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Note

Combination of different methods for detection of *Campylobacter* spp. in young children with moderate to severe diarrhea

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ABSTRACT

Campylobacter spp. were detected - using culture, ELISA, PCR, and qPCR - among children (0–36 months) with moderate to severe diarrhea in Northeastern Brazil. Our data showed that either the qPCR alone or PCR along with ELISA might be an alternative to culture to diagnose *Campylobacter* due to their enhanced sensitivity.

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1. Introduction

Campylobacter spp. are important etiological agents of gastroenteritis, especially in children living in developing countries (Fernández, 2011). Food-borne campylobacteriosis is reported as zoonotic infection, and the poultry's farm-to-fork chain is the main source of infection (Wassenaar, 2011).

Previous reports have shown an association between *Campylobacter* spp. diagnosis/detection and malnutrition or worse developmental scores in children living in Chile (Fernández et al., 2008) and Northeastern Brazil (Quetz et al., 2010). In a multisite cohort study, called MAL-ED, *Campylobacter* spp. were the most frequently detected pathogens and had the highest impact in cases of diarrhea in Brazil, Peru and South Africa in the first year of life (Platts-Mills et al., 2014, 2015).

Campylobacter diagnosis is hampered by the fastidious characteristics of this microorganism, and by its ability to reach a viable-non-cultivable state (Jackson et al., 2009). Molecular and immunoenzymatic methods, which have greater sensitivity, are challenging the conventional diagnosis by culture (Bessède et al., 2011; Lehours et al., 2012).

In the present work, we aimed to investigate the best alternative methods (PCR, qPCR and ELISA) to detect *Campylobacter* spp. compared with gold standard test, culture, regarding their agreement, specificity and sensitivity in stools samples.

2. Materials and methods

This study was part of a project entitled: Diarrhea Enteric Card. It was approved by local and national Ethical Committee in Brazil (HIAS 80/06 and CONEPE 13523/2007, respectively). Children (0–36 months age/58.66% male and 41.34% female) from urban area of Fortaleza received medical care because of a diarrheal event in the emergency rooms of two pediatric hospitals in Fortaleza, Ceara, Brazil: Albert Sabin Pediatric Hospital (HIAS) and Center for Child Assistance Lucia de Fatima R.G. Sa (CROA). Exclusion criteria consisted of prior administration of antibiotics or breastfeeding.

Stool samples were collected from May 2008 to April 2009. 153 of them were tested for *Campylobacter* spp. using microbiologic diagnosis, Enzyme Linked Immuno Sorbent Assay (ELISA) and molecular tests (PCR and qPCR). Besides *Campylobacter* species, other bacterial pathogens, such as *Escherichia coli* non-O157:H7, *Salmonella* and *Shigella*, were investigated simultaneously. For the purpose of microbiological analysis, the samples were processed within a 4 h period. For the isolation of *Campylobacter* spp., fecal samples were cultivated in Columbia agar supplemented with 5% sheep blood and Campylobacter Growth Supplement (Oxoid, Hampshire, UK, SR0232). The selectivity of the medium was obtained by adding Campylobacter Selective Supplement Blaser-Wang (Oxoid, SR0098) containing vancomycin, polymyxin B,

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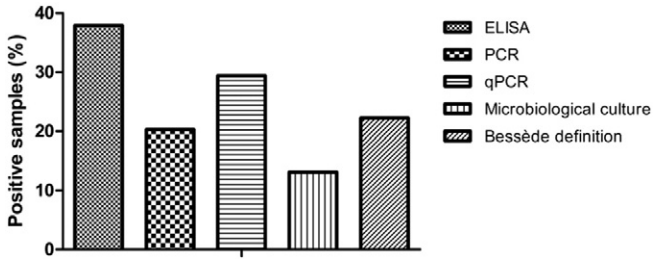

Fig. 1. Positive percentage found with different methods for detection of *Campylobacter* spp.

Table 1
Positive distribution profile of *Campylobacter* cases detected through the use of different methods.

