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Research Article

ISOLATION, MOLECULAR CHARACTERIZATION AND *IN-VITRO* ANTIBIOTIC SENSITIVITY OF *CAMPYLOBACTER* SPP. FROM CHICKEN MEAT

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ABSTRACT

In the present study, 80 chicken meat and chicken meat product samples collected from the local market were examined by culture method for presence of *Campylobacter* spp. Culture method for isolation of Campylobacter from chicken meat sample included broth enrichment followed by inoculation on Skirrow agar which resulted in isolation of 14 suspected *Campylobacter* spp. On the basis of morphological and biochemical tests, all the 14 isolates were characterized as *Campylobacter jejuni*. A multiplex PCR assay using three sets of primers (including a universal primer for genus *Campylobacter*, one for *C. jejuni* and one for *C. coli*) was employed for amplification of 16S rDNA gene, hippuricase gene and aspartokinase gene, respectively. All 14 isolates yielded a specific band for 16S rDNA and hippuricase genes suggesting all the isolates to be *C. jejuni*. Antibiogram revealed that all the isolates were 100% sensitive to amoxyclav and doxycycline and 100% resistant to nalidixic acid while variable results were obtained with other 13 antibiotics. Among the 14 isolates, five isolates showed multiple drug resistance to more than seven antibiotics.

Key words: *Campylobacter jejuni, hipO* gmene, 16S rDNA gene, aspartokinase gene, Campylobacteriosis

Thermophilic *Campylobacter* spp have been implicated as the most frequent cause of bacterial gastroenteritis in humans worldwide (Man, 2011). Campylobacteriosis cases exceed the total number of cases caused by *Salmonella, Shigella and Escherichia coli O157:*H7 in humans (EFSA, 2011). Reports from Asia, Africa and Middle East indicate that *Campylobacter* is endemic in these areas (Kaakoush *et al.*, 2015). Poultry has been recognized as the primary reservoir and source of transmission of campylobacteriosis to humans. In India, isolation of *Campylobacter* spp has been reported from different foods of animal origin including chicken meat, milk (Malik *et al.,* 2014; Rajagunalan *et al.,* 2014; Modi *et al.*, 2015; Pallavi *et al.*, 2015; Monika *et al.*, 2016). The most common antimicrobial agents used in the treatment of *Campylobacter* infections are fluoroquinolones and macrolides. Resistance to both groups of antimicrobials is increasing among *Campylobacter* spp. isolated from livestock, food and humans (Stone *et al.,* 2013; Mukherjee *et al*., 2014; Nguyen *et al.*, 2016). Keeping in view the public health significance of *Campylobacter* spp. as foodborne pathogen with poultry being the primary reservoir, the present study was taken up to determine the prevalence and antibiotic resistance of *Campylobacter* spp from chicken meat in the area.

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MATERIALS AND METHODS

A total of 80 chicken meat samples comprising of 50 raw chicken meat, 10 frozen chicken meat, five each of frozen chicken heart, frozen chicken liver, chicken wings and chicken balls from Hisar, Haryana were collected in sterile sample container with all aseptic precautions and transported to the laboratory under cold conditions. These samples were processed immediately for isolation of *Campylobacter* spp.

Selective Enrichment and Plating: A total of 25 gm of sample was homogenized with 100 ml Preston broth using sterile scissors, pestle and mortar. This initial homogenate was transferred in a 100 ml conical flask and incubated at 42°C for 48h under microaerophilic conditions. Following incubation a loop full of inoculated Preston broth was streaked on to Skirrow agar and incubated at 42°C for 48h under microaerophilic conditions. Presumptive *Campylobacter* isolates having characteristic round convex dew-drop like non-haemolytic colonies on Skirrow agar were purified on blood agar and examined initially for their morphology and Gram's staining reaction followed by motility/wet mount, catalase, oxidase, aerobic growth at 42°C for identification at genus level. For differentiation of *Campylobacter* species, biochemical tests like hippurate hydrolysis, indoxyl-acetate hydrolysis and H_2S production on TSI slants were employed.

