

1 Cultural Isolation and PCR Based Assay for 2 Detection of *flaA* gene of *Campylobacter jejuni* from 3 Acute Diarrheic Patients in Tertiary Care Hospital at 4 Dhaka, Bangladesh.

5 ABSTRACT

Aim: Among bacterial causes of acute diarrhea *Campylobacter* species is frequently responsible. *Campylobacter* spp. are the leading agents of bacterial gastroenteritis in developed as well as developing countries. This study was conducted to determine the frequency and antimicrobial susceptibility pattern of *Campylobacter* spp. isolated from stool sample of acute diarrheic patients.

Study design: This was a cross-sectional study.

Place and Duration of Study: Two hundred stool samples of children of age between 6 months to 5 years with diarrhea/dysentery were taken from outpatient and inpatient department of Dhaka Medical College Hospital (DMCH) and Dhaka Shishu Hospital from the 1st January, 2011 to 31st December, 2011. The study was carried out at the department of Microbiology, Dhaka Medical College (DMC).

Methodology:

The samples were inoculated on Campy-Bap media and MacConkey's agar media and were incubated at 42°C under microaerophilic conditions. The growth after 48 hours was provisionally identified by colonial morphology, oxidase test, catalase test, Gram staining and motility. The organisms were identified to species level by hippurate hydrolysis and resistance to cephalothin. All isolates of *Campylobacter jejuni* by using conventional bacteriological method were also positive using the Polymerized Chain Reaction (PCR) assay by detecting *flaA* gene specific for *Campylobacter jejuni*. Susceptibilities of 27 *Campylobacter* isolates were determined for seven antimicrobial drugs using the disk diffusion assay.

Results:

Using conventional bacteriological methods, 27 (13.5%) of 200 stool samples were positive for *Campylobacter* spp. Among isolated *Campylobacter* spp., *Campylobacter jejuni* was (88.89%); the remaining isolates was *Campylobacter coli* (11.11%). Peak age of children with *Campylobacter* spp. infection was 6-12 months. The male and female ratio was 1.5:1. Resistance to co-trimoxazole was the most common finding, followed by resistance to nalidixic acid, and ciprofloxacin.

Keywords: Diarrheal disease, Polymerized Chain Reaction, Campy-Bap media, *flaA* gene, antimicrobial susceptibility.

6 1. INTRODUCTION

7 Diarrheal disease is the second leading cause of death and causes 1.3 million deaths every year in
8 children under five years [1]. The causes of diarrhea include a wide range of viruses, bacteria and
9 parasites [2]. *Campylobacter* spp. are universally acknowledged as the most frequently isolated
10 bacterial pathogens associated with human gastroenteritis, particularly in young children. Among the
11 *Campylobacter* spp., *C. jejuni*, accounting for about 90% of infections and rest 10% infection is
12 caused by *C. coli* [3]. In Bangladesh, the frequency of *Campylobacter* spp. causing acute childhood
13 diarrhea varies from 12.3% to 12.9% [4,5]. In the Middle East countries, about 5% to 10% of acute
14 diarrhea cases are caused by *Campylobacter* spp. [6] and in Pakistan the frequency is about 18% [7].

Various pathogenic mechanisms like production of cholera like enterotoxin and cytotoxin and ability to adhere and invade epithelial cells have been proposed to play the role in cases of enteritis [8] caused by *Campylobacter* spp. The prevalence of *flaA* gene in all the isolates indicates pathogenic potential since the *flaA* gene plays an important role in *Campylobacter* pathogenesis [9]. The *flaA* gene is the pathogenicity determinants of *Campylobacter* and a popular target of genotyping especially in epidemiological studies[10].

Campylobacter gained more importance particularly during last 30 years as it has also been recognized as a major cause of human illnesses ranging from gastroenteritis to Guillain-Barre Syndrome [11, 12]. In Bangladesh, few studies have been done for detection of *C. jejuni* by PCR [13]. Antimicrobial resistance in both human and animal *Campylobacter* isolates has become increasingly common in developing countries [14]. *Campylobacter* spp. also is generally susceptible to aminoglycosides, chloramphenicol, clindamycin, nitrofurantoin and imipenem[15].

The present study was designed to estimate the frequency of *C. jejuni* infection among acute diarrheal children by culture and genotype detection by PCR and identify their antimicrobial susceptibility pattern.

2. MATERIALS AND METHODS

This cross sectional study was performed on two hundred stool samples of children of age between 6 months to 5 years with diarrhea/dysentery in outpatient and inpatient department of Dhaka Medical College Hospital (DMCH) and Dhaka Shishu Hospital from the 1st January 2011 to 31st December, 2011. Stool of acute watery diarrhea or dysentery mixed with or without blood or mucus were collected in a clean, dry, wide mouth, leak proof container. Before collection the containers were labelled with full name of patient, age, sex, serial number, date and time of collection. Then stool samples were carried in ice box to Department of Microbiology, DMC within 1 to 2 hours for further procedures. Chi-square (χ^2) test was carried out to determine the relative importance of various variables. The Ethical Review Committee (ERC) approved the protocol. Informed written consent was taken from guardian before collection of sample.

2.1 Microbiological Study

The samples were inoculated in Campy-Bap media containing plates and kept in candle jar and incubated at 42^o C (microaerophilic condition) for at least 48 hours. If no growth after 48 hours, then the culture plates were reincubated for next 24 hrs. The growth was identified after 48 hours by colony morphology, Gram staining, motility test, catalase test, oxidase test (old DC, 1996), and cephalothin sensitivity test (16, 17). The organisms were identified to species level by hippurate hydrolysis. *C. jejuni* gave positive reaction while *C. coli* gave negative reaction.

2.2 Molecular Detection

Colonies of *Campylobacter* spp. were taken from blood agar media and bacterial pellets were formed after incubation the colony at 42^oC in sterile eppendorf tube. Then 300 μ l distilled water was mixed with bacterial pellet and was vortexed until mixed well. The eppendorf tube was kept in block heater (DAIHA Scientific, Seoul, Korea) at 100^oC for 10 minutes for boiling. After boiling the tube was immediately kept on ice. Then the tube was centrifuged at 4^oC at 14000 rpm for 10 minutes. Finally supernatant containing extracted DNA was taken using micropipette. Amplification was performed in a final reaction volume of 25 μ l. The 2 μ l extracted DNA from *Campylobacter* spp. was mixed in 12.5 μ l master mix - PCR buffer, dNTP, Taq polymerase enzyme, MgCl₂ and loaded dye (Promega Corporation, USA), together with 4 μ l primer of *flaA* gene (forward and reverse). The primer sequence of *flaA* gene C16S-F 5'CTAGCTTGCTAGAACTTAGA3' and C16-R 5'GTCCACACCTTCCTCCTC3' and the size of amplified product is 155bp [18]. Volume of the reaction mixture was adjusted by adding 6.5 μ l filtered deionized water (nuclease free water). After a brief vortex, the tubes were centrifuged in a microcentrifuge machine for few seconds.

PCR assays were performed in a DNA thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg, Germany). Each PCR run comprised of preheat at 94^oc for 10 minutes followed by 36

cycles of denaturation at 94°C for 1 minute, annealing 58°C for 45 seconds, extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes. Amplified products were run on to horizontal gel electrophoresis in 1.5% agarose (Bethesda Research Laboratories) in 1X TBE buffer at room temperature at 100 volt (50 mA) for 30 to 35 minutes. Five µl amplified DNA mixed with tracking dye was then loaded into an individual well of the gel (5 mm thick). One hundred bp DNA molecular size marker was loaded into well at the middle or at two sides of the gel for comparing with the base pair of identified band. DNA bands were detected by staining with ethidium bromide (0.5 µl/ml) for 30 minutes at room temperature and then destained with distilled water for 15 minutes. Photographs were taken using digital camera with UV transilluminator (Gel Doc, Major science, Taiwan).

2.3 Antimicrobial susceptibility test

All the identified *Campylobacter* spp. was tested for antimicrobial susceptibility performed by Kirby Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute [17]. The antimicrobial disks were used according to the standard antibiotic panel for specific sample and isolated organisms. Antibiotic disks were obtained from commercial sources (Oxoid, UK). The isolated organisms were tested against ampicillin (10 µg), tetracycline (30 µg), cotrimoxazole (25 µg), nalidixic acid (30 µg), tetracycline (30 µg), cephalothin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg). Antibiotic sensitivity testing of identified *C. jejuni* and *C. coli* was performed in blood agar media at 42°C for 24 hours.

3. RESULTAND DISCUSSION

Campylobacters are one of the most frequent causes of foodborne gastroenteritis in developing as well as developed countries [15]. It is noteworthy that *C. jejuni* and *C. coli* are the two main species isolated from the stool of diarrheal children[19].

In the present study, among 200 patients, 27 (13.50%) were positive for *Campylobacter* spp. In Bangladesh, Blaser *et al.*, Albert *et al.* and Huda reported 12.3%, 17.4% and 12.9% *Campylobacter* spp. respectively from diarrheal children. These are in accordance with the present study.[4, 5, 20] Various types of *Campylobacter* spp. were isolated from diarrheic stool samples such as 13% in Thailand, [21] 17.7% in Algeria, [22] 18% in Tanzania, [23] 18% in Pakistan, [7] 12.5% in the US [24] and 18.6% in Peru [25] which support the present study. The difference in frequency of *C. jejuni* in different parts of the world is probably due to varying standards of living conditions, water supply and feeding habits as the infection occurs through water and food. In both developed and developing countries poultry is an important source of Campylobacteriosis. Strains isolated from human and chickens were matched both phenotypically and genotypically, confirming that chickens are an important source of human Campylobacteriosis [26].

In the present study, 5 (2.5%) patients had both *Campylobacter* and DEC. *Campylobacter* is isolated frequently with another enteric pathogen in patients with diarrhea in developing countries. In some cases half or more patients with *Campylobacter* enteritis also had other enteric pathogens [5].

Table 1: Age and sex distribution of *Campylobacter* spp. among the study population.

Age (Months)	<i>Campylobacter</i> sp. Positive cases N (%)		Total N (%) (n = 200)	
	Male (n =27)	Female	Male	Female
6-12	8 (29.63%)	5 (18.52%)	50 (25%)	48 (24%)

13-24	3 (11.11%)	3 (11.11%)	33 (16.5%)	14 (7%)
25-36	2 (7.41%)	1 (3.70%)	12 (6%)	10 (5%)
37-48	2 (7.41%)	1 (3.70%)	9 (4.5%)	6 (3%)
49-60	1 (3.70%)	1 (3.70%)	10 (5%)	8 (4%)
Total	16 (59.26%)	11 (40.74%)	114 (57%)	86 (43%)

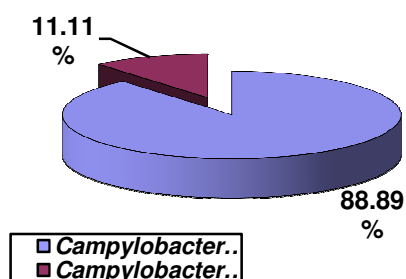


Figure 1: Distribution of *Campylobacter* spp. among the culture positive and biochemically identified positive cases (n=27).

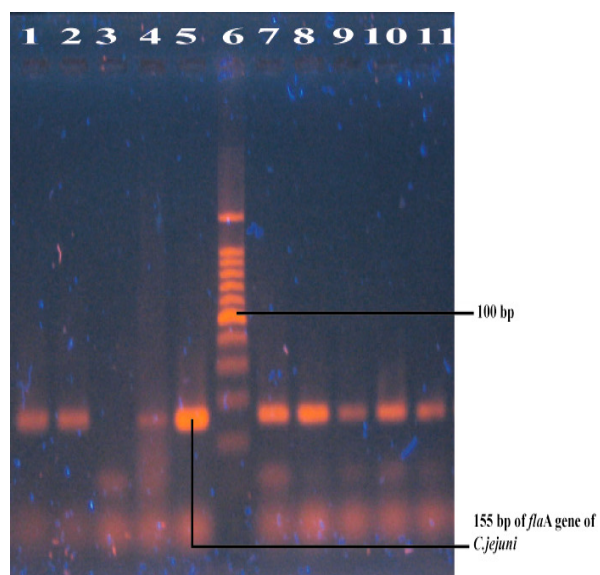


Figure 2: Photograph shows bands of amplified DNA of *flaA* gene of *C. jejuni* (lanes: 1, 2, 4, 5, 7, 8, 9, 10, 11) and 100 bp DNA ladder (lane: 6).

Table 2: Identification of *Campylobacter jejuni* by PCR using species specific primer.

<i>Campylobacter</i> spp.	PCR	
	Positive N (%)	Negative N (%)
<i>Campylobacter jejuni</i> (n = 24)	24 (100)	0 (0)
<i>Campylobacter coli</i> (n = 3)	0 (0)	3 (100)

Table 3: Antimicrobial susceptibility pattern of identified *Campylobacter jejuni* and *Campylobacter coli* (n=27).

Name of the antimicrobials	<i>Campylobacter jejuni</i> N (%)	<i>Campylobacter coli</i> N (%)
Ampicillin	17 (69.96%)	0 (0.00)
Cephalothin	0 (0.00)	0 (0.00)
Nalidixic acid	12 (44.44%)	2 (7.40%)
Tetracycline	22 (81.48%)	3 (11.11%)
Erythromycin	18 (66.66%)	1 (3.70%)
Cotrimoxazole	2 (7.41%)	0 (0.00)
Ciprofloxacin	14 (51.85%)	1 (3.70%)

In the present study, among 27 *Campylobacter* spp., 13 (48.15%) were from 6 months to 12 months of age group and 6 (22.22%) from 13 to 24 months of age group and 8 (29.63%) were above 24

months. This difference in *Campylobacter* infection in different age groups is not statistically significant ($p>0.05$). In Bangladesh, Haq and Rahman[27] reported that *Campylobacter spp.* infection was 38.8% in children of ≤ 12 months of age and 15.9% in children of >12 months of age.[29] These findings agree with the findings of the present study. Ali *et al.* and Feizabadi *et al.* reported peak incidence of *C. jejuni* infection in <24 months of age group, which is also similar to the present study.[7, 28] In this study, 8 (4%) cases were from above 24 months of age group. Similarly, Haque *et al.* reported that 5.8% *C. jejuni* were isolated from 2 years to 5 years of diarrhea patients. [29]

In the present study, among 27 *Campylobacter* positive cases, 16 (59.26%) were male, 11 (40.74%) were female and ratio of male and female was 1.5:1. In Bangladesh, Huda and Alam *et al.* reported male and female ratio of *Campylobacter spp.* were 1.4: 1 and 1.8: 1 respectively which are in accordance with the present study[20, 30]. Ali *et al.* reported the ratio between male and female was 1.7:1 in Rawalpindi which correlate with the present study [7]. Tardy *et al.* showed male and female ratio of 1:1.2 in France which was not in agreement with the present study [18]. There is a preponderance of males among *Campylobacter* infected persons but the reason of this type of sex distribution remains unknown [31, 15].

In this study, among 27 isolated *Campylobacter spp.*, 24 (88.89%) were *Campylobacter jejuni* and 3 (11.11%) were *Campylobacter coli*. This differentiation was done by hippurate hydrolysis test and cephalothin susceptibility test. Mshana *et al.* reported in Uganda among isolated *Campylobacter spp.*, 80.9% were *C. jejuni*, 4.5% were *C. coli* and 14.6% were other species of *Campylobacter* which correlate with the present study [32]. Similar study was performed by Linton *et al.* in UK and reported that 80% were *C. jejuni* and 10% were *C. coli* and rest 10% comprised of mixed infection [33]. Feizabadi *et al.* also showed that 85.8% were *C. jejuni* and 14.2% were *C. coli* from Iran [28]. The colonies with typical morphology were further identified using gram stain, catalase test, oxidase test, susceptibility to nalidixic acid and cephalothin[32].