Number of cases	Results			
	Culture	ELISA	qPCR	PCR
Positive microbiological diagnosis (n = 20)				
13	+	+	+	+
4	+	+	+	−
1	+	+	−	+
1	+	+	+	NR
1	+	+	−	NR
Others positive diagnosis (n = 42)				
12	−	+	+	+
1	−	+	−	+
1	−	+	+	−
1	−	−	−	+
3	−	−	+	+
24	−	+	−	−

Abbreviations: NR – no results.

trimethoprim, amphotericin B, and cephalothin. Incubation occurred at 42 °C ± 1 °C for 72 h in a microaerophilic atmosphere. The suggestive phenotypic identification of *Campylobacter* spp. was determined by their morphological characteristics: growth of non-hemolytic, translucent or colorless colonies. The activity of cytochrome oxidase was tested, using the reagent *N*, *N*-dimethyl-*p*-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA). It was also performed the investigation of Gram-negative bacilli in the form of "gulls wings" by optical microscopy after smear slide and gram stain with carbol fuchsin (Quetz et al., 2012).

The remaining fecal material was diluted for the ELISA and directly used for the DNA extraction (Quetz et al., 2012). *Campylobacter ProSpect Microplate Assay* from REMEL™ (Lenexa, KS, USA) was the chosen enzyme immunoassay for the qualitative detection of *Campylobacter* specific antigen in fecal specimens performed according the manufacturer guidelines.

We performed genomic DNA extraction using *QIAamp DNA stool Mini kit* (QIAGEN, Valencia, USA). The DNA for positive controls amplifications were extracted from *Campylobacter jejuni* ATCC 33291, and *Campylobacter coli* INCQS 00263 (kindly provided by Oswaldo Cruz Foundation, Rio de Janeiro, Brazil).

The gene used for the molecular diagnosis of *Campylobacter* spp., in both techniques, was the gyrA (DNA gyrase subunit A). SYBER Green-based Real-Time PCR (qPCR) was assayed at PATH (USA) facilities, using the following primers, synthetized by Micronics (Redmonds, USA): 5′-GAGTGTTATTATAGGTCGTGCTTTG-3′ (Forward), and 3′-CTATAACAGCACCCACTATACGG-5′ (Reverse). After a fast step of initial denaturation (50 °C, 2 min.; 90 °C, 10 min.), 40 cycles of amplification (95 °C, 15 s.; 60 °C, 1 min.; 72 °C, 45 s.) took place. Results were expressed as positive (≤35 cycles), marginal (between 35 and 40 cycles) or negative (>40 cycles) amplification.

The PCR was performed using GoTaq Green Master Mix 2× (Promega), and the primer design was the same mentioned above for the qPCR. After a step of initial denaturation (95 °C, 5 min.), 40 cycles of amplification (95 °C, 20 s.; 55 °C, 20 s.; 72 °C, 45 s.) took place. PCR were performed in duplicate and the results were coincident. Each reaction product was run on 2% agarose gel in Tris/Acetate EDTA buffer. The reaction was flushed with Blue Green loading dye (LGC Biotechnology, São Paulo, Brazil), and photographed using a ChemiDoc XRS UV transilluminator (Bio-Rad).

Data were entered twice by two independent persons, validated using Excel software (Microsoft Corp., Cupertino, USA) and analyzed using SPSS (Statistical Package for the Social Sciences) version 20.0 (SPSS Inc.™, Chicago, IL, USA). The graphics were performed using the GraphPad Prism version 5.0 (GraphPad™ Software, San Diego, CA). Significance level was set at $P < 0.05$.

3. Results and discussion

The microbiological diagnosis showed 13.07% (20/153) positive samples for *Campylobacter* spp. On the other hand, the ELISA was able to detect 37.9% (58/153) positive samples, and just one sample was not definitively diagnosed. Among the positive samples diagnosed by culture, all of them were also positive for the ELISA. Regarding the molecular methods, 29.4% (45/153) of samples were positively diagnosed by qPCR, while only 20.3% (31/150 - loss of 3 samples) positive samples were detected by PCR.

