A Comparative Study of Conventional and Molecular Techniques in Diagnosis of Campylobacter Gastroenteritis in Children

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Abstract. **Background:** Campylobacter species are a significant cause of gastroenteritis among children worldwide. Conventional methods for detection of Campylobacter spp. based on cultural isolation and biochemical tests are cumbersome and time consuming. Because of their superior sensitivity and cost effectiveness, molecular methods are often used for identification of the pathogens. **Aims:** To evaluate different diagnostic methods for identification of Campylobacter. **Materials and Methods:** Faecal samples were collected from 585 children (age ≤ 12 years) with acute diarrhoea admitted in a tertiary-care hospital, excluding children already on antimicrobial therapy. All samples were examined by four methods: Grams’ staining, culture methods, Enzyme-Immuno Assay, and Polymerase Chain Reaction (PCR). After Grams’ staining, samples were inoculated on modified charcoal cefoperazone deoxycholate agar. ProSpecT® Microplate Assay® and PCR assay using cadF gene was done for detection of Campylobacter specific antigen and DNA, respectively, in faecal samples. McNemar’s test was used to compare the results wherever applicable. **Results:** 197 cases (33.67%) were found to be positive for Campylobacter by at least one method. But only 121 (20.78%) out of the 585 stool specimens tested fulfilled the positivity criteria, i.e., positive either by culture or by any two tests among other three. Culture had very low sensitivity (37.19%), whereas PCR had the highest (96.69%) sensitivity but lowest positive predictive value (86.03%). Rapid Grams’ staining technique (sensitivity 63.64%) was found to be better than culture. Detection by PCR and ELISA was significantly better than by culture on selective media and Grams’ staining (p<0.0001). **Conclusions:** Molecular techniques significantly increased detection rates of Campylobacter in children with diarrhoea. However, enzyme-immuno assay with high accuracy has the advantage of applicability in resource-poor settings.

**Key words:** Campylobacter, Diarrhoea, PCR, Enzyme-immuno assay, and Grams’ staining. **Abbreviations:** CCDA, charcoal cefoperazone deoxycholate agar; ELISA, enzyme linked immunosorbent assay; PCR, polymerase chain reaction

Introduction

**Campylobacter enteritis** is becoming a leading cause of acute bacterial gastroenteritis worldwide. Consumption of contaminated poultry, meat, or water are the primary sources of infection. An overall incidence rate of 14.3 per 100,000 had been documented in United States by FoodNet in 2012 [1]. In developing countries, the rate of isolation is 5-20% [2]. Close proximity to animals, uncovered garbage in cooking areas, lack of safe water supply, and lack of knowledge about sanitary disposal of faeces are the main risk factors for acquiring infections. **Campylobacter jejuni** and **Campylobacter coli** have been isolated from chicken, goats, sheep, and pigs in developing countries. Children, especially during their first 5 years of life, are affected most frequently by **Campylobacter** gastroenteritis [3]. The most common symptom of **C. jejuni** infection is diarrhoea of abrupt onset accompanied by severe abdominal pain. Infection is more severe in young (<2 years old), elderly, or immunocompromised patients and may require antibiotic therapy [1,4].
Gullain-Barre’ Syndrome (GBS, acute polyneuropathy) and Reiter’s Syndrome (reactive arthropathy) are remarkable systemic complications following \textit{C. jejuni} infection. GBS is gaining notice as a cause of acute flaccid paralysis (AFP) as the world is on the verge of Poliovirus eradication. A high titre of serum antibodies against \textit{Campylobacter} among patients with GBS has been documented by many studies in the Indian sub-continent [5]. Continuous surveillance of \textit{Campylobacter} infection cases, especially in children, is therefore necessary to know the prevalence rate and to formulate control measures.

