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Low Prevalence of Campylobacteriosis in the Northern Region of India

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Abstract

***Campylobacter* is one of the most common bacterial enteropathogens of food borne origin in industrialized countries with *C. jejuni* being the most common species followed by *C. coli*. The prevalence of *Campylobacters* in and around Chandigarh, India was studied by phenotypic and genotypic methods. Fecal samples from 1145 diarrheal patients and 102 healthy subjects from hospital and community were cultured on *Campylobacter* media and identified by Gram stain, biochemical investigations and serotyping. Molecular identification of *Campylobacter* isolates was done using specific primers to unique regions of 16S rRNA, *Campylobacter jejuni* (*hip*O), *Campylobacter coli* (*asp*K), *Campylobacter lari* (*gly*A) and *Campylobacter upsaliensis* (*lpx*A) genes. Identification of specific genes to look for resistance to nalidixic acid, ciprofloxacin, tetracyclin and streptomycin was also done. *Campylobacters* were isolated from 2.6% of patients with diarrhea. Campylobacteriosis was more prevalent in children ≤5 years old and during summer season. The most frequent serotypes were S:27, B:2, Z_5:52 and V:32. All the *Campylobacters* isolated by culture were confirmed genotypically by identification of 16S rRNA, *hip*O and *asp*K genes. Of the 30 isolates, 27 were *C. jejuni* and 3 were *C. coli*. No *C. lari* or *C. upsaliensis* were detected. Antibiotic resistance was 40% for nalidixic acid, 23.3% for ciprofloxacin, 50% for tetracyclin and 20% for streptomycin. *Campylobacter* prevalence is low in the region with *C. jejuni* being the most common species. A high degree of resistance was found for nalidixic acid and tetracyclin but moderate for ciprofloxacin and streptomycin.**

Keywords

Antibiotic Resistance, *Campylobacter* Diarrheas, Molecular Investigation, Phenotypes, Serotyping

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1. Introduction

Campylobacter is an important cause of acute bacterial diarrhea worldwide. The most common species causing campylobacteriosis in human beings is *C. jejuni* (95%) followed by *C. coli* (5%). Other species like *C. fetus*, *C. upsaliensis* and *C. lari* rarely cause human infections. Most of the isolates causing human gastroenteritis are thermo-tolerant variety and can grow even at incubation temperatures of 42˚C. *Campylobacter* is generally transmitted to humans by domestic animals, unpasteurized milk and contaminated water. The infectious dose is very small and as little as 500 cells are sufficient to produce gastroenteritis. Campylobacteriosis is characterized by fever, bloody diarrhea, headache and severe abdominal pain. When diarrhea is minimal but abdominal pain is present, it can lead to a mistaken diagnosis of acute abdomen and subsequently unnecessary laparotomy. Fever persisting up to 1 week is reported by >90% of the patients. However, 50% of persons infected with *Campylobacters* are asymptomatic [1].

Most *Campylobacter* infections are sporadic in nature but some outbreaks have also been reported at times [2]. There is a seasonal peak for *Campylobacter* infections. In temperate climates, the incidence of infection is roughly twice as high in summer as in winter, and the incidence is high in rural than in urban communities [2]. *Campylobacter* is isolated from infants and young adults more frequently than from persons in other age groups and more frequently from males than from females [3]. Most people who develop campylobacteriosis recover completely within 2 - 5 days, but in some cases recovery can take up to 10 days [4]. Common long term complications of campylobacteriosis are Guillain Barré syndrome, rheumatoid arthritis, bacteremia and inflammatory bowel disease. Local complications of *Campylobacter* infections occur as a result of direct spread from gastrointestinal tract and can include cholecystitis, pancreatitis, peritonitis [4], massive gastrointestinal hemorrhage [5], thyroiditis [6] and prosthetic joint infection [7]. Timely treatment can reduce the duration and severity of the infection and reduce the chances of serious complications.