In this study all suspected colonies of *Campylobacter spp.* were resistant to cephalothin and considered as *C. jejuni* and *C. coli*. Present study showed that among 27 *Campylobacter spp.*, 24 were positive by hippurate hydrolysis test and all 24 were also positive by polymerized chain reaction by using species specific primer of *C. jejuni*. Three samples were negative by hippurate hydrolysis test and identified as *C. coli* and they were also negative by PCR using *C. jejuni* specific primer.

C. jejuni and *C. coli* are closely related by phylogenetic and by genetic criteria [34] so identification of *Campylobacter* at species level is difficult. Although the hippurate hydrolysis test is widely used to differentiate *C. jejuni* from other species of *Campylobacter* [35]. This test is not entirely reliable because *C. jejuni* hippurate - negative strains have been isolated [36, 37] which leads to misidentification. So, for reliable and absolute identification, molecular tests due to their relative ease, low cost and potential application in large-scale screening programs, by means of automated technologies, appear to be attractive candidates [38]. Serotyping alone may be insufficient, cross reactivity may occur and expensive to characterize *C. jejuni* and *C. coli* but may be useful in conjunction with other typing schemes such as PCR [39]. In present study, primer of *flaA* gene specific for *C. jejuni* was used for identification of the desired strain.

Most cases of Campylobacteriosis do not require antimicrobial treatment since they are clinically mild and self-limiting in nature, although antimicrobial therapy is required for serious enteritis and systemic infections. Macrolides and fluoroquinolones are considered as drugs of choice for the treatment of enteric infections and intravenous aminoglycoside for those cases present with systemic manifestations [40, 41]. There are some previous reports that have shown a marked increase in resistance to quinolones in both developed and developing countries [42, 43]. In the present study, the sensitivity of *C. jejuni* was 91.67% to tetracycline, 75% to erythromycin, 70.83% to ampicillin, 58.33% to ciprofloxacin 50% to nalidixic acid. Twenty four (100%) *Campylobacter jejuni* were resistant to cephalothin and 91.67% to co-trimoxazole.

Similarly in a study in Thailand it was reported by Boonma *et al.* that the sensitivity pattern of *C. jejuni* to erythromycin was 88%, to ampicillin 56%[44]. Engber *et al.* also showed that, over time erythromycin resistance have remained low and stable in Japan, Canada and Finland [45]. Erythromycin is considered the optimal drug for treatment of *Campylobacter* infections. In this study, sensitivity of *C. jejuni* to tetracycline was 91.67%. Similarly, Feizabadi *et al.* reported that 75.7% *C. jejuni* was sensitive to tetracycline [28]. In Canada Gaudreau and Michaud reported that ciprofloxacin

and erythromycin resistant *C. jejuni* were susceptible to tetracycline which supports the high sensitivity pattern to tetracycline in the present study [40]. On the contrary, Boonmar *et al.* showed 38% of isolate were susceptible to tetracycline. In Thailand Boonmar *et al.* also reported that *C. jejuni* was resistant to cephalothin (100%) which is in agreement with the present study [44]. In the present study, 58.33% *C. jejuni* was sensitive to ciprofloxacin. Similarly, in Iran, Feizabadi *et al.* reported that 41.4% *C. jejuni* was sensitive to ciprofloxacin [28]. In Thailand and Nigeria the resistance to ciprofloxacin increased from 0% to 84% (1991-1996) as a result of inappropriate use of quinolones. Resistance to quinolones is contributed to the indiscriminate use of drug with the initiation of diarrhea. [32]

In the present study, all *C. coli* were sensitive to tetracycline, 66.66% to nalidixic acid, 33.33% to both ciprofloxacin and erythromycin. All (100%) *C. coli* were resistant to ampicillin, cephalothin and co-trimoxazole. Increasing antimicrobial resistance among *Campylobacter* was noted which may be due to frequent use of these antimicrobials, self-medication, inappropriate and incomplete dose schedule in developing countries. [44]

As *Campylobacter jejuni* has now become one of the frequent causes of diarrhea and PCR should be used routinely in diagnostic laboratory to detect *Campylobacter spp.*

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