\textit{Campylobacter}, a slender, curved/spiral, motile gram negative rod, needs microaerophilic conditions for growth. \textit{Campylobacter} is highly infectious, with infective doses as low as 500 to 800 CFU [6]. As \textit{Campylobacters} are known to have a low survival rate when exposed to room temperature and atmospheric air, the presence of viable but non-cultivable (VBNC) forms in refrigerated food may be the cause of lower recovery by culture method. In recent years, several enzyme immunoassays (EIAs) and molecular methods, which allow detection of \textit{C. jejuni} and \textit{C. coli} antigen and DNA directly from samples, have been increasingly employed due to their high sensitivity and cost-effectiveness. Many previous studies have evaluated amplification techniques for the detection of \textit{Campylobacter} directly on stool samples using different target genes including: GTPase-based PCR-reverse hybridization assay, [7] the \textit{ceuE} gene, [8] the 16S rRNA gene, [9,10,11] the 23S rRNA gene, [12] the \textit{gyrA} gene, [13] the \textit{gyrB} gene, [14] the flagellin gene (\textit{flaA}), [15] the lipid A gene \textit{lpxA}, [16] or random, [17] or a multiplex of the 16S rRNA, \textit{hipO} (hippuricase), and aspartokinase genes [18].

A wide range (3.1-13.5\%) of isolation rates of \textit{Campylobacter} has been reported by numerous Indian studies from different regions [19,20,21]. Recently, \textit{Campylobacter} spp. were detected in 4.5\% children with diarrhoea in Vellore, Tamilnadu, India using a PCR-based tool, and the majority of these patients were co-infected with other enteropathogens [22]. \textit{C. jejuni} (82.3\%), \textit{C. coli} (12.9\%), \textit{C. lari} (3.2\%), and \textit{C. upsaliensis} (1.6\%) were various species isolated in a study conducted in north India [23]. Studies evaluating the association of \textit{Campylobacter} spp. with paediatric diarrheal patients in Indian population are scarce. This scarcity necessitates the evaluation of newer detection methods to fill the diagnostic gap in diarrheal diseases by decreasing the significant proportion of missed cases.

**Materials and Methods**

Stool samples were collected from 585 children (≤12 years of age) having acute watery diarrhoea (≤ 14 days duration) admitted to the paediatric diarrhoea ward of Lok Nayak Hospital, Maulana Azad Medical College, New Delhi, India. Children who had received prior antimicrobial therapy were excluded. The study was approved by the Institutional Ethics Committee, and informed consent was obtained from the parents/guardians/person attending the study subject after a description of the study was given. All samples were screened for \textit{Campylobacter} by four methods.

1) **Direct examinations:** Gram’s staining of stool samples was done using carbol fuchsin or 0.1\% aqueous basic fuchsin as counter stain [24].

2) **Cultural and biochemical examination:** All the faecal samples were inoculated on modified charcoal cefoperazone deoxycholate agar (CCDA) (Oxoid®, Hampshire, United Kingdom) with campylobacter selective supplement (Butzler) (Oxoid®, Hampshire, United Kingdom) containing bacitracin (12,500IU), cycloheximide (25mg), colistin sulfate (5,000IU), cephaiazolin sodium (7.5mg), novobiocin (2.5mg), and growth supplement (Oxoid®, Hampshire, United Kingdom). The plates were incubated along with a control strain of \textit{C. jejuni} for 48 hours at 42°C under microaerophilic conditions generated by ANOXOMAT AN2OP system (Mart Microbiology®, Drachten, Netherlands). Cultures were discarded as sterile after 7 days of incubation. Suspected moist, translucent colonies were identified using a modified Gram’s stain and the oxidase test. The isolates were speciated using biochemical tests such as catalase, nitrate reduction, hippurate hydrolysis, hydrolysis of indoxyl acetate, growth in 1% glucose & 1.5% NaCl, H₂S production on triple sugar iron agar, growth at 25°C and 42°C, and susceptibility to nalidixic acid (30μg) and cephalothin (30μg).

3) **Enzyme-Immunno Assay:** \textit{Campylobacter} specific antigen was detected in the faecal samples using ProSpecTTM Campylobacter Microplate Assay* (Oxoid Ltd, UK) following the manufacturer’s instructions.

4) **Molecular detection of Campylobacter:** Polymerase chain reaction (PCR) is a process used to amplify DNA via a temperature-mediated DNA polymerase using a specific primer to detect the presence of \textit{Campylobacter} in faecal samples.
DNA extraction. DNA was extracted from stool samples using QIAamp DNA Stool Mini Kit (QIAGEN, Gmbh, Germany) as per the manufacturer’s instruction manual. DNA extracts were then stored at -20°C until needed for PCR.

Amplification of extracted DNA. PCR was performed to amplify a 400 bp region of cadF, a genus-specific virulence gene of Campylobacter, using the following DNA primers: 5’-TTG AAG GTA ATT TAG ATA TG-3’ (forward primer) and 5’-CTA ATA CCT AAA GTT GAA AC-3’ (reverse primer). This segment of Campylobacter DNA corresponds to sequences described by Nayak et al [25]. The PCR reaction mixture (25μl) consisted of 6μ extracted DNA, 3μ PCR reaction buffer, 50 pM of cadF forward and reverse primer, 0.25mM concentration of each dNTP , and 1U of Taq DNA polymerase. Positive (ATCC 29428) and negative controls were included in each PCR run. Cycling conditions used were: pre-heating at 94°C for 4 minutes, denaturation at 94°C for 1 minute, annealing at 47°C for 1 minute, extension at 72OC for 7 minutes, and final extension at 72°C for 5 minutes. Thirty-six cycles were performed using the thermocycler (Mycycler™, Bio-Rad, USA).

Amplified PCR products were electrophoresed on 2% ethidium bromide-stained agarose gel, along with a molecular weight marker (1000-bp DNA ladder, Bio-basic inc.). The electrophoresis was carried out at a constant voltage of 90V for 2 hours, and a band of 400-bp was taken to be positive. The bands in the gel were photographed under UV transillumination (Figure 1).

Statistical analysis. All data obtained were analyzed using SPSS statistical software, and sensitivity, specificity, positive and negative predictive values were calculated. McNemar’s test was used wherever applicable to compare the results.

Results

Stool samples from 585 subjects were analysed using four diagnostic methods for the detection of Campylobacter spp. as a cause of diarrhoea. In the present study, 197 cases were positive for Campylobacter by at least one method. All four methods were positive in only 43 cases.

Definition of a Campylobacter-positive stool sample. A sample was taken as positive for Campylobacter if it was either culture positive OR positive by any two tests among Grams’ staining, antigen detection by ELISA, and PCR.

Based on the case definition, 121/585 (20.68%) cases were positive for Campylobacter, of which only 45 yielded growths on culture and 76 were culture-negative but fulfilled the positivity criteria. Of the 45 culture-positive samples, only one case each was missed by PCR and ELISA. Grams’ staining failed to detect the bacilli in two culture-positive cases. Out of 76 culture-negative samples, 25 were positive by the other three methods, while 51 were positive by any two of the two methods used (Table 1). In our study, spiral bacilli were identified in 77 cases by Grams’ staining, out of which 43 were culture-positive and 34 were positive by ELISA and/or PCR.

PCR had the highest sensitivity (96.69%), but its positive predictive value (85.40%) was the lowest. ELISA was able to detect the Campylobacter antigen in 114 stool samples, and thus had high sensitivity (94.21%). Culture and Grams’ staining both had 100% PPV (Table 2). McNemar’s test was applied to analyse the statistical significance of differences in the detection rates of the four methods. The difference in the rates of detection was significant for culture and ELISA (p<0.0001), for selective culture and PCR (p<0.0001), for Grams’ staining and PCR (p<0.0001) and for Grams’ staining and ELISA (p<0.0001). However, the difference between ELISA and PCR or culture and Grams’ staining was not statistically significant.

Figure 1. PCR amplification of cadF gene from representative samples for detection of Campylobacter. Lane 1: DNA ladder, Lane 2: Negative control, Lane 3: Positive control, Lanes 4 and 5: Diarrhoeic stool.
According to WHO estimates, diarrhoeal diseases are the third-most common cause of infectious disease deaths worldwide, with about 1.7-2.5 million deaths per year [26]. India alone loses 600,000 children each year due to diarrhoea, and it causes the death of almost 23% of Indian children who die before the age of 5 years [27]. The wide diversity of microbial infections causing diarrhoea creates a huge diagnostic gap, especially in developing, resource-poor countries with little or no access to modern laboratory procedures. Several pathogens go undetected or are detected very late due to their fastidious and delicate nature, and failure to grow on routinely-used culture methods. Newer, sophisticated, and sensitive molecular methods have provided ways to overcome the drawbacks of etiological diagnosis and surveillance procedures.