Campylobacteriosis is estimated to affect over 2.4 million persons annually, or 0.8% of the population [3] making it a highly important organism from a socio-economic perspective but many of the cases go undiagnosed. It is envisaged that campylobacteriosis has been in existence for many years but has only recently been recognized as a common infection due to improved laboratory methods. As *Campylobacter* is a major health burden for both developed and developing countries, *Campylobacter* infections remain a high research priority in order to improve the strategies for prevention and management of the disease. The present work was carried out to study the prevalence of campylobacteriosis in and around Chandigarh, which is in the northern region of India.

2. Methods

The study was approved by the Institute Ethical Committee and was conducted between May 2009 and January 2013.

2.1. Study Population

Both adult and paediatric patients with complaints of diarrhea were investigated. They included outpatients and inpatients from wards of hospitals in Chandigarh and those situated in slum areas of various colonies. Patients from community areas like Mauli Jagran and Indira Colony in the region around Chandigarh, India were also investigated by door-to-door visits. A total of 1145 patients were enrolled and informed, consent was taken from all the patients or their wards involved in the study. Detailed enquiry regarding their clinical symptoms was recorded in a pre-printed proforma. Healthy subjects (n = 102) from among the attendants of patients, who had no diarrhea of any kind, were also included in the study.

2.2. Collection and Transportation of the Fecal Samples to the Laboratory

Sterile stool containers (Stericol, Himedia, India) with 3 ml of Campy-thioglycollate media were given to patients and healthy subjects. They were instructed to collect approximately 4 - 5 grams of the stool sample especially from mucoid or bloody area and transport the same immediately to the Microbiology Division of the department for processing. Fecal samples from community were collected by door-to-door visit by the research staff and transported in an ice-bucket to the laboratory. The samples were collected from the community either on the same day of visit or the next day depending on the frequency of diarrhea in the cases.

2.3. Culture for *Campylobacters*

The stool samples were inoculated directly either on selective *Campylobacter* agar base media containing vancomycin, polymyxin B, trimethoprim and 5% - 7% defibrinated sheep blood or charcoal cefoperazone deoxycholate agar by quadrant streaking. For culture using filtration technique, stool samples were homogenized in physiological saline as 1:1 ratio and the homogenate was passed through membrane filters (0.45 µm and 0.65 µm) placed on media plates. The filters were removed after an hour and discarded. The plates were incubated under microaerophilic conditions at 37˚C and 42˚C for 72 h.

2.4. Phenotypic Identification of *Campylobacters*

Predominant or pure growth of grey to ceramic colonies on culture media were picked up and identified for *Campylobacters* as oxidase positive, catalase producing gram negative spiral rods. *Campylobacter* diarrhea was suspected when the isolate gave positive reaction with oxidase and/or hippurate in addition to any other positive identification tests. Serotyping of *Campylobacter* isolates was done based on the Penner scheme by the hemagglutination technique for heat stable antigens of *C. jejuni* using 25 *C. jejuni* specific antisera (Denka-Seiken, Japan) against the following heat-stable serotypes: (1, 44), 2, 3, (4, 13, 16, 43, 50), 5, (6, 7), 8, 10, 11, 12, 15, 18, 19, 21, (23, 36, 53), 27, 31, 32, 37, 38, 41, 45, 52, 55, and 57 with grouped antisera given in parentheses.

2.5. Molecular Identification of *Campylobacter* Isolates and Antibiotic Resistance

Molecular identification of *Campylobacter* isolates was done using specific primers to the unique regions of *Campylobacter* genus and different species as well as detection of resistance to antibiotics after isolation of DNA as follows.

2.5.1. DNA Isolation

For DNA isolation, a heavy growth of *Campylobacter* was suspended in Tris-EDTA buffer and boiled at 100˚C for 10 mins. The boiled suspension was immediately transferred to an ice bath and incubated for 1 h. Next, the suspension was centrifuged at 10,000 rpm for 2 mins and the supernatant was separated and checked for DNA by 0.8% agarose gel electrophoresis. DNA was also extracted from whole stool samples by QIAmp DNA mini kit (Qiagen, Netherlands) to tap any *Campylobacter* that could have been inadvertently lost.

