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ORIGINAL ARTICLE

Incidence of *C. jejuni/coli* in Cases of Diarrhea and Utility of PCR and EIA as Rapid Alternatives for Routine Culture Technique

Amal Amin[1], Marian Gerges[2]
[1]Department of Microbiology and Immunology, Faculty of Medicine, Fayoum University, Egypt
[2]Department of Microbiology & Immunology, Faculty of Medicine, Zagazig University, Egypt

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Corresponding Author:
Amal Amin, MD
Lecturer of Microbiology and Immunology
Tel: 201114636000
areemrasmy@yahoo.com

ABSTRACT

Background: *Campylobacter is an invasive microorganism associated with diarrheal and systemic diseases. Campylobacter jejuni and coli frequently cause intestinal infections worldwide. Routine detection of Campylobacter species is primarily and traditionally based on growth followed by phenotypic identification. Nucleic acid based methods, particularly polymerase chain reaction (PCR), have emerged as promising techniques for the rapid, reliable, and sensitive detection and diagnosis of infections. Furthermore, several enzyme immunoassays (EIAs) have demonstrated excellent sensitivity and specificity compared with culture results.* ***Objectives****: This study aimed to determine the incidence of C. jejuni and C. coli isolation in cases of diarrhea and to assess the utility of PCR and EIA as rapid alternatives for routine culture technique.* ***Methodology****: A total of 343 stool samples and rectal swabs collected from patients (n=193) and matched controls (n=150) were cultured on two selective media and phenotypically identified by conventional methods. Eighty cases were selected for multiplex PCR and EIA examination.* ***Results:*** *Campylobacter was isolated from 5.7% of the patients and 0.7% of the controls. Compared to culture, PCR had 100% specificity and 91.7% sensitivity, and EIA had 89.7% specificity and 91.7% sensitivity.* ***Conclusion****: Selective culture remains the optimum method for detection of Campylobacter spp. from stool samples. EIA has poor specificity and needs to be redeveloped. Although PCR offers increased specificity, it is preferred for epidemiologic studies.*

INTRODUCTION

Campylobacter is an invasive microorganism that is usually associated with diarrheal and systemic diseases globally [1]. Of the *Campylobacter* species causing human disease, *Campylobacter jejuni* and *coli* are frequent causes of intestinal infections [2, 3]. *C. jejuni* is the prototye for enteric pathogen and the most important cause of enterocolitis, particularly in developing countries [4]. *Campylobacter* infections typically manifest as diarrhea of abrupt onset with severe abdominal pain [5]. In developing countries, *C. jejuni* mostly causes a non-inflammatory diarrhea manifested by watery stools, fever, abdominal pain, vomiting, and dehydration [6, 7]. Infection can lead to subsequent sequelae that include Guillain-Barré syndrome (GBS) and reactive arthritis. *Campylobacter* infections most often result from eating contaminated food [8, 9]. *Campylobacter* infections have a complex epidemiology [10], with the contribution of components including food, water, and environmental sources [11]. Routine detection of *Campylobacter* species in clinical laboratories is based on the traditional "gold standard" of diagnostic culturing of a stool specimen on selective media, followed by phenotypic identification [12]. It is a rather complex and time-consuming process. Nucleic acid based methods, particularly polymerase chain reaction (PCR), enables the rapid and precise detection and diagnosis of an infection [13-15]. Enzyme immunoassays (EIAs), which directly detect *C. jejuni and C. coli* antigens from samples, has become more widely used and has been reported to have excellent sensitivity and specificity as compared with culture results [11, 16]. A test used for patients must be capable of detecting the target pathogen sensitively and accurately for the rapid initiation of treatment and, in the case of an illness outbreak, to prompt epidemiological investigation.

In our locality (Al Sharqia, Egypt) the isolation rates of *C. jejuni* and *C. coli* from cases of diarrhea are unclear. This study aimed to determine the incidence of *C. jejuni* and *C. coli* isolation in cases of diarrhea and to assess the utility of using PCR and EIA as rapid alternatives for routine culture technique

METHODOLOGY

This study was conducted from April 2012 to August 2014 at the Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt. A total of 193 patients suffering from diarrhea or dysentery were selected from the Pediatric, Tropical, and General Medicine outpatient clinics and departments in Zagazig University Hospitals. A total of 150 age-matched, apparently healthy individuals (not suffering from diarrhea for at least 3 weeks) were enrolled as the control group. Informed written consent was obtained from every participant or his/her caregiver before sample collection.