Molecular Characterization: A multiplex polymerase chain reaction (m-PCR) assay was carried out on extracted DNA samples from *Campylobacter* isolates for the identiûcation of *C. coli* and *C. jejuni* as described by Perrson and Olsen (2005). In this m-PCR method, *C. coli* speciûc *asp*-primers (Linton *et al.*, 1997) which result in a 500 bp amplicon; primers designed to amplify a 344 bp fragment of the *hipO* gene characteristic of *C. jejuni*, and universal primers used to amplify a 1062 bp fragment of the *16S rDNA* gene, specific of genus *Campylobacter* were included (Perrson and Olsen, 2005). Details of the primers are given in Table 1. The reaction mixture (25µl) was prepared by adding 9.5µl of nuclease free water (NFW), 10.5µl master mix and 0.5µl of all three sets of primers and after proper mixing it was distributed in the tubes (23µl in each tube). Two microlitre of genomic DNA was added in all the tubes. All these steps were carried out at 4°C. The 25 µl reaction mix was kept for amplification in a programmed thermo cycler. After the final extension step, the PCR products were stored at 4°C for further analysis.

A 100 bp ladder was used as marker in each run. The extraction of genomic DNA from broth culture of *Campylobacter* isolates was carried using the DNA extraction mini kit (QIAamp) following the manufacturer's recommendations. The PCR amplification was achieved with initial denaturation of DNA at 94°C for 6 min with Hotstar Taq master mix, Qiagen. It was followed by 35 cycles of denaturation, annealing and extension at 94°C for 50 sec, 59°C for 40 sec and 72°C for 50 sec respectively and final extension was carried out at 72°C for 3 min. The PCR products were analysed on 1.5% agarose in gel electrophoresis unit and photographed using gel documentation system (Alphaimager, HP).

In- vitro Antibiotic Sensitivity: The *in vitro* single disc diffusion technique (Bauer *et al*., 1966) was employed using Muller-Hilton agar medium with 5% sheep blood and various antibiotic discs viz., amoxyclav (30 mcg), ampicillin (10 mcg), azithromycin (15 mcg), cefdinir (5 mcg), cefotetan (30 mcg), cefoxitin (30 mcg), cefpodoxime (10 mcg), chloramphenicol (10 mcg), ciprofloxacin (30 mcg), clarithromycin (15 mcg), doxycycline hydrochloride (30 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), norfloxacin (10 mcg), piperacillin/tazobactam (10 mcg), tetracycline (30 mcg) were used. The results were interpreted on the basis of inhibition zone as per the manufacturer's recommendations (Hi-Media, Bombay) after 24 h incubation at 37°C under microaerophilic conditions.

RESULTS AND DISCUSSION

The cultural examination of 80 chicken meat and chicken meat product samples resulted in the isolation of 14 (17.5%) *Campylobacter* isolates. Fourteen (28%) of 50 raw chicken meat samples were found positive by culture method and all of the 30 chicken meat products samples (frozen chicken meat, frozen chicken liver, frozen chicken heart, chicken wings and chicken balls) were found negative for *Campylobacter* spp. All the 14 *Campylobacter* isolates produced characteristic round convex dew-drop like non-haemolytic colonies on Skirrow agar. On the basis of biochemical tests all the isolates were characterised as *C. jejuni*. All the isolates did not show any growth following aerobic incubation at 42°C, were found positive for catalase and oxidase tests, hydrolysed hippurate and indoxyl-acetate and did not produce H_2S on TSI slants. The prevalence of *Campylobacter* spp. of 17.5% from chicken meat is similar to the recovery rate of 19.09% reported by Sharma *et al*. (2011) from raw poultry and ready to eat poultry meat product samples and 17.33% by Pallavi and Kumar (2014) in chicken meat samples. On the contrary, higher prevalence (22.72% to 32%) of *Campylobacters* from poultry hasbeen reported (Singh *et al*., 2008). The reported prevalence of 17.5% of *C. jejuni* in the present study was also different than the prevalence of 12.13% of *C. coli* and 3.76% of *C. jejuni* as reported by Rajagunalan *et al*. (2014) from poultry and 6.58% by Monika *et al*. (2016) in meat. Significant variations in prevalence rates have been reported in studies in different countries (Suzuki and Yamamoto, 2009; Ilida and Faridah, 2012; Skarp *et al*., 2016). Several factors including