In recent times, Campylobacter has become a leading cause of acute bacterial gastroenteritis, and may be responsible for as many as 400-500 million cases worldwide each year [28]. There are quite a few Indian studies showing the remarkable potency of this microbe as a pathogen of diarrhoea in different regions [19-21]. In North India, the hot and humid climate, dense population, below-average literacy rate, and poor knowledge of hygiene makes the condition favourable for this food and water-borne pathogen to spread. Our previous study had shown 10.28% of children with acute diarrhoea were positive for Campylobacter by culture, and isolates were highly resistant (86.11%) to ciprofloxacin [29]. Very few researchers have highlighted the actual prevalence of this microaerophilic pathogen and compared the molecular techniques with other conventional methods for its diagnosis.

In the present study, a total of 197 (33.67%) specimens were positive by at least one method amongst four (direct microscopic examination, culture on selective media, ELISA for detection of Campylobacter antigen, and PCR for detection of Campylobacter DNA). Based on the case definition, 121 (20.68%) specimens were taken as positive for Campylobacter: 45 were positive by culture, and 76 were culture-negative but positive by any two tests among Grams’ staining, antigen detection by ELISA, and PCR. A higher isolation rate was seen in the present study as compared to previous studies from India in which the isolation rate varied from 3.1% to 13.5% in acute diarrheal cases [19-21]. The variation in results may be due to the use of different techniques of detection. In the present study, newer methods including PCR were used, while the other studies used only culture as the method of detection. A recent study from the southern part of India reported a 4.5% detection rate from children using PCR-based tool [22].

<table>
<thead>
<tr>
<th>No. of cases (n=121)</th>
<th>Grams’ staining</th>
<th>ELISA</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of culture positive specimens (n=45)†</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Number of culture negative specimens (n=76)

| Number of culture negative specimens (n=76) | + | + | + |
| 25                                           |   |   |   |
| 6                                            |   |   | + |
| 3                                            |   | + |   |
| 42                                           |   | - |   |

*, + positive; - negative
† All isolates were identified as C. jejuni

Discussion

According to WHO estimates, diarrhoeal diseases are the third-most common cause of infectious disease deaths worldwide, with about 1.7-2.5 million deaths per year [26]. India alone loses 600,000 children each year due to diarrhoea, and it causes the death of almost 23% of Indian children who die before the age of 5 years [27]. The wide diversity of microbial infections causing diarrhoea creates a huge diagnostic gap, especially in developing, resource-poor countries with little or no access to modern laboratory procedures. Several pathogens go undetected or are detected very late due to their fastidious and delicate nature, and failure to grow on routinely-used culture methods. Newer, sophisticated, and sensitive molecular methods have provided ways to overcome the drawbacks of etiological diagnosis and surveillance procedures.

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of 16S rRNA gene as the target of PCR and regional difference may be the cause of lower detection rate. Studies from neighbouring countries like China, Pakistan and Bangladesh have revealed an isolation rate of 11.8%, 18%, and 25.5% respectively, results similar to those of the present study [30,31,32]. However, a very high prevalence (62%) of Campylobacter has been reported from Thailand [33].

It is striking that among the 121 cases that tested positive for Campylobacter, only 45 yielded growths on culture. All of the 45 strains isolated on culture were identified as C. jejuni based on biochemical tests. This low sensitivity (37.19%) of culture may be due to certain limitations such as the fastidious nature of the organism, use of highly selective media with antimicrobial agents, and presence of viable but non-cultivable bacilli. Therefore, the true incidence of Campylobacter enteritis may remain underestimated if culture is used as the only method of diagnosis. Similar results have been shown by Lawson et al and Besse’de et al, where some cases were missed by the culture method [34,35]. Nevertheless, isolation of Campylobacter on culture is important as it allows precise identification of bacteria and antimicrobial susceptibility testing.

Grams’ staining is an inexpensive and rapid method for presumptive identification of Campylobacter in stool samples. The sensitivity of Grams’ staining technique was found to be 63.64% with 100% specificity and PPV. However a higher sensitivity of 89% has been reported by Wang et al [36]. The variation may be due to subjectivity in the identification of the organism and differences in the staining methods used.

Several commercially available enzyme immunoassays have been developed for the direct detection of C. jejuni and C. coli in stool specimens. In our study, EIA [ProSpecT™ Campylobacter Microplate Assay* (Oxoid Ltd, UK)] showed high sensitivity (94.21%). Previous reports also documented a similar performance of this method in comparison with both culture and PCR [37,38,39]. Cross-reactivity to other bacteria and various components found in stool specimens may explain false-positive EIA results [39].

Molecular methods are increasingly being used to overcome these problems. Our result showed that PCR could detect three times as many positive cases (117) as culture (45) with 96.69% sensitivity. The superiority of PCR as a method for detection of Campylobacter has been reported by Al Amri et al and Kulkarni et al [40,41]. Singh et al also reported a higher rate of detection by PCR (13.3%) versus culture (10.5%)[41]. The high sensitivity of PCR for detection of C. jejuni both from pure culture (as few as 50 cells per PCR reaction) and artificially spiked samples has also been reported [42]. However, Lawson et al did not find a statistically significant difference between PCR and culture detection (9.5% and 8% isolation rates, respectively) [34]. In another study, the presence of C. jejuni was identified in 6.7% of culture-negative specimens using cadF gene[40]. Higher sensitivity of PCR to culture may be due to the ability of molecular methods to detect the presence of viable but non-cultivable and also dead organisms, which are missed by conventional culture methods. Hence it may not be appropriate to use culture as a sole reference technique for statistical evaluation. In this study, PCR was negative in one culture-positive

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### Table 2. Statistical Characteristics of Different Techniques Used for Campylobacter Detection.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV*</th>
<th>NPV†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>37.19%</td>
<td>100%</td>
<td>100%</td>
<td>85.93%</td>
</tr>
<tr>
<td>Grams’ staining</td>
<td>63.64%</td>
<td>100%</td>
<td>100%</td>
<td>91.34%</td>
</tr>
<tr>
<td>ELISA</td>
<td>94.21%</td>
<td>96.34%</td>
<td>87.02%</td>
<td>98.46%</td>
</tr>
<tr>
<td>PCR</td>
<td>96.69%</td>
<td>95.91%</td>
<td>86.03%</td>
<td>99.11%</td>
</tr>
</tbody>
</table>

* Positive predictive value
† Negative predictive value
case. This may be due to cells being lysed in situ in the interval between collection and nucleic acid extraction, due to the presence of diverse nucleases in faecal matter [34]. In a study by Hoang et al., PCR did not yield positive results in 8% of culture-positive stools that became positive after dilution of samples, thereby indicating that some substances in faecal samples may be inhibitory to PCR amplification [43].

In the present study, cadF gene was chosen for Campylobacter identification as the gene encodes a 37-kDa outer membrane protein that promotes the binding of the organism to intestinal epithelial cells and is an important virulence factor [25,40]. The gene and its product have also been shown to be conserved among a number of C. jejuni isolates [44,45].

Stool represents a heterogeneous specimen and makes DNA extraction technically challenging. This has hindered the direct application of molecular techniques to faecal material [40]. The use of the QIAamp DNA stool mini kit produced satisfactory results for extracting the DNA of Campylobacter spp. and was used in the present study also [40].

Conclusions. The present study has shown that a large number of patients with acute diarrhoea have Campylobacter in faecal samples. Campylobacter is an established cause of gastroenteritis in children. The presence of an important virulence gene (cadF) revealed the pathogenic potential of the organism as well. The study also highlights the superiority of molecular methods in detecting Campylobacter spp. directly from faecal samples in comparison with other conventional methods. However, the high accuracy of enzyme-immuno assay offers the advantage of wider applicability in resource-poor settings where molecular tests are not a viable option.

References