Bacteriological examination:

Stool samples or rectal swabs were collected from all patients and controls. Those samples were cultured on Skirrow's medium and modified charcoal cefoperazone desoxycholate agar (mCCDA) medium, which are selective for *Campylobacter* spp. The inoculated media were incubated for 48 h at 42˚C (Skirrow's medium) or 37˚C (mCCDA medium). Presumptive identification of *C. jejuni and C. coli* was based on colony appearance, microscopic examination, and biochemical activities (catalase test, oxidase test, hippurate hydrolysis test, and susceptibility to nalidixic acid and cephalothin). Identified colonies were maintained in tryptic soy broth containing 15% glycerol at -40˚C until required for DNA extraction and PCR examination.

Enzyme immunoassay:

Campylobacter specific antigen was detected in the fecal samples using Ridascreen Campylobacter (R-Biopharm AG, Darmstadt, Germany) following the manufacturer's instructions.

Polymerase chain reaction:

DNA extraction from fecal samples was carried out using QIAamp DNA Stool MiniKit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. DNA extracts were kept at -20˚C until required. PCR was performed in a T gradient thermal cycler (Biometra, Göttingen, Germany) using the following primer sets: 5'-AGA GTT TGA TCA TGG CTC AG-3' and 5'-GGA CTA CCA GGG TAT CTA AT-3' for 16S rRNA gene (as an internal control), 5´-GAA GAG GGT TTG GGT GGT G-3' ,5´-AGC TAG CTT CGC ATA ATA ACT TG-3' for *hipO*gene (specific for *C. jejuni*), and *5`-GGTATGATTTCTACAAAGCGAG-3`,5`-ATA AAAGAC TAT CGT CGC GTG-3`)* for *asp* gene (specific for *C. coli*). All primers were supplied by Operon Biotechnologies GmbH (Cologne, Germany). PCR was performed using the *Taq* PCR Master Mix Kit (Qiagen GmbH) in 25 µl of a solution containing 5 µl template DNA and 0.2 µM *hipO* primers, 0.4 µM *asp* primers, and 0.05 µM 16S rRNA primers. An initial denaturation step at 94˚C for 6 min was performed, followed by 35 cycles of denaturation at 94˚C for 50s. Afterwards, annealing at 57˚C for 40 s was done followed by an extension at 72˚C for 50 s and a final extension for 10 min at 72˚C. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. Each run included a positive control containing DNA extracted from *C. jejuni* ATCC 33291 and *C. coli* ATCC 43478, and a negative control containing distilled water instead of template DNA.

RESULTS

This study analyzed 343 stool samples and rectal swabs collected from two matched groups. The patient group included 193 patients (mean age 58.7±10.3 months) suffering from diarrhea or dysentery. The control group included 150 apparently healthy individuals (mean age 58 ± 10.6 months) who, with the exception of neonates, had not suffered from diarrhea for at least 3 weeks.

The age and sex distribution of both groups are presented in table 1. Infants comprised the majority of cases, forming 66.3% and 64.7% of the patient and control group, respectively.

Table 1: Age and sex distribution of patient and control groups

	Category	Patient group (n=193)		Control group (n=150)	
		No.	Percent	No.	Percent
Age	**< 2 years**	128	66.3%	97	64.7%
	2 to < 18 years	51	26.4%	41	27.3%
	≥ 18 years	14	7.3%	12	8%
Sex	**Male**	89	46.1%	66	44%
	Female	104	53.9%	84	56%

Stool samples from both groups were cultured on the aforementioned selective media to isolate *C. jejuni* and *C. coli*. Twelve isolates were obtained. Eleven were in the patients group, corresponding to an isolation rate of 5.7%. The remaining isolate was from the control group, corresponding to an isolation rate of 0.7%. Nine of the 12 isolates were identified as *C. jejuni* with three identified as *C. coli* phenotypically by conventional methods. Eight of the *C. jejuni* isolates were from the patient group (4.1% isolation rate) and one strain was isolated from the control group (0.7% isolation rate) (Table 2). All three *C. coli* isolates were obtained from the patient group (1.6% isolation rate). These results were statistically significant (p =0.01).