2.5.2. Polymerase Chain Reaction for *Campylobacter* Identification

Polymerase chain reaction (PCR) assay was done using specific primers to the unique regions of *Campylobacter* genus and to the unique regions of different *Campylobacter* species. For genus identification of *Campylobacter*, 16S rRNA was amplified using C412F and C1228R primers. Species-specific identification of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* was done by amplifying the hippuricase (*hip*O) gene, the aspartokinase (*asp*K) gene, the serine hydroxymethyltransferase (*gly*A) gene and lipopolysaccharide (*lpx*A) gene respectively (**Table 1**).

2.5.3. Polymerase Chain Reaction for Detection of Antibiotic Resistance

Nalidixic acid resistance was identified by amplifying specific *gyr*A gene by PCR using primers *gyr*A F and *gyr*A R. Ciprofloxacin (*gyr*A) resistance in *Campylobacter* isolates was investigated by Mismatch Amplification Mutation Assay (MAMA) using a conserved forward primer, Campy MAMA *gyr*A1 and a mutation detection reverse primer, Campy MAMA *gyr*A5. An annealing temperature of 50˚C was used to give 265 bp products which indicated the presence of the Thr-6 to Ile (ACA → ATA) mutation in the *Campylobacter gyr*A gene. Tetracyclin resistance was detected by using specific primers to amplify *tet*O gene. Streptomycin resistance was detected by amplifying the *str*A gene. All the PCR products were analyzed by electrophoresis on 1.8% agarose gel containing 0.1 µg/ml ethidium bromide.

2.6. Statistical Analysis

Data was analyzed by SPSS version 16.0.

Table 1. Primers used for molecular identification of *Campylobacter* and antibiotic resistance genes.

S. No.	Target Gene	Primer Sequence
1	16S rRNA	F- 5' AATCTAATGGCTTAACCATTA 3' R- 5' GTAACTAGTTTAGTATTCCGC 3'
2	Hippuricase (*hip*O) (*C. jejuni*)	F- 5'-GGAGAGGGTTTGGGTGGT-3' R- 5'-AGCTAGCCTCGCATAATAACTTG-3'
3	Aspartokinase (*asp*K) (*C. coli*)	F- 5' GGTATGATTTCTACAAAGCGAG-3' R- 5' ATAAAAGACTATCGTCGCGTG-3
4	*gly*A (*C.lari*)	F- 5' TAGAGAGATAGCAAAAGAGA 3' R- 5' TACACATAATAATCCCACCC 3'
5	*lpx*A (*C. upsaliensis*)	F- 5' CGATGATGTCAAATTGAAGC 3' R- 5' TTCTAGCCCCTTGCTTGATG 3'
6	Tetracycline (*tet*O)	F- 5' AACTTAGGCATTCTGGCTCAC 3' R- 5' TCCCACTGTTCCATATCGTCA 3'
7	Nalidixic acid (*gyr*A)	F- 5' GCT CTT GTT TTA GCT TGATGCA 3' R- 5' TTG TCG CCA TC CTA CAGCTA 3'
8	Ciprofloxacin (MAMA *gyr*A)	F- 5' TTT TTA GCA AAG ATT CTG AT 3' R- 5'CAA AGC ATC ATA AAC TGC AA 3'
9	Streptomycin (*str*A)	F- 5' CCAATCGCAGATAGAAGGCAAG 3' R- 5' ATCAACTGGCAGGAGGAACAGG 3'

3. Results

There were 734 males and 411 females with age range of 17 days old to 87 years old in the diarrheal patient group. The clinical symptoms present in them at the time of examination were diarrhea in 959 (83.75%), fever in 469 (40.96%) and abdominal pain in 406 (35.45%). Of the 102 age-matched healthy control subjects, there were 69 males and 33 females with age range of 2 months old to 77 years old.

3.1. Phenotypic Methods

Of the 1145 stool samples from diarrheic cases, *Campylobacter* was isolated from 30 (2.6%) of them, but none from the control samples. *Campylobacter* positivity was found in 19 male patients and 11 female patients. The preponderance of infection was more in summer season (76.7%) than in winter season (23.3%). Fifty percent prevalence was seen in children ≤5 years old and the remaining 50% were scattered in the age group 10 - 66 years old with predominant positivity between 29 - 40 years old. The clinical symptoms fever and abdominal pain were found to be statistically significant ($p = 0.000$) in the *Campylobacter* positive patients compared to the negative ones. With the serotyping of *Campylobacter* isolates, 93.3% reacted to multiple antisera and thus belonged to multiple serotypes (**Table 2**). Two of the *Campylobacter* isolates were non-typeable. The most frequent serotypes in descending order were Groups S:27, B:2, Z_5:52 and V:32.

3.2. Molecular Methods

All the *Campylobacters* isolated by culture (100%) were also found to be positive for genus *Campylobacter* by PCR based on detection of species-specific 16S rRNA (**Figure 1**). Of the 30 *Campylobacter* isolates, 27 were *C. jejuni* and 3 were *C. coli* based on detection of *hip*O gene (**Figure 2**) and *asp*K gene (**Figure 3**) respectively. No co-infection of *C. jejuni* and *C. coli* were seen. However, molecular investigation of DNA from isolates and from whole fecal samples were found to be negative for *gly*A gene (*C. lari*) and *lpxA* gene (*C. upsaliensis*) depicting the absence of these two species. Antibiogram resistance study showed 40% (12/30) of the isolates resistant to nalidixic acid (**Figure 4(a)**), 23.3% (7/30) to ciprofloxacin (**Figure 4(b)**), 50% (15/30) to tetracyclin (**Figure 5(a)**) and 20% (6/30) to streptomycin (**Figure 5(b)**).

Table 2. Number of *C. jejuni* isolated showing multiple serotypes in alphabetical order.

Serotypes	No. of *C. jejuni* isolates showing multiple serotypes
A:1, 44	4
B:2	**14**
C:3	3
D:4, 13, 16, 43, 50	3
E:5	9
F:6, 7	7
G:8	3
I:10	4
J:11	4
K:12	4
L:15	6
N:18	7
O:19	6
P:21	1
R:23, 36, 53	7
S:27	**13**
U:31	3
V:32	**10**
Y:37	3
Z:38	7
Z_2:41	5
Z_4:45	1
Z_5:52	**11**
Z_6:55	2
Z_7:57	8

Footnote: The most frequent serotypes are marked in bold.

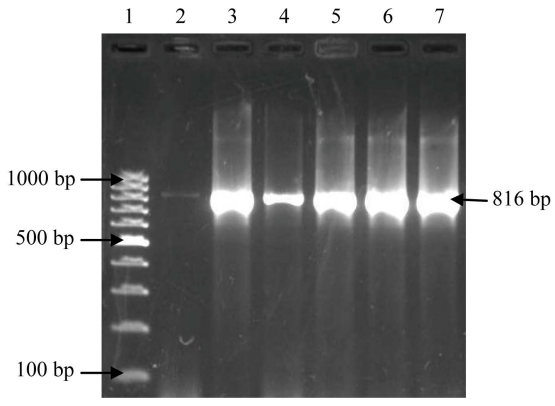

Figure 1. PCR detection of 16S rRNA gene (816 bp) of *C. jejuni*. Lane 1—100 bp Marker; Lane 2—Positive control; Lane 3 to 12—*Campylobacter* isolates.

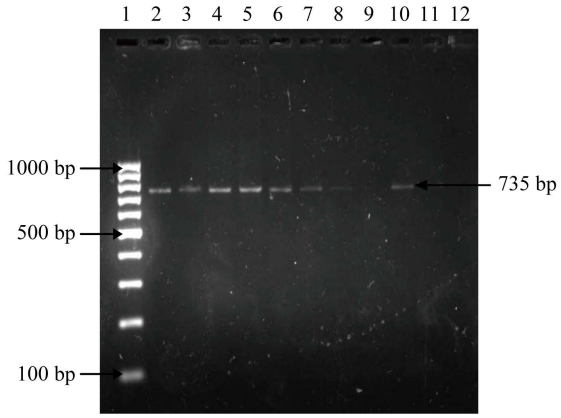

Figure 2. PCR detection of *hip*O gene (735 bp) of *C. jejuni*. Lane 1—100 bp Marker; Lane 2—Positive control; Lanes 3 to 5—*Campylobacter* isolates; Lane 12—Negative control.

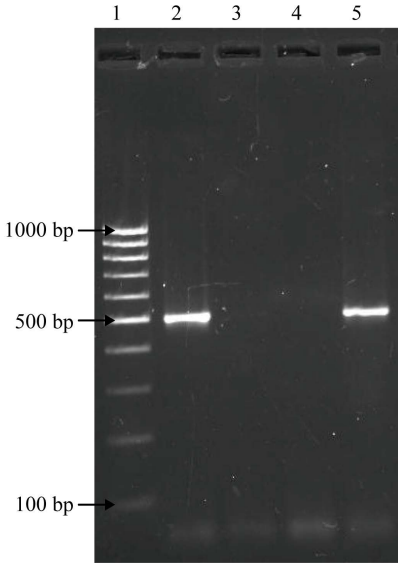

Figure 3. *asp*K gene of *C. coli* (500 bp). Lane 1—100 bp Marker; Lane 2—Positive control; Lanes 3 to 5—*Campylobacter* isolates.

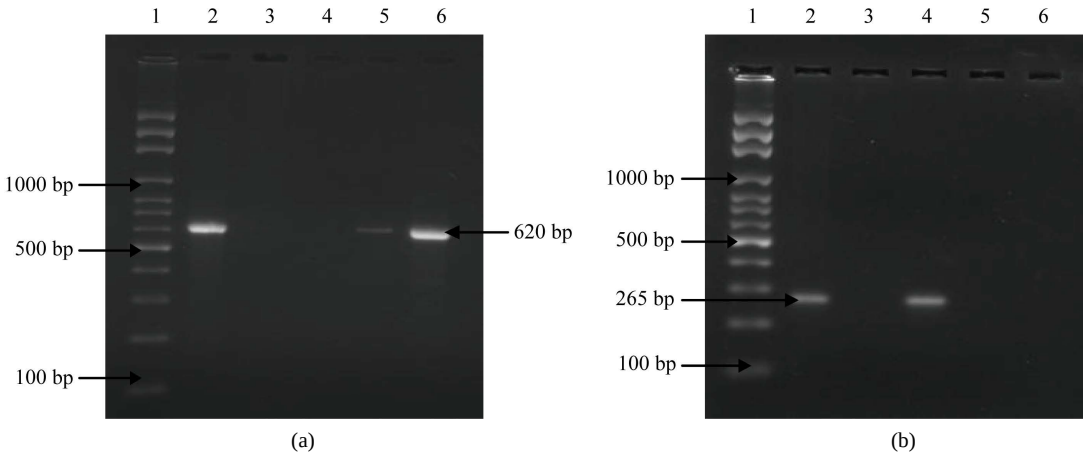

Figure 4. PCR detection of *gyr*A genes for resistance of nalidixic acid (620 bp) and ciprofloxacin (265 bp) respectively. Lane 1—Marker; Lane 2—Positive control; Lanes 3 to 6—*Campylobacter* isolates.

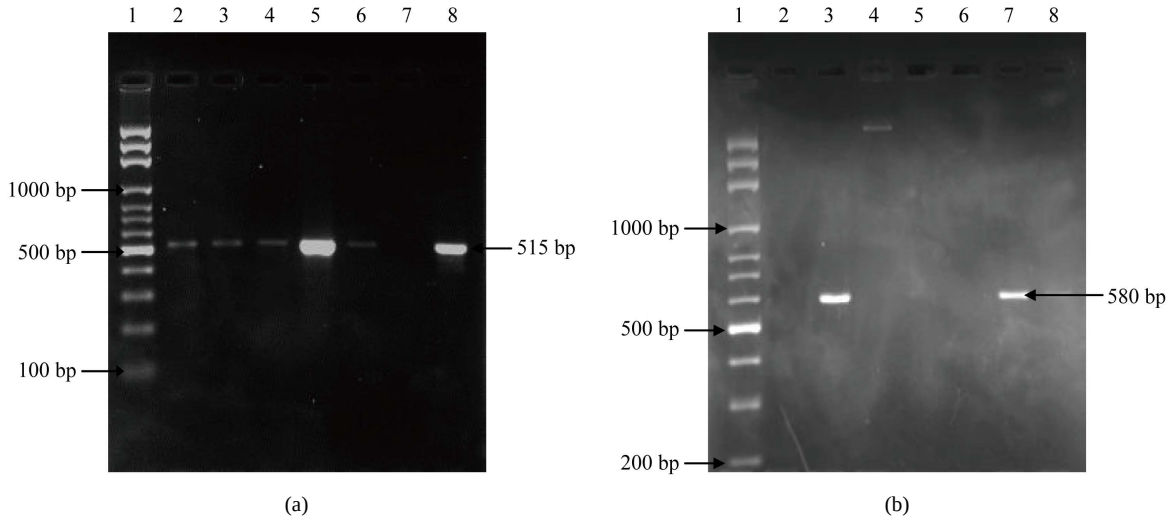

Figure 5. PCR detection of *tet*O and *str*A genes for resistance of tetracycline (515 bp) and streptomycin (580 bp) respectively. (a) Lane 1—100 bp Marker; Lane 2—Positive control; Lanes 3 to 8—*Campylobacter* isolates; (b) Lane 1—Positive control; Lane 2 Negative control; Lane 3 Positive control; Lanes 4 to 8—*Campylobacter* isolates.

4. Discussion

Although 14 *Campylobacter* species have been identified, in the United States >99% of reported infections with *Campylobacter* are with *C. jejuni* [8]. The frequency of isolation of *C. jejuni* in various parts of the world varies due to the varying standards of living conditions, water supply and feeding habits. The isolation rate of *Campylobacter* reported from different regions of the world are 9.5% (France) [9], 6.7% (United Kingdom) [10], 9% (Central Africa) [11], 44% (South Africa) [12], 17.7% Bangladesh [13], 8% (Tehran) [14], 12% (Lahore) [15], and 18% (Rawalpindi) [16]. Reports from India include 16% from rural population in Mumbai, 8.6% from Ranchi [17] and 14.8% from Vellore [18]. Salim *et al.* [3] isolated *Campylobacter* in 10% of the 50 fecal samples investigated from children suffering from diarrhea, dysentery or acute gastroenteritis. In the present study, *Campylobacters* were isolated in only 2.6% samples from hospital and community and none from 102 healthy subjects from the same community. This shows a very low level of prevalence of *Campylobacter* in the region and can be attributed to availability of good drinking water sources and better hygiene practices. However, interestingly, the patients with *Campylobacter* positivity also had significant clinical symptoms of fever and abdominal pain compared to those who were *Campylobacter* negative.

Campylobacter more frequently affects children. In tropical developing countries, *Campylobacter* infections are hyperendemic among young children especially those aged >2 years old. Salim *et al.* [3] reported the minimum age for *Campylobacter* isolation is 2 days old and the maximum is 4 years old respectively. The minimum and maximum age of isolation reported from Rawalpindi was 3 months old and 48 months old. Studies from Ranchi [17] and Vellore [18] showed maximum isolation from >6 years old and from pre-school children respectively. In the present study, the minimum age of isolation of *Campylobacters* was 5 months old and the maximum was 66 years old with 50% prevalence in children ≤5 years old, especially those living in rural regions of Chandigarh. In the remaining 50% predominant positivity was seen in patients aged 29 - 40 years old.

In industrialized nations, there is a preponderance of males among *Campylobacter* infected persons [19], the reasons for which are unknown. In the present study also, *Campylobacter* infections were more common in males than in females. Campylobacteriosis occurs much more frequently in summer than in winter [20]. In the present study also a 3-fold higher frequency of *Campylobacter* diarrhea was seen during the summer seasons.

Outbreaks of *Campylobacters* have been reported from several countries. Yu *et al.* [21] reported the first recognized major *C. jejuni* outbreak was associated with contaminated chicken among middle school students in Korea where an attack rate of 11.6% occurred with 40.3% stool samples positives for *C. jejuni*. Karagiannis *et al.* [5] reported a *C. jejuni* outbreak in Crete in which most cases originated from rural areas and had a strong epidemiological evidence that tap water was the vehicle of the outbreak. Longenberger *et al.* [22] reported a multistate outbreak of *Campylobacter* infections was associated with unpasteurized milk in US which resulted in

148 illnesses. Taylor *et al.* [23] reviewed reports of campylobacteriosis in US from 1997 to 2008 where 262 outbreaks with 9135 illnesses, 159 hospitalizations and 3 deaths were recorded. Though sporadic infection of *Campylobacter* has been observed from time to time in India, no outbreak as such has been reported.

In our study, *C. jejuni* was present in 90% and *C. coli* in 10% of the patient samples with no co-infection seen amongst them. Both *C. lari* and *C. upsaliensis* were not found among the isolates. The prevalence of a particular serotype differs across countries based on their geographic locations. In Japan, the most predominant serotypes are B, D, and L, while those in Denmark are serotypes B, A, and D [24] [25]. Ishihara *et al.* [26] identified 18 serotypes with major serotypes being B, D and R among the *C. jejuni* isolates from humans. In another study from Thailand [27], *C. jejuni* isolates from humans were classified into 10 Penner serotypes viz. B, C, R, E, G, A, K, D, I and L. The most predominant serotype was B (9 strains), followed by serotype C (8 strains), R (5 strains), E (4 strains), G (3 strains), A (2 strains), K (2 strains), D (1 strain), I (1 strain), and L (1 strain). In the present study, the most frequent serotypes were S:27, B:2, Z_5:52 and V:32 in descending order. Serotype B appears to be present everywhere as observed from reported studies.

Most *Campylobacter* diarrhea is generally self-limiting, but antimicrobial treatment may be required for patients with severe, prolonged or systemic infections or to control infection in high-risk groups. The antimicrobial agents of choice are macrolides and fluoroquinolones. However, antibiotic management of *Campylobacter* has become more complex as *C. jejuni* has undergone a rapid increased resistance to fluoroquinolone [28] due to their use in food animals [4]. The clinical impact of antibiotic resistance in *Campylobacter* infections are reported in epidemiological studies. Patients with quinolone-resistant *C. jejuni*, treated with fluoroquinolones in Minnesota in 1997 had a median duration of diarrhea of 10 days compared to 7 days in those with sensitive strains [29]. Gupta *et al.* [30] reported 31% patients with ciprofloxacin-resistant *Campylobacter* were hospitalized for gastroenteritis compared to 3% patients with sensitive strains. In a case control study carried out in 1998-1999 in 290 patients who did not take anti-diarrheal medication for campylobacteriosis, those with ciprofloxacin-resistant strains had a mean duration of diarrhea of 9 days, compared to 7 days in those with sensitive isolates [31]. Of 85 persons who took fluoroquinolone antimicrobials only, diarrhea lasted a mean of 8 days in those with resistant strains but 6 days in those with sensitive isolates. In 63 patients who took no antimicrobials, those with ciprofloxacin-resistant isolates had diarrhea for a mean of 12 days versus 6 days for sensitive strains. This suggests that those infected with resistant *Campylobacters* have a prolonged and more severe infection than those with sensitive strains.

