

Isolation of *Campylobacters* from Intestinal Tract of Poultry in Northern Region of India

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Abstract

Campylobacter is one of the most common food-borne bacterial enteropathogens. We planned to investigate the prevalence and antibiotic resistogram of *Campylobacter* in poultry in and around Chandigarh. Poultry samples (n = 127) were obtained from slaughter houses/retail outlets and cultured microaerophilically on *Campylobacter* media. The isolates were identified phenotypically and by molecular investigation. Identification of specific genes to look for resistance to nalidixic acid, ciprofloxacin, tetracyclin and streptomycin was also done. *Campylobacter* was isolated from 57/127 (44.9%) of the samples. The most frequent serotypes identified were B: 2, S: 27, Z₅: 52 and Z₇: 57. All culture isolates (100%) were reconfirmed as *Campylobacter* by 16S rRNA polymerase chain reaction. Molecular identification of isolates revealed the presence of *C. jejuni* in 45 (79.0%), *C. coli* in 1 (1.8%) and co-infection of *C. coli* and *C. jejuni* in 11 (19.3%). No *C. lari* and *C. upsaliensis* were detected. Antibigram typing showed nalidixic acid resistance in 36.8%, ciprofloxacin resistance in 35.0% and 31.5% resistance for both streptomycin and tetracyclin. A high level of *Campylobacter* prevalence was found among the poultry with *C. jejuni* being the most commonly isolated species. Resistance to major antibiotics among *Campylobacter* isolates from poultry was also very high. The study of prevalence of *Campylobacter* in poultry and its resistance to major antibiotics will help to plan risk burden strategies throughout the food chain.

Keywords

Antibiotic Resistance, *Campylobacter*, Molecular Investigation, Phenotypic Identification, Poultry, Serotyping

1. Introduction

Campylobacter has long been known as a part of the normal flora in the intestine of most animals including poultry due to their high body temperature which provides an optimum growth milieu for this thermotolerant

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genus [1]. The organism has been found to be responsible for causing abortion in cattle and sheep and diarrhea in cattle and pigs [2]. Human beings can acquire *Campylobacter* through consumption of raw or undercooked meat and poultry [3], contaminated water and vegetables [4], unpasteurized milk [5], or by contact with fecal matter from infected domestic pets or people [6]. As poultry meat is a good source of animal protein, it is easily appealing to consumers and the consumption of which leads to infections. Poultry is therefore considered to be the main reservoir for campylobacteriosis in humans. Poultry meat is of prime importance in food associated disease in developed countries [7]. The most common *Campylobacters* causing human diseases are *C. jejuni* and *C. coli* with rarely other species like *C. fetus*, *C. upsaliensis* and *C. lari* [8] [9]. *C. jejuni* has been reported to be the most commonly isolated species in chickens [1]. Thus, *Campylobacter* is one of the most common agents of bacterial gastroenteritis and a major health burden for both developed and developing countries [10].

Campylobacter is an important bacterial cause of food-borne disease outbreaks in the United States [11]. Studies on antibiotic resistant pattern of *Campylobacters* from developing countries are sparse, due to lack of funding and facilities for culture. There is a paucity of information from India regarding *Campylobacter* and their antibiotic resistance pattern in poultry, probably due to the absence of surveillance in our region. *Campylobacter* infections remain a high research priority for improvement of strategies related to prevention and management of the disease [9]. As poultry is the main reservoir of *Campylobacters* for human transmission, there is clearly a need for local surveillance and control measures based on quantitative data of *Campylobacter* colonization in poultry. The present work was carried out to study the prevalent antibiotic profile of *Campylobacters* isolated from poultry in Chandigarh, a northern region of India.

2. Materials and Methods

The study was approved by the Institute Ethics Committee of Post Graduate Institute of Medical Institute and Research, Chandigarh, India and was conducted from May 2009 to January 2013.