Table 2: Isolation rate of *Campylobacter* spp. among patient and control groups

No. of *Campylobacter* isolates	Patient group (n=193)	Percent	Control group (n=150)	Percent	*P*	X^2
***C. jejuni* (n=9)**	8	4.1%	1	0.7%	0.01	6.3
***C. coli* (n=3)**	3	1.6%	0	0.0%		
Total	11	5.7%	1	0.7%		

Because of limited financial resources, we use only one kit of EIA that can test a total of 80 cases. Therefore all *C. jejuni* and *C. coli* culture-positive cases (n=12) and 68 culture-negative cases (63 from patients and five from the control group) were subjected to EIA that detects *Campylobacter* antigen in stools in a qualitative manner. Complete agreement was found between the visual and the spectrophotometric readings. A total of 18 cases were positive in EIA testing (22.5%). All the detected cases were from patients (18/74, 24.3%). Among the 12 culture positive cases, 11 (91.7%) were detected by EIA (all were from the patient group). On the other hand, seven cases (10.3%) were detected among the culture negative cases (all were from the patient group as well). This corresponded to a sensitivity of 91.7% and a specificity of 89.7% in comparison to culture. Positive predictive value (PPV) and negative predictive value (NPV) was 61.1% and 98.4%, respectively.

All cases that were tested by EIA (n=80) were subjected to DNA extraction directly from stools and multiplex PCR targeting 16S rRNA, *hipO*, and *asp*.

Out of the 12 culture positive cases, 11 were positive in PCR (91.7%), while one culture positive case was negative in PCR (among the patient group). No positive cases were detected among the culture negative cases giving the test 100% specificity and 91.7% sensitivity, with a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 98.6% (Table 3).

Table 3: Specificity and sensitivity of PCR and EIA for the detection of *C. jejuni* and *C. coli* in stools among culture-positive and culture-negative cases

Culture	PCR			EIA		
	Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Positive	11	0	11	11	7	18
Negative	1	68	69	1	61	62
Total	12	68	80	12	68	80
Specificity	100%			89.7%		
Sensitivity	91.7%			91.7%		
Positive Predictive Value	100%			98.6%		
Negative Predictive Value	61.1%			98.4%		

DISCUSSION

The diverse microbial infections that can cause diarrhea create a huge diagnostic gap. This is especially true for developing countries with poor resources and little or no access to modern laboratory procedures. Pathogens may go undetected or are detected extremely late. This is due to their fastidious and delicate nature or their failure to grow on routinely-used culture methods. Newer, more sophisticated, and sensitive molecular methods have offered ways to overcome the drawbacks of etiological diagnosis and surveillance procedures. *Campylobacter* enteritis is a clear example in this regard. Detection is hampered by the organism's special growth requirements, low dose required for infection, and possibility of entering a viable but non-cultivable state. Molecular methods based on PCR amplification

have several advantages over classical bacteriology, concerning detection limits, species identification level, speed, and potential for automation. The molecular methods may be alternatives to culture methods for the detection of *Campylobacter*, particularly for epidemiological studies, where many samples are examined [17-19]. This study aimed to detect the isolation rate of *C. jejuni* and *C. coli* in stool samples among cases with diarrhea using traditional culture, PCR, and EIA.

The overall isolation rate of *C. jejuni* and *C. coli* among cases with diarrhea was 5.7%, with rates of 4.1% for *C. jejuni* and 1.6% for *C. coli*. Only one isolate (*C. jejuni*) was obtained from control samples, representing a 0.7% isolation rate. These results agree with two prior studies in Egypt. One reported an isolation rate of 5.5% among children below 60 months of age in El-Fayoum [20]. The other study carried out at Ain Shams reported an isolation rate of 5.8% [21]. The present rates are lower than the 17.2% rates recorded among Egyptian infants in Alexandria [22] and 9% among rural Egyptian children in Abu Homos [23]. The results clearly indicate that *Campylobacter* infection may be more prevalent in certain regions in Egypt. Looking at studies conducted outside Egypt, our results agree with rates of 6% reported in Tehran, Iran [24] and with 5.4% in Turkey [25]. Our results are also comparable with the rate of 4.7% reported among children in Gaza, Palestine [26]. However, the present findings are lower than the rates of 4.8% reported from diarrheal patients in China [27], 8.6% for Japan[28], and 8.2% from Nigeria [29]. Globally, the isolation rates have varied markedly from 2.8-29%) [4, 8, 30-35], clearly indicating that differences in the methods used for detection, populations, geographic factors, and sample size can greatly influence the isolation rate of *Campylobacter*.

Presently, the isolation rate of *C. jejuni* in the control group was 0.7%. This nearly coincides with the results found by Olesen et al. [33] in young children in Denmark, where the rate among patients was nearly 7-times more than the control group (2.9% compared to 0.4%). On the other hand, our result differs from the 7.2% reported by Josiane et al. [36] in the control group compared to 9.6% in the patient group. Interestingly, Samuel et al. [29] did not detect *Campylobacter* in their control group.

