ORIGINAL RESEARCH

Study of Prevalence of *Campylobacter* Gastroenteritis among Pediatric Population Using a Multiplex PCR in a Tertiary Care Hospital in Puducherry, South India

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Abstract

Background and objectives: *Campylobacter* is one of the four common causes of diarrheal illness worldwide. *Campylobacter* infection is more common in developing than in developed countries. As conventional methods pose a great difficulty for the isolation and identification of this organism, molecular methods are much preferred.

Materials and methods: A total of 133 stool samples were collected from children less than 13 years presenting to pediatric outpatient department and the emergency department in a tertiary care hospital in Puducherry. The stool samples were extracted, and the DNA was subjected to multiplex PCR to detect *Campylobacter* species, followed by sequencing.

Results: *Campylobacter* species was detected in 13 children (9.7% of the study population) (95% CI: 5.5–16.4), with *Campylobacter jejuni* (11 of 13) being the predominant species. The prevalence was higher in children less than 24 months (18.18%) with a higher predisposition to girls (14.29%). The most common clinical presentation was found to be acute watery diarrhea (10%). *Campylobacter* detection was higher from August to November (62%), with the highest incidence in October (22.3%). *Campylobacter* was detected in six (21.4%) children who had contact with pets.

Conclusion: The study reveals that the prevalence of *Campylobacter* infection was high in Puducherry. There is an increased need to implement molecular assays for the routine detection of *Campylobacter* in all clinical pediatric stool samples.

Keywords: *Campylobacter*, Children, Gastroenteritis, Multiplex PCR.

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Introduction

Campylobacter is one of the four common causes of diarrheal illness worldwide.[1] CDC estimates that about 1.5 million U.S. residents are infected by *Campylobacter* every year.[2] In India, diarrhea is the third common cause of mortality, with a mortality rate of 13%, and is responsible for the death of nearly 300,000 under 5 years children in India every year.[3] *C. jejuni* and *C. coli* are the primary agents of gastroenteritis in humans. *Campylobacter* is a slender, curved/spiral, "S"-shaped motile gram-negative, nonspore-forming microaerophilic organism with polar flagella.[4–6] Ingestion of contaminated food and water remains the primary mode of transmission.

The traditional method for the isolation of *Campylobacter* species from stool samples is by inoculating the sample onto the selective media such as blood or charcoal-based media and incubating the plates at 42°C in a microaerophilic (5% O_2, 10% CO_2, 85% N_2) environment.[7,8] Despite the public health problems posed by this organism, the routine isolation and identification of this organism are often missed due to the inappropriate diagnostic modalities. Therefore, this study was conducted to utilize multiplex PCR to identify *Campylobacter*, thereby determining the prevalence of *Campylobacter* infection among the pediatric population in a tertiary care hospital in Puducherry.

Materials and Methods

The study was a hospital-based cross-sectional descriptive study and was approved by the Institute Ethics committee (IEC No JIP/IEC/2018/0398). The duration of the study was from January 2019 to June 2020.

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Conflict of interest: None

Study Population

Stool samples from children less than 13 years of age with acute diarrhea and dysentery were included in the study after obtaining informed written consent from the parents/guardians. Stool samples from children with hospital-acquired (the onset of loose or watery stools at least 48 hours after hospital admission), persistent, and chronic diarrhea were excluded.