Goodchild *et al.* [32] in a study of 200 *Campylobacters* isolates from New Zealand, reported resistance to ciprofloxacin in 4% of the *C. jejuni* isolates against 20% resistance in "non-jejuni" isolates, with only one of the isolates being resistant to tetracyclin. Korolik *et al.* [33] studied antibiotic profile on 81 strains of *C. jejuni* and 8 of *C. coli* collected from 1989-1990 and 79 of *C. jejuni* and 6 of *C. coli* collected from 1994-1995. During the second period, doxycycline resistance fell from 10% to 2.5% in *C. jejuni* and from 25% to 16% in *C. coli*. This could be because though tetracyclins help in the treatment of clinical campylobacteriosis, in practice they are rarely used as the *tet*O gene has shown to confer extremely high-levels of tetracyclin resistance [34]. Huysman and Turnidge [35] in a study of 100 strains (79 *C. jejuni*, 19 *C. coli* and 2 *C. lari*) found all strains were sensitive to erythromycin, gentamicin, nalidixic acid and ciprofloxacin and 9 strains resistant to tetracyclin. A study in the Hunter region of New South Wales of Australia investigating resistance of 180 isolates of *C. jejuni* found 11% of isolates resistant to tetracyclin, 3.4% to nalidixic acid, 2.9% to ciprofloxacin, 64% to ampicillin, 3.4% to erythromycin and 48% to roxithromycin with none resistant to gentamicin [36].

In the present study, antibiotic resistance was 40.0% for nalidixic acid, 23.3% for ciprofloxacin, 50% for tetracyclin and 20% for streptomycin, indicating the development of multiple resistances. Molecular techniques such as MAMA-PCR [37] successfully detect ciprofloxacin-resistant *C. jejuni* and *C. coli* isolates by identifying a mutation Thr-86-Ile in the *gyr*A gene, commonly associated with ciprofloxacin resistance in *Campylobacters*. However, the major disadvantage of this technique is that it may not be able to detect resistance if a new, unexpected resistance mechanism occurs [38].

5. Conclusion

Antibiotic resistance rates are rising worldwide with the development of multiple resistances to several classes of antibiotics. Therefore, the decision to use antibiotics should be made judiciously. Routine use of antibiotic pro- phylaxis to prevent *Campylobacter* infections is not recommended. From the study, it is concluded that *C.*

jejuni is most commonly isolated among the *Campylobacter* diarrheas with high degree of resistance for nalidixic acid and tetracyclin and moderate degree of resistance for ciprofloxacin and streptomycin.

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Transparency Declaration

There are no conflicts of interest to report.

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List of Abbreviations

1) *asp*K—aspartokinase
2) *C. coli*—*Campylobacter coli*
3) *C. fetus*—*Campylobacter fetus*
4) *C. jejuni*—*Campylobacter jejuni*
5) *C. lari*—*Campylobacter lari*
6) *C. upsaliensis*—*Campylobacter upsaliensis*
7) DNA—Deoxyribonucleic acid
8) EDTA—Ethylenediamine tetraacetic acid
9) *gly*A—Glycine
10) *gyr*A—Gyrase A
11) *hipO*—hippuricase
12) *lpx*A—lipopolysaccharide
13) MAMA—Mismatch Amplification Mutation Assay
14) PCR—Polymerase chain reaction
15) %—Percentage
16) rRNA—Ribosomal ribonucleic acid