Table 1
Detailed description of primers used in the present study

Primers used	Target gene	Primers sequence	Amplicon size (base pairs)	Reference
Universal primer specific to Genus *Campylobacter*	*16S rDNA*	F-5'-GGAGGCAGCAGTAGGGAATA-3' R-5'-TGACGGGCGGTGAGTACAAG-3'	1062	Persson and Olsen (2005)
Species specific primer for *C. jejuni*	*hipO*	F-5'-GACTTCGTGCAGATATGGATGCTT-3' R-5'-GCTATAACTATCCGAAGAAGCCATCA-3'	344	Persson and Olsen (2005)
Species specific primer for *C. coli*	*asp*	F-5'-GGTATGATTTCTACAAAGCGAG-3' R-5'-ATAAAAGACTATCGTCGCGTG-3'	500	Linton *et al.* (1997)

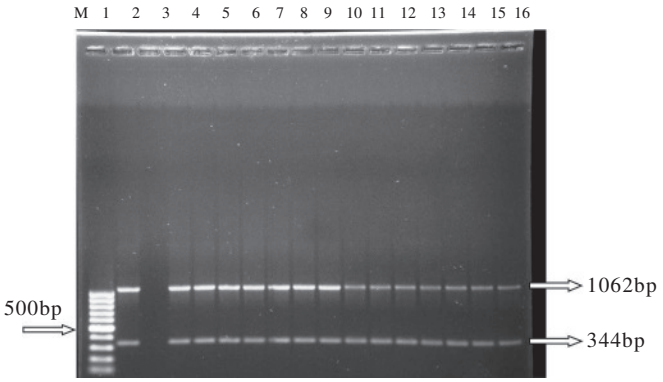

Fig 1. Multiplex PCR results amplifying *16s rDNA* gene (1062bp), *hipO gene* (344bp) and *asp* gene (500bp) of *Campylobacter* isolates from chicken meat
Lane M=Ladder (100bp); Lane 1=C. *jejuni* positive control; Lane 2=*E. coli* negative control; Lane 3-16=chicken meat isolates

difference in infection rates in food animals and food production system can be responsible for these variations. Further the stages starting from production at rearing farms, transport to slaughter, the slaughter process and subsequent processing of chicken meat products, selling products at retail level, handling and consumption of meat at home, all have role in transmission of *Campylobacters* (Kaakoush *et al*., 2015).

In the present study all frozen poultry meat and chicken meat product samples were found negative for *Campylobacters*. Though there are reports of isolation of *Campylobacter* spp. from frozen chicken meat and poultry products (Suzuki and Yamamoto, 2009), but the absence of *Campylobacters* in poultry products in the present study may be due to the effect of freezing or heat process on viability of *Campylobacters*. Some of the studies have reported lower prevalence of *Campylobacters* in frozen meat and by products as compared to raw poultry meat and by products (Shuzuki and Yamamoto, 2009).

All 14 isolates when amplified using genus and species specific primer were found to be positive for *C. jejuni*. PCR using genus specific and *C. jejuni* species specific primers produced amplicons of 1062 bp and 344 bp in all the isolates, respectively (Fig 1.). There are reports of PCR based molecular characterization of biochemically confirmed *Campylobacter* isolates from different samples (Altun *et al*., 2014; Monika *et al*., 2016).

All the 14 chicken meat isolates of *Campylobacter* were tested against 16 (Table 2). All the isolates were found sensitive to amoxyclav and doxycