate of 4.8 % using stool culture, in their study evaluating *C. jejuni* infections preceding Guillain–Barré syndrome (Sinha *et al.*, 2004).

The increased detection rate seen with PCR-based methods in the present study is comparable to several other studies worldwide, the findings of which have demonstrated rates of *Campylobacter* infection in cases of childhood diarrhoea ranging from 7 % in Tehran and Pakistan (Jafari *et al.*, 2009; Soofi *et al.*, 2011) to 30.8 % in Canada (Kaboré *et al.*, 2010), even reaching 52 % in children <4 years old in Bulgaria (Ivanova *et al.*, 2010). In South America, the prevalence of *Campylobacter* isolation in children with diarrhoea varied between 2.3 % and 23.0 % according to Fernández (2011).

Information from ELISA tests

From the 22 faecal samples identified by culture methods as suggestive of *Campylobacter* infection (MicD⁺), four were PCR-negative for *C. jejuni* and *C. coli*. Therefore, an ELISA test (ELISA ProSpecT *Campylobacter* Assay) was performed for all MicD⁺ samples, which revealed that 100 % of these samples (22/22) had ELISA-positive results. We attribute the failure of PCR detection to the very watery characteristic of these four samples, a factor that may negatively influence the DNA extraction process. We also performed the ELISA test on an additional 52 samples,

Fig. 1. Amplified products from PCR and multiplex PCR reactions targeting *C. jejuni* VAGs *ciaB*, *dnaJ* and *racR* (b) and *pldA* and *flaA* (c) using the *rrs* gene (a) as an internal control. Reactions were performed using DNA samples from children with diarrhoea who were admitted to emergency rooms in Fortaleza, Brazil. Numbered columns represent amplified products from DNA samples. +, Positive control – commensal *E. coli* strain HS (a), *C. jejuni* ATCC 33291 (b, c); –, negative control (autoclaved MilliQ water used as template). M, 100 bp molecular marker.

chosen at random, according to the availability of 300 mg faecal material. Based on the results obtained, we were able to demonstrate a concordance between the PCR protocol and the ELISA test, which was regular ($Kappa=0.411 \pm 0.112$). A previous study determined that the ProSpecT *Campylobacter* assay presents 89.1 % sensitivity and 97.7 % specificity for both *C. jejuni* and *C. coli* (Dediste *et al.*, 2003). If it had been possible to apply the ELISA to all our samples, it would have improved the concordance between our proposed molecular protocol and this immunoassay.

C. jejuni virulence-associated genes

C. jejuni VAGs have, to our knowledge, not been examined previously in Brazil. *C. jejuni* (*hipO*)-positive samples were further tested using PCR or multiplex PCR in order to evaluate the prevalence of VAGs, the results of which are summarized in Table 4 and Fig. 1(b, c).

A combination of six VAGs was detected in 18 of the 60 (30 %) *hipO*⁺ samples, five VAGs were detected in 32 (53.3 %) samples, four VAGs were detected in seven (11.7 %)

samples and at least three VAGs (minimum detected) were detected in three (5 %) samples. In accordance with our findings, other studies regarding the prevalence of VAGs worldwide also found a very high proportion of these genes in different types of clinical and animal samples (Datta *et al.*, 2003; Al-Mahmeed *et al.*, 2006; Talukder *et al.*, 2008; Ripabelli *et al.*, 2010; Andrzejewska *et al.*, 2011; Hamidian *et al.*, 2011), especially *ciaB* (95–98 %) and the *cdtABC* operon (85–100 %).

We did not detect pVir among any of the *hipO*⁺ samples, not even in samples from children whose guardians reported bloody diarrhoea (18/60), a scenario that has also been reported previously (Tracz *et al.*, 2005). The absence of pVir may be due to lost plasmid material during the DNA extraction process; it may also be explained by the geographical characteristics of *Campylobacter* infection, as previously suggested (Louwen *et al.*, 2006).

In cases of campylobacteriosis, the most studied *C. jejuni* virulence factor is CDT. Recently, a Danish group studied clinical samples from 30 patients with confirmed *Campylobacter* infections (Mortensen *et al.*, 2011). As part of their analyses, they determined CDT activity *in vitro* and also sequenced the *cdtB* portion of the *cdt* operon gene, an interesting strategy that could be helpful in furthering our own studies, since we observed a difference in the prevalence of *cdtABC* genes between the present study (57/60, 95.0 %) and our previous case-control study (8/14, 57.1 %; da Silva Quetz *et al.*, 2010).

Clinical symptoms of the disease did not appear to be associated with the detection of either *C. jejuni* or *C. coli* or with any particular combination of VAGs (Fisher's test, $P>0.05$). This differed from a previous report that found a higher rate of detection of two VAGs in one particular symptomatic group (Al-Mahmeed *et al.*, 2006). Once again, this lack of association may be caused by geographical factors or individual circumstances relating to the *Campylobacter* infections. Further analysis will be necessary in order to investigate the proteins expressed during *Campylobacter* infection, since studies of the expressed protein products of enteric pathogens are crucial in determining the mechanisms by which they are able to cause disease and spread through the population.

Table 4. Molecular detection of *Campylobacter* spp. and *C. jejuni* VAGs in DNA samples extracted directly from the 366 stool samples

Gene detected (target)	No. positive samples (%)
<i>Campylobacter</i> diagnosis	
<i>hipO</i> (<i>C. jejuni</i>)	60 (16.4)
<i>ask</i> (<i>C. coli</i>)	5 (1.4)
<i>C. jejuni</i> VAGs*	
<i>ciaB</i>	57 (95.0)
<i>dnaJ</i>	52 (86.7)
<i>racR</i>	59 (98.3)
<i>flaA</i>	48 (80.0)
<i>pldA</i>	27 (45.0)
<i>cdtABC</i>	57 (95.0)
pVir	0 (0.0)

*Tested on the 60 *hipO*-positive samples.

Conclusions

C. jejuni and its VAGs were detected in a high number of DNA samples extracted from faecal material of children who required medical care in two emergency rooms of Fortaleza, Brazil as a result of a diarrhoeal event. Using molecular tools, we were able to detect a considerable proportion of *Campylobacter*-positive faecal samples that had, at first, been declared as negative by culture-based methods. Further efforts will be directed to better determine the sensitivity of our protocol and to elucidate whether genetic factors or related expressed proteins play a role in *Campylobacter* pathogenesis.

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