2.1. Poultry Sample Collection

One hundred and twenty seven poultry samples from slaughter houses/retail outlets in and around Chandigarh formed the basis of investigation. Lower intestinal portions of poultry were collected in campy-thioglycollate media (HiMedia, India), transported in an ice bucket to the Microbiology Division of the Department of Gastroenterology and were immediately processed for the isolation of *Campylobacters*. This was the same time period (May 2009 and January 2013) and geographical area (Maui Jagran and Indira colony) from where the community fecal samples investigating for *Campylobacters* were collected in an earlier study [9].

2.2. Culture for *Campylobacters*

Poultry intestine pieces were homogenized in physiological saline and vortex mixed for 1 min under sterile conditions. The suspension was inoculated directly either on selective *Campylobacter* agar base media (Oxoid Ltd., Cheshire, England) containing antibiotics and 5% - 7% defibrinated sheep blood or on charcoal cefoperazone deoxycholate agar (Oxoid Ltd., Cheshire, England) by quadrant streaking. The suspensions were also passed through filter membrane of pore size 0.45 μm and 0.65 μm (Millipore, USA) placed on media plates and kept as such for one hour. The filters were then removed and discarded. All the plates were incubated under microaerophilic conditions at 37°C and 42°C for 72 h.

2.3. Phenotypic Identification of *Campylobacters*

Predominant or pure growth of grey to ceramic colonies (Figure 1) was investigated by Gram stain and by biochemical tests viz. catalase, oxidase and hippurate hydrolysis. *Campylobacter* species was suspected when the isolate gave positive reaction with oxidase and/or hippurate (Figure 2) in addition to any other positive identification tests.

2.4. Serotyping of *C. jejuni*

The pathogenic *C. jejuni* was serotyped on the basis of its heat stable “O” antigen extract and a passive hemag-



Figure 1. Colonies of *Campylobacter* on *Campylobacter* agar base media.

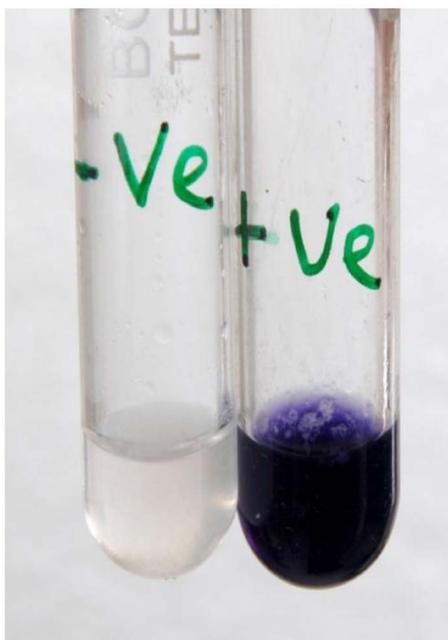


Figure 2. Formation of purple color indicative of positive hip-purate hydrolysis.

glutination assay using 25 *C. jejuni* specific antisera (Denka-Seiken, Japan). For this procedure, antigen was prepared by suspending a loopful of the organisms in physiological saline, heating at 100°C for 1 h and centrifuging at 3287 g for 5 mins. The extracted antigen was suspended in 500 µl phosphate buffered saline. This suspension was mixed with an equal volume of washed suspension of sheep erythrocytes (1% v/v). After sensitization for 1 hour at 37°C, erythrocytes were washed and resuspended to 500 µl. Twenty-five microliter each of 25 antisera were added in U-bottom microplates and an equal volume of sensitized sheep erythrocyte suspension was added to each well. The plates were observed for agglutination after incubation at 37°C for 30 mins.