Sample Size Calculation

The approximate sample size calculated was 133 using sample size for sensitivity specificity studies by Naing.[9] This was calculated

considering the detection rate of *Campylobacter* using PCR to be 34% (0.34), the sensitivity of PCR to be 97% (i.e., 0.97) from a study conducted in New Delhi with a precision of 5% (0.05) and with a confidence level of 95% (0.95).[10]

Stool Processing

All the stool samples underwent routine processing as per our laboratory standard operating procedures upon reception at the laboratory. Briefly, each sample was examined macroscopically for the consistency, presence of obvious blood/mucus, and visible worms/proglottids. This was followed by microscopic examination of saline wet mount preparation to look for the presence of pus cells, red blood cells (RBCs), ova, cyst, and trophozoites. Subsequently, each sample was plated onto MacConkey agar (MAC), xylose lysine deoxycholate agar (XLD), and inoculated into selenite F enrichment broth. Watery samples were subjected to an additional plating on thiosulfate citrate bile salt sucrose agar (TCBS) and alkaline peptone water (APW). The plates and the broth were incubated aerobically at 37°C for 18 hours. After incubation the plates were read for the presence of any suspected colonies of Salmonella/*Shigella*/Vibrio and Aeromonas on XLD and MAC agar. Subculture from selenite F broth was done after 16–18 hours of incubation on MAC and XLD agar and incubated at 37°C for 18 hours. Subculture from APW was done within 6–8 hours of incubation on TCBS agar and incubated at 37°C for 18 hours. The remaining stool samples were preserved at 2–8°C till the DNA extraction is performed.

Stool DNA Extraction and PCR

Genomic DNA was extracted directly from the feces using QIAamp® Fast DNA Stool Mini Kit obtained from (Qiagen, Germany). The steps of extraction were done as per the manufacturer's protocol. Every extraction set was carried out along with the extraction of nuclease-free water, which served as the negative extraction control to rule out the carry over contamination during the extraction. The yielded DNA was stored at −80°C till further analysis.

Multiplex PCR was performed in a 25 µL reaction mixture containing a 2× master mix, 1.0 µL of each primer of the targets[11,12] and the internal control.[13] The target genes for the multiplex PCR were *16S rRNA* (genus *Campylobacter*), *mapA* (*C. jejuni*), *ceuA* (*C. coli*), and *actB* for internal control. β-*actin* was used as the internal control to rule out the presence of PCR inhibitors in the stool samples. Amplification reactions were as follows: one cycle of 10 minute at 95°C followed by 35 cycles each consisting of 30 seconds at 95°C, 90 seconds at 59°C, and 60 seconds at 72°C. The PCR was terminated by a final extension step of 10 minutes at 72°C. Amplification was expected to generate 857, 589, 462, and 619 base pair DNA fragments specific for the genus *Campylobacter* and for *C. jejuni*, *C. coli*, and β-*actin* genes, respectively. Post PCR, the PCR products were subjected to 1.5% gel electrophoresis and were visualized under ultraviolet light.[11] A 100 bp DNA ladder was used in gel electrophoresis that aided in determining the basepair size of the bands that are formed. Reference strain (ATCC *C. jejuni* 33291) was used as the positive control. Each set of multiplex PCR reactions was carried out along with a no template control, negative extraction control, and a positive control. The primers used in the study are mentioned in Table 1.

Sequencing of PCR Products

The products of PCR amplification were subjected to sequencing using an ABI 3730 XL sequencer (Applied Biosystem, Foster, California, USA) at Eurofins Genomics Private Limited, India. The sequences generated were assembled and compared using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information. Translation of DNA sequences into protein sequences was done using Expert Protein Analysis System translate. The translated protein sequences were assembled and compared using the protein BLAST of the NCBI.

Statistical Analysis

All the data were entered and analyzed using STATA 2.0 statistical software. Continuous variables, like age, were summarized as median depending on the distribution of the data. Categorical variables like gender, duration of symptoms, blood, or mucus in stools, duration of antibiotic use were expressed as a percentage. The Chi-square test was used to compare the two groups. A *p*-value of <0.05 was considered statistically significant. Outcome variable like detection of *Campylobacter* was expressed in percentage with 95% confidence interval.

Results

A total of 133 children were included in our study. Out of this, 77 (57.8%) were boys, and 56 (42.1%) were girls. The age distribution of these 133 children were 66 children (49.6%) less than 24 months, 22 (16.5%) between 25 and 60 months, 34 (25.5%) between 61 and 120 months and 11 children (8.2%) between 121 and 156 months. Of these 133, 111 (83.4%) presented with acute watery diarrhea and 22 (16.5%) with dysentery. 105 children (79%) had no contact with pets, and 28 (21%) had contact with pets. Of them, 96 (72.1%) presented in 3–5 days, 32 (24%) presented in 2 days, 5 (3.7%) presented after 5 days. Among the 133 children, 11 (8.3%) were already started on antibiotics, of which nearly nine were under antibiotic coverage for less than 48 hours, and two were between 48 hours to 120 hours. Out of the 133 stool samples, 55 (41.4%) were watery, 49 (36.8%) were semi-formed, 21 (15.8%) were blood-tinged, and 8 (6%) were formed. Microscopically, 100 (75.2%) samples had no pus cells, 26 (19.6%) had pus cells, 3 (2.3%) had RBC, 4 (3%) had both pus cells and RBC. Stool culture had grown enteric pathogens in 23 samples accounting for about 17%. The most common pathogen was found

Table 1: Details of multiplex PCR for the detection of members of the genus and of the thermophilic species of *Campylobacter*[*]

Target gene	*Sequence 5′–3′ of primers*[*]	*Amplicon length specificity*	*References*
16S rRNA	ATC TAA TGG CTT AAC CAT TAA AC GGA CGG TAA CTA GTT TAG TAT	857 bp *Campylobacter*	Denis et al., 1999[12*]
mapA	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	589 bp *Campylobacter jejuni*	Denis et al., 1999[12*]
ceuA	TGATTTTATTATTTGTAGCAGCG AATTGAAAATTGCTCCAACTATG	462 bp *Campylobacter coli*	Denis et al., 1999[12*]
actb	GCACCACACCTTCTACAATG TGCTTGCTGATCCACATCTG	619 bp beta actin	Glare et al., 2002[13]

to be *Salmonella* species (10.3%), followed by *Shigella* species (6.7%) in the stool culture.

All these stool samples were subjected to multiplex PCR, of which 13 children were tested positive for campylobacteriosis, among which 8 (14.3%) were girls and 5 (6.5%) were boys ($p = 0.13$). The majority (18.9%) were less than 24 months which was found to be statistically significant. ($p \leq 0.05$). Acute watery diarrhea was found to be the common clinical presentation accounting for about 10%.

Distribution and association of campylobacteriosis with demographic and clinical variables is depicted in Table 2.

Table 2: Distribution and association of campylobacteriosis with demographic and clinical variables*

Variables	*Total no. of samples (n)%*	*No. of campylobacter positive cases, n (%)*	*95% CI*	*p value*
Gender				
Boys	77 (57.8%)	5 (6.5)	2.1–14.5	1
Girls	56 (42.1%)	8 (14.3)	6.4–26.2	0.13
Age				
<24 months	66 (49.6%)	11 (16.7)	8.6–27.9	0.12
25–60 months	22 (16.5%)	0	—	—
61–120 months	34 (25.5%)	2 (5.9)	0.7–19.7	1
121–156 months	11 (8.2%)	0	—	—
Clinical presentations				
Acute watery diarrhea	111 (83.4%)	11 (9.9)	5.1–17.0	0.9
Dysentery	22 (16.5%)	2 (9.1)	1.1–29.2	1
Fever				
Yes	37	6 (16.2)	6.2–32.0	0.12
No	96	7 (7.2)	3.0–14.4	1
Abdominal pain				
Yes	51	5 (9.8)	3.3–21.4	0.99
No	82	8 (9.8)	4.3–18.3	1
Nausea/vomiting				
Yes	27	5 (18.5)	6.3–38.1	0.08
No	106	8 (7.5)	3.3–14.3	1
Duration of symptoms				
<2 days	32 (24%)	3 (9.4)	2.0–25.0	0.7
3–5 days	96 (72.1%)	8 (8.3)	3.7–15.8	1
>5 days	5 (3.7%)	0	—	—
Contact with pets				
Yes	28 (21%)	6 (21.5)	8.3–40.1	0.9
No	105 (79%)	7 (6.7)	2.7–13.3	1
Under antibiotic coverage				
Yes	11 (8.3%)	0	—	—
<48 hours	9 (6.8%)	0	—	—
48–120 hours	2 (1.5%)	0	—	—
No	122 (91.7%)	13 (10.7)	5.8–17.5	—
Stool macroscopy				
Watery	55 (41.4%)	8 (14.5)	6.5–26.7	0.16
Semi-formed	49 (36.8%)	3 (6.1)	1.3–16.9	1
Formed	29 (21.8%)	2 (6.9)	8.5–22.8	0.89
Stool microscopy				
Pus cells+	26 (19.6%)	3 (11.5)	2.4–30.2	0.56
RBC+	3 (2.3%)	2 (66.7)	9.4–99.2	<0.001
Pus cells and RBC+	4 (3%)	0	—	—
Nil	100 (75.2%)	8 (8.0)	3.5–15.2	1
Stool culture				
Salmonella species	13 (10.3%)	0	—	—
Shigella species	10 (6.7%)	1 (10.0)	0.2–44.5	1
Nil	110	12 (10.9)	5.8–18.3	0.92

Of 133 samples, 13 (9.8%) were positive for *16S rRNA* gene, which corresponds to the genus *Campylobacter*, and 11 (84.6%) among the 13 samples were positive for the *mapA* gene confirming the species *jejuni*. The two remaining samples detected *Campylobacter* species other than *C. jejuni* and *C. coli*. The internal control, β *actin* gene, had flagged positively in all the stool samples ruling out the presence of PCR inhibitors.

Aligned sequences were searched in NCBI-BLAST (megablast) for the similarity of significant matches in the database. The sequences of *16S rRNA* showed 99.75% similarity with *C. jejuni* strain BfR-CA-12970 chromosome (Accession ID-CP054848.1) and *mapA* sequences showed 99.45% homology with *C. jejuni* strain 129108 chromosome (Accession ID-CP053854.1).

Discussion

Campylobacteriosis is common in developing countries. The overall burden was estimated to be 9.8% in the present study (95% CI: 5.5–16.4). This is in concordance with an earlier study from our institute in 2011, which showed a prevalence of 10%[14] and with the study in Assam and Nagaland in 2014–2016.[15] A report from Bhubaneswar from 2016 to 2017 found that 16.77% were positive for *Campylobacter* PCR.[16] A study conducted in Kolkata in 2008–2010 observed campylobacteriosis was 7% by culture[17] and 16.2% by real-time PCR. The prevalence of *campylobacter* was observed to be 8.5% in Meghalaya, Assam in 2010.[18] In 2012, the prevalence of campylobacter reported from Vellore[19] was 4.5%. In 2015, a low prevalence of campylobacteriosis (2.6%) from 1,145 diarrheal samples was reported from North India with *C. jejuni* as the most common species detected by culture and molecular investigation.[20] In a study from South India, the prevalence of *C. fetus* subsp. *jejuni* was found to be 14.8% isolated from the feces of healthy individuals.[21]

Asian studies showed an isolation rate of 14.9% from China,[22] 17.7% from Bangladesh, 8% from Tehran, 12% from Lahore, and 18% from Rawalpindi.[14] European studies revealed an isolation rate of 71.4% in the Netherlands, 31.9% in Portugal, 4.7% in Southern Ireland, 9.6% in North Poland.[23] Moreover, African studies stated a prevalence of 21% in Malawi, 8.9% in Madagascar, 5.8% in Kenya,[24] and 0.5% in Nigeria.[25]

The median age of presentation in our study was 36 months. Campylobacteriosis was higher (18.9%) in children less than 24 months which is in concordance with the study conducted earlier in our institute.[14] Similar findings were observed in Odisha, Israel,[15,26] and Bangladesh[27] and was also in line with the World Health Organization, indicating that the *Campylobacter* infection is common in children less than 2 years in the developing countries.[1] Another study from Denmark found a higher incidence in 1–4 years and young adults (15–24 years).[28] Kappareud et al.[29] found a higher incidence of *Campylobacter* in 0–4 years of age. This increased incidence in infants and toddlers could be because of the immature immune system, poor hand hygiene practices and contact with soil, water, and pets.[23]

Campylobacteriosis was found to be more in girls (14.3%) than boys (6.5%). This is in concordance with the study from Odisha, which showed a female preponderance (20%),[16] whereas in an earlier study conducted in our institute males had a higher prevalence.[14] This difference is not attributable to any host-specific or pathogen-specific factors related to virulence or manifestations of the disease.

The clinical presentation varies between developing countries and developed countries. In the former, it was watery diarrhea, whereas in the latter, it was bloody diarrhea. The most common presentation was found to be acute watery diarrhea (10%) in the present study. This is in concordance with the earlier study conducted in our institute.[14] The duration of symptoms was 3–5 days in 13.2% of the children. The mean duration of symptoms was 3 ± 1.1 days which is in line with the WHO data, suggesting that the symptoms typically last for 3–6 days.[1] In our study, the detection of *Campylobacter* was higher in watery stools (61.5%), followed by semi-formed stools (23%) and the least in formed stools (15%). Nearly 70% of the stools were devoid of pus cells and RBC.

The positivity rate was found to be higher (21.4%) in children who had contact with pets, similar to a Denmark study which found 52% isolation in children with pets. A study in North Poland found an isolation rate of 8.1% from pets.[23] Pintar et al.[30] found that the prevalence of *Campylobacter* in pet animals and petting zoo animals was 24.7 and 6.5%, respectively. Increased contact with pets has been associated with increased disease manifestations because the pets are known to serve as reservoirs.

Campylobacteriosis was highly detected during August–November (62%), with the highest in October (22.3%) as depicted in Figure 1. This is in concordance with a study from Denmark where the highest prevalence was from June to October.[28] In China, the isolation rate was the highest (6.29%) during June–August,[31] and in Beijing, it was in June.[22] This could be attributed to the monsoon changes as diarrheal episodes are relatively higher in the rainy season because of possible contamination of the ingested food and water with potential enteric pathogens.

None of the children with *Campylobacter* infection was started on antibiotics before sample collection. This is because fluid and electrolyte replacement is of prime importance for treating all forms of acute diarrhea. Antibiotics were not routinely recommended for all diarrhea cases in our hospital. This is in line with the existing guidelines for treating diarrheal diseases where antibiotics are indicated for dysentery and immunocompromised patients.[32]

Only 1 (7.6%) out of the 13 samples had grown *Shigella sonnei* in culture. There was no co-infection of other diarrheal disease-causing bacteria with *Campylobacter* observed in our study. Co-infection of *Campylobacter* with *Shigella* 0157 (2.2%) and

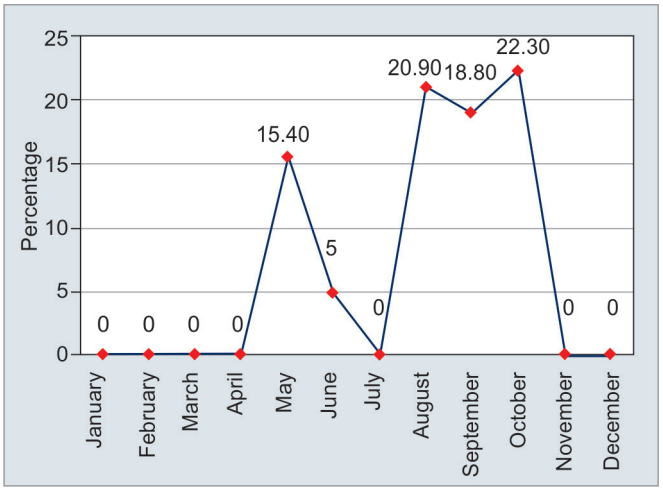

Fig. 1: Seasonal trends of campylobacteriosis from January to December 2019

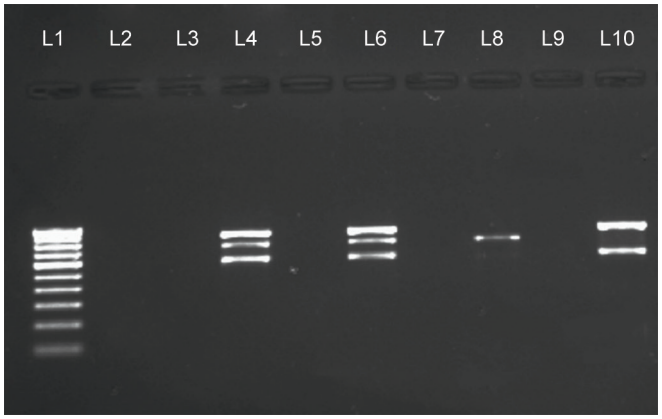

Fig. 2: Gel electrophoresis image showing bands for genus *Campylobacter* (857 bp) and species *jejuni* (589 bp) β actin gene (619 bp). Lanes: L1, 100 bp DNA ladder; L2, No template control (NTC); L3, Negative extraction control (NEC); L4, L6, Samples positive for *Campylobacter jejuni*; L8, Sample negative for *Campylobacter* species with detection of internal control; L5, L7, L9, Blank; L10, Positive control (ATCC *C. jejuni* 33291) without internal control

Rotavirus (2.2%) was observed in an earlier study.[16] Rotavirus was not investigated in the present study.

Multiplex PCR detected the genus *Campylobacter* in 13 (9.8%) samples, of which 11 (84%) were *C. jejuni*. Figure 2 depicts the Gel electrophoresis image showing bands for Genus *Campylobacter* (857 bp) and species *jejuni* (589 bp) β actin gene (619 bp). Species identification could not be made in the other two samples. The reason for this could be attributed to issues with multiplexing, where amplification of one target could have had a suppressive effect on the amplification of the other target. *C. coli* or other species were not detected in any of the samples. A similar picture was observed in an earlier study from Assam, where 80% ($n = 41$) of the positive samples had detectable *C. jejuni*.[15] Nadeem et al. in Kolkata and Chen et al. in China observed 70% ($n = 142$) and 89% ($n = 142$) of the positives were *C. jejuni*, respectively.[17,22] In a study from North India, 27 (90%) out of 30 positive samples were *C. jejuni* and 3 (10%) were *C. coli*.[20] This finding suggests that *C. jejuni* is the most predominant species in humans than the other species.

The strength of our study is that multiplex PCR was performed where genus and species level identification of *Campylobacter* was done in a single step. However, there were some limitations in the present study, i.e., (i) lesser sample size, (ii) inability to retrieve detailed history as the population comprised largely of infants and toddlers, and (iii) real-time PCR could have been a better choice for detecting pathogens in clinical samples because of its increased sensitivity and least contamination rate. We could not do that in the present study because of the limited financial resources.

Conclusion

As there are a subtle number of children with undiagnosed *Campylobacter* infection, there is a need for the implementation of molecular tests for diagnosis. The advantage of this multiplex PCR is that the detection and species differentiation can be done in a single step with a relatively less turnaround time. This aids in the early diagnosis of the infection and better patient management. This study highlights the prevalence of *Campylobacter* as a common agent of gastroenteritis and a need to employ sensitive and robust PCR-based tools for its detection.

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