In the present study, multiplex PCR was used for the detection from stool samples of *C. jejuni* (*hip O*) and *C. coli* (*asp*) with 16S rRNA as an internal control. PCR was negative in a single culture-positive specimen. A similar finding was reported by Roumi et al. [5], who also explained this by the possibility of cell lysis in situ in the interval between sample collection and nucleic acid extraction by diverse nucleases in the feces. Moreover, the presence of inhibitory substances in stools was also suggested as a factor that could affect the PCR results. Tribble et al. [37] and Houng et al. [38] demonstrated that PCR did not yield positive results in 8% of culture-positive stools, which subsequently tested positive after dilution of the samples.

Presently, PCR had a sensitivity of 91.7% and specificity of 100% specificity, with 100% and 98.6% PPV and NPV, respectively, compared with the results from selective culture. Low sensitivity has also been reported previously [39]. These authors concluded that selective culture remains the optimum method for detection of campylobacters from stool samples. Furthermore, it was reported that the delay in DNA extraction from stools can result in DNA degradation due to freezing and may lead to PCR failure. Iijima et al. [40] reported that the detection limit of PCR was lower than the detection limit of culture methods. They concluded that selective culture is superior to PCR when fresh stools are analyzed. However, our results contrast with other studies that demonstrated the significantly good performance of PCR on stools, with 100% sensitivity for *Campylobacter* identification [8, 12, 21, 24].

Negative PCR results were obtained from all culture negative cases, including patient and control samples in our study. The findings demonstrate the species specificity of the assay and corroborates previous studies that used *hipO* alone or both *hipO* and *asp* [24]. In contrast, lower specificity of 95.9% was reported in another study using PCR that targeted the same two genes [5]. The latter difference could reflect the different primers used that targeted different areas of the genes of interest.

Theoretically, same-day results allow for earlier initiation of treatment. For microbiology laboratories with no campylobacter culture capability, a sensitive EIA would provide an option for detection of *Campylobacter* spp. EIA is not a time-consuming procedure. Other available rapid tests are either nonspecific, such as Gram and acridine orange staining, or are typically available only in a research setting, such as PCR. Furthermore, when different EIA versions were compared to culture, each performed better than culture in detecting *C. jejuni* and *C. coli* in stool specimens. However, false-negative and false-positive results were observed [41].

Compared to selective culture, EIA had 91.7% sensitivity, 89.7% specificity, 98.4% NPV, and 61.1% PPV. A negative EIA result was recorded for a single culture-positive specimen. Nevertheless, EIA yielded positive results with seven culture-negative stool samples, perhaps due to cross-reactivity with other *Campylobacter* spp. that went undetected by culture. The present findings are similar to those of Thomas and Ralf [42], who reported 96.8% sensitivity and 98% NPV. However, these authors reported a much higher specificity (97.2%) and PPV (96.8%). The better specificity may reflect the smaller number tested samples in our study and the prior use of a newer generation EIA. The same version of EIA as we used was utilized in another study and showed a specificity

(87%) and NPV (97%) similar to our values [43]. However, these authors reported lower sensitivity and PPV (69% and 36%, respectively), suggesting that the choice of antibodies can yield better results. In other studies, values have been diverse, and include 92% sensitivity [36], 89% sensitivity and 99% NPV [1], 89.1% sensitivity and 99% NPV [16], and 94.1% sensitivity and 98.46% NPV [5]. All these studies concluded that the high accuracy of EIA offers the advantage of wider applicability in resource-poor settings where molecular tests are not an available option. It was also concluded that EIA targeting different antigens can affect the specificity of the test.

CONCLUSION

In conclusion, the results of this study demonstrate that selective culture remains the optimum method for detection of *Campylobacter* spp. from stool samples. However, the choice of the assay depends on whether it is used for the etiologic diagnosis of diarrhea or for surveillance. EIA offers the potential for providing same-day results, eliminating the need and expense associated with the utilization of special devices for the creation of an optimum microaerophilic environment. The assay is rather simple to perform in small laboratories. However, it has poor specificity for the detection of *C. jejuni* and *C. coli*, in part due to detection of other *Campylobacter* spp. Although it can detect more than 91% of *Campylobacter* infections, it should be redeveloped to be more specific. Nucleic acid-based diagnostics offer increased specificity and have the ability to determine the presence of infection. Also, *Campylobacter* at the species level can be distinguished. Therefore, PCR, if feasible, is a preferred diagnostic method for detection of *Campylobacter* infection for epidemiologic studies in the developing world.

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