2.5. Molecular Identification of *Campylobacter* Isolates

Campylobacter isolates were subjected to molecular identification for different species by polymerase chain reaction (PCR). For isolation of DNA, heavy growth of *Campylobacter* isolate was suspended in 500 µl Tris-EDTA buffer. The suspension was boiled at 100°C for 10 mins and immediately transferred to an ice bath and incubated for 1 h. Next, the suspension was centrifuged at 6710 g for 2 mins and the supernatant was separated and

checked for DNA by 0.8% agarose gel electrophoresis. The primers (**Table 1**) used to identify *Campylobacter* species and antibiotic resistance by amplifying specific genes are the same as those mentioned in an earlier publication [9]. All the PCR products were analyzed by electrophoresis on 1.8% agarose gel containing 0.1 µg/ml ethidium bromide.

(1) Identification of *Campylobacter* species: PCR assay was done using specific primers to the unique regions of *Campylobacter* genus and to the unique regions of different *Campylobacter* species. Species-specific identification of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* was done by amplifying the hippuricase (*hipO*) gene, the aspartokinase (*aspK*) gene, the serine hydroxymethyl transferase (*glyA*) gene and lipopolysaccharide (*lpxA*) gene respectively.

(2) Detection of antibiotic resistance: Nalidixic acid resistance was identified by amplifying specific *gyrA* gene using primers *gyrA* forward and *gyrA* reverse. Ciprofloxacin (*gyrA*) resistance gene in *Campylobacter* isolates was investigated by Mismatch Amplification Mutation Assay (MAMA) using a conserved forward primer, Campy MAMA *gyrA1* and a mutation detection reverse primer, Campy MAMA *gyrA5*. 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 gyrA* gene. Tetracyclin resistance was detected by using specific primers to amplify *tetO* gene. Streptomycin resistance was detected by amplifying the *strA* gene.

3. Results

3.1. Phenotypic Methods

Campylobacter was isolated from 57/127 (44.9%) of the poultry samples by culture. Eighteen (31%) of the *C. jejuni* isolates were serotyped. The serotype of the tested organism was determined as positive based on agglutination obtained over the bottom of well and as negative when button formation occurred (**Figure 3**). If the organism reacted to multiple antisera, it was determined as multiple serotypes. The most frequent serotypes of *C. difficile* from poultry isolates in descending order were B: 2, S: 27, Z₅: 52, V: 32 and Z₇: 57 (**Table 2**).

3.2. Molecular Methods

All the 57 (100%) culture positive isolates were reconfirmed as *Campylobacter* species by molecular investigation for 16S rRNA, *hipO*, *aspK*, *glyA* and *lpxA* genes. PCR revealed the presence of *C. jejuni* in 45 (79.0%), *C. coli* in 1 (1.8%) and co-infection of *C. coli* and *C. jejuni* in 11 (19.3%) of the cultures but were negative for *C. lari* and *C. upsaliensis*. Antibiogram typing done by molecular methods showed nalidixic acid resistance in 36.8% (21/57), ciprofloxacin resistance in 35.0% (20/57) and 31.5% (18/57) resistance each for streptomycin and tetracycline (**Figure 4**).

Table 1. Primers used for amplification of target genes of *Campylobacter*.

S. No.	Target genes	Primer Sequences	References
1	16S rRNA	F-5' AATCTAATGGCTTAACCATTA 3' R-5' GTAAC TAGTTTAGTATTCCGC 3'	Linton et al. [32]
2	<i>hipO</i> (<i>C. jejuni</i>)	F-5' GGAGAGGGTTTGGGTGGT-3' R-5'-AGCTAGCCTCGCATAATAACTTG-3'	Linton et al. [32]
3	<i>aspK</i> (<i>C. coli</i>)	F-5' GGTATGATTCTACAAAGCGAG-3' R-5' ATAAAAGACTATCGTCGCGTG-3'	On and Jordan [33]
4	<i>glyA</i> (<i>C. lari</i>)	F-5' TAGAGAGATAGCAAAAAGAGA 3' R-5' TACACATAATAATCCACCC 3'	Yamazaki-Matsune et al. [34]
5	<i>lpxA</i> (<i>C. upsaliensis</i>)	F-5' CGATGATGTCAAATTGAAGC 3' R-5' TTCTAGCCCCCTTGCTTGATG 3'	Yamazaki-Matsune et al. [34]
6	Tetracyclin (<i>tetO</i>)	F-5' AACTTAGGCATTCTGGCTCAC 3' R-5' TCCCACTGTTCCATATCGTCA 3'	Ng et al. [35]
7	Nalidixic acid (<i>gyrA</i>)	F-5' GCT CTT GTT TTA GCT TGATGCA 3' R-5' TTG TCG CCA TC CTA CAGCTA 3'	Jesse et al. [36]
8	Ciprofloxacin (MAMA <i>gyrA</i>)	F-5' TTT TTA GCA AAG ATT CTG AT 3' R-5' CAA AGC ATC ATA AAC TGC AA 3'	Zirnstein et al. [37]
9	Streptomycin (<i>strA</i>)	F-5' CCAATCGCAGATAGAAGGCAAG 3' R-5' ATCAACTGGCAGGAGGAACAGG 3'	Maidhof et al. [38]

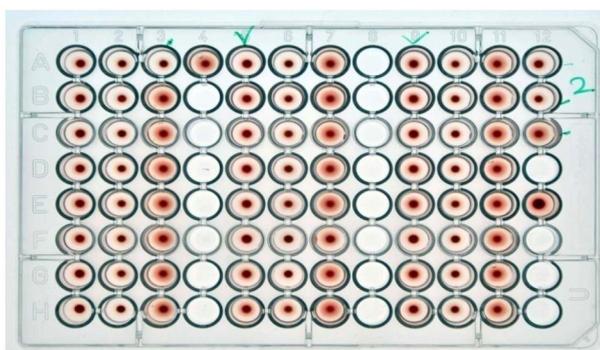


Figure 3. Microplate showing agglutination and button formation for *C. jejuni* isolates.

Table 2. Number of *C. jejuni* isolates showing multiple serotypes (serotypes are given in alphabetic order and the most frequent ones are marked in bold).

Serogroup	No. of <i>C. jejuni</i> isolates (multiple serotypes)
Group A: 1, 44	3
Group B: 2	19
Group C: 3	6
Group D: 4, 13, 16, 43, 50	6
Group E: 5	3
Group F: 6, 7	6
Group G: 8	4
Group I: 10	6
Group J: 11	7
Group K: 12	6
Group L: 15	7
Group N: 18	6
Group O: 19	6
Group P: 21	5
Group R: 23, 36, 53	6
Group S: 27	12
Group U: 31	6
Group V: 32	9
Group Y: 37	5
Group Z: 38	4
Group Z ₂ : 41	7
Group Z ₄ : 45	7
Group Z ₅ : 52	12
Group Z ₆ : 55	6
Group Z ₇ : 57	9

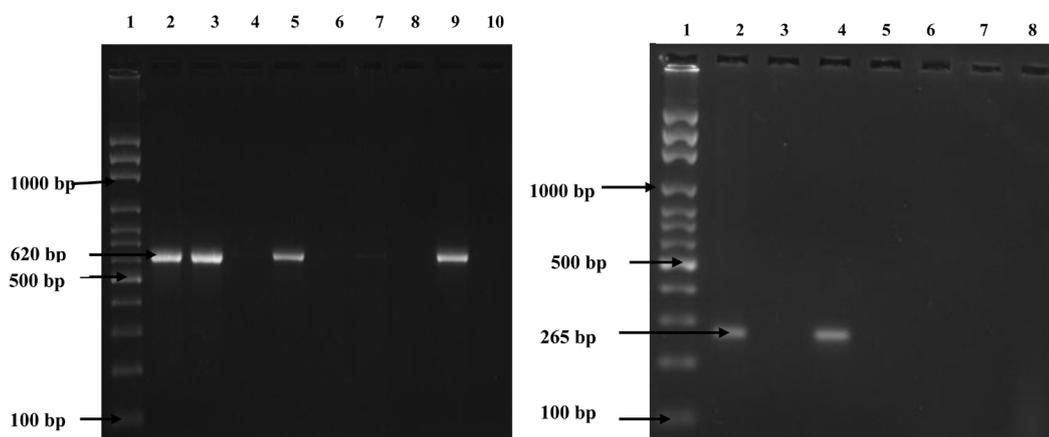


Figure 4. PCR detection of *gyrA* genes for resistance of nalidixic acid (620 bp) and ciprofloxacin (265 bp) respectively. Lane 1—Marker; Lane 2—Positive control; Lanes 3 to 10—*Campylobacter* isolates.

4. Discussion

Campylobacter is a major health burden for both developed and developing countries. Most of the *Campylobacter* isolates causing human gastroenteritis are thermo-tolerant variety and can grow even at incubation temperatures of 42°C. Farm animals and wild birds are the primary sources contributing to human infections of *Campylobacter* due to consumption of contaminated water. Drinking water gets contaminated from septic seepage and waste water intrusion into ground water sources. Handling and eating undercooked poultry have consistently been shown to be important risk factors in food-borne illness due to *Campylobacter* [3]. A survey of raw poultry demonstrated that 50% - 70% of raw chickens tested at the retail level were contaminated with *Campylobacter* [12].

C. jejuni has been reported to be the most isolated species in chicken [1]. Yu *et al.* [13] reported the first recognized major *C. jejuni* outbreak in a middle School in Incheon, associated with contaminated chicken in Korea where an attack rate of 11.6% occurred with 40.3% stool samples positive for *C. jejuni*. The authors state that the raw chickens used in the chicken soup with ginseng were supplied frozen (−6°C) in the morning of July 1 by a company which was free of food hygiene violations. The chickens had been slaughtered and processed on June 29 and were deemed acceptable for use until July 9 under refrigeration. Despite this, the chicken soup prepared and consumed on July 1, 2009 was considered to be the source of human infection [13]. Yano *et al.* [14] monitored *C. jejuni* in four chicken farms during the period 2003 to 2006 to elucidate the mechanisms of transmission. A total of 206 *C. jejuni* isolates were obtained with *C. jejuni* coming from common sources external to the farms. In the present study, prevalence of *Campylobacters* was 44.9% in the poultry in and around Chandigarh region and *C. jejuni* was the most prevalent *Campylobacter*. This is in contrast to another study from the same region carried out during the same time period among human beings where a low prevalence of *Campylobacter* was seen [9].

Typing methods have a significant role in the identification, monitoring, and prevention of *Campylobacter* infections. Combination of phenotypic and genotypic methods can improve species discrimination of pathogens such as *Campylobacter*. A genotyping method like PCR has been found to be an efficient and reliable typing method with greater discriminatory power providing superior results [15]. Lawson *et al.* [16] used genetic targets *aspK* and *hipO* and Bang *et al.* [17] used 16S rRNA to identify *C. coli* and *C. jejuni*. The same molecular methods were also used in the present study.

Serotyping methods can be used to differentiate clonally related isolates from unrelated ones due to different characteristics. Penner hemagglutination assay is a serotyping method widely used for characterizing *Campylobacter* isolates. The *C. jejuni* capsular polysaccharides (CPS) are the primary serodeterminant of the Penner scheme. On and off CPS expression by *C. jejuni* suggests that this tactic might have a role in *Campylobacter* virulence [18]. Based on their geographic locations, the serotype prevalence of *Campylobacter* differs across countries. The most predominant serotypes in Japan are B, D, and L, while those in Denmark are serotypes B, A, and D [19] [20]. Ishihara *et al.* [21] identified 18 serotypes among the *C. jejuni* isolates from humans with major

ones being B, D and R. In another study from Thailand [22], *C. jejuni* isolates from poultry were classified into 10 Penner serotypes viz. A, C, I, K, B, E, S, D, L and R. The most frequent serotypes in the present study were B: 2, S: 27, Z₅: 52 and Z₇: 57 in descending order. Thus serotype B seems to be more common among the *Campylobacter* isolates as also seen in our earlier study in humans [9] and *Campylobacter* from poultry could be the source of infection among the human population studied from the same region [9].

Another problem of great concern at global level is the acquisition of resistance to multiple antibiotics by *Campylobacters* due to unregulated use of antimicrobial agents in food animal production [23]. Several resistance genes underlie the emergence of multidrug-resistant *Campylobacter*. Macrolides and fluoroquinolones are commonly prescribed for campylobacteriosis. But, resistance to these and other antibiotics also occurs. In Netherlands almost 30% of *Campylobacter* isolates were resistant to fluoroquinolones [24]. An increase in fluoroquinolone resistance in *Campylobacter* spp. from Europe and USA has also been reported [25] due to their use in food animals [26]. Since erythromycin is the drug of choice for the treatment of *Campylobacter* infections the prevalence of resistance to this antimicrobial, especially among strains isolated from food, is a cause of concern. Previous studies on the susceptibility of *Campylobacter* to macrolides showed that the rate of resistant isolates was at a low level and did not exceed 1% [27]-[29]. However, a relatively high level of resistance to streptomycin (22.8%) was reported in Poland [30]. In the present study antibiotic resistance was 36.8% for nalidixic acid, 35.0% for ciprofloxacin, and 31.5% for both streptomycin and tetracyclin. The findings of the present investigation are consistent with previous results as a relatively high level of resistance to streptomycin was seen. Isolates from chicken broilers were 67% resistant to tetracyclin [31]. The MAMA PCR has been considered as a valuable and reliable alternative technique to sequencing for detection of the Thr-86 → Ile mutation for ciprofloxacin [31].

5. Conclusion

This study indicates that in northern region of India, there is an increasing emergence of antibiotic resistance among *Campylobacter* strains in poultry. It is important to reduce the contamination rates by *Campylobacter* in poultry by planning risk burden strategies throughout the food chain. One limitation of the present study is that we cannot investigate *Campylobacters* in domestic poultry rearing, which can also be a source of infection to human beings. New strategies for containing *Campylobacter* infections will likely include limiting consumption of antibiotics by animals, disinfection of their food and water, treatment of their manure, and isolation of the contagiously ill. Other strategies like irradiation of foods of animal origin may become a feasible method of control of the bacterial contamination of foods. Integrated efforts must be done in order to encourage the appropriate use of antimicrobials in food animals and for the implementation of a surveillance system of drug resistance in *Campylobacters*.

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Transparency Declaration

There is no conflict of interest.

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List of Abbreviations

- 1) %—Percentage
- 2) μg —Microgram
- 3) μl —Microliter
- 4) *aspK*—aspartokinase
- 5) *C. coli*—*Campylobacter coli*
- 6) *C. fetus*—*Campylobacter fetus*
- 7) *C. jejuni*—*Campylobacter jejuni*
- 8) *C. lari*—*Campylobacter lari*
- 9) *C. upsaliensis*—*Campylobacter upsaliensis*
- 10) CPS—Capsular polysaccharides
- 11) DNA—Deoxyribonucleic acid
- 12) EDTA—Ethylenediaminetetraacetic acid
- 13) g—gravity
- 14) *glyA*—Glycine
- 15) *gyrA*—Gyrase A
- 16) h—hour
- 17) *hipO*—hippuricase
- 18) Ile—Isoleucine
- 19) *lpxA*—lipopolysaccharide
- 20) MAMA—Mismatch Amplification Mutation Assay
- 21) mins—minutes
- 22) $^{\circ}\text{C}$ —Degrees Celsius
- 23) PCR—Polymerase chain reaction
- 24) rRNA—Ribosomal ribonucleic acid
- 25) *strA*—streptomycin A gene
- 26) *tetO*—tetracyclin O gene
- 27) Thr—Threonine