Bessède et al. (2011) considered stool samples as positive for *Campylobacter* spp. when either culture was positive, or in the case of negative culture, but both the molecular and the immunoenzymatic methods were positive. Based on this definition, 22.2% (34/153) of the samples were positive for *Campylobacter* spp. The variation between the positive percentages found for each diagnostic test can be observed in Fig. 1.

According to the results of all techniques, 13 samples were positive for all methods of diagnosis, and 12 samples were positive for all methods except for conventional microbiology. Among the positive results for culture, 4 were not positive for PCR, and 2 were not positive for qPCR. The different combinations are presented in Table 1.

The culture was not able to detect *Campylobacter* species in 14 samples, which can be explained by the low sensitivity of the method as

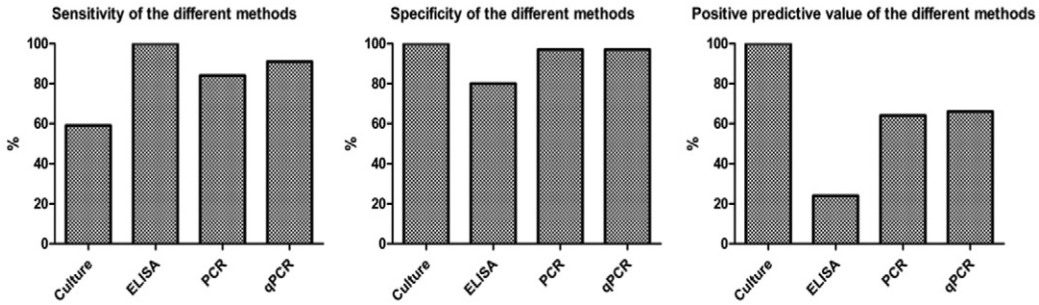

Fig. 2. Simple measures of sensitivity, specificity and predictive positive values (PPV), of culture, ELISA, qPCR and PCR.

shown in Fig. 2. However, these samples were positive for the ELISA and some of the molecular methods used. One possible explanation for the lack of diagnosis by culture is the limitation of this test due to the fastidious characteristic of *Campylobacter* spp., which reduces substantially the sensitivity of the test (Jackson et al., 2009). Errors in measuring the sensitivity and specificity of a particular test will arise especially if the gold standard test itself does not have 100% sensitivity and 100% specificity, which is not a rare case among the infectious diseases (Banoo et al., 2006).

Seeking to avoid the underestimation of positive cases as a result of low sensitivity of culture, in a multisite birth cohort study (MAL-ED), the diagnosis of *Campylobacter* spp. was performed by ELISA, achieving a high detection index. Bessède et al. (2011) have reported the high sensitivity of immunoassays compared to other methods, including molecular methods and conventional culture. Similarly, we found 100% sensitivity for ELISA, which was higher than the sensitivity found for the other methods. However, its specificity and positive predictive value (PPV) were significantly reduced, 80% and 24%, respectively. The 6% prevalence value of *Campylobacter* spp. described by Quetz et al. (2012) was used to calculate the PPVs (Fig. 2).

Bessède et al. (2011) have also mentioned that the molecular methods are not used as an alternative to the microbiological culture due to their limited specificity. Conversely, in this study, both the PCR and the qPCR showed a great specificity (97%). However, the qPCR had a higher sensitivity (91%) when compared to the PCR (84%). The combination of elevated sensitivity and specificity of qPCR suggests that, after culture, it can be considered as the best method to diagnose *Campylobacter* species. On the other hand, the lower sensitivity of PCR may be overcome by combining this method with ELISA, which have shown 100% sensitivity, but lack of specificity.

In conclusion, cases of infection with *Campylobacter* spp. were underestimated using microbiological culture as the gold standard test for diagnosis. Besides culture, either qPCR alone or PCR along with ELISA might be good alternatives for the diagnosis of this pathogen. However, the culture is still the only method capable to give 100% specificity and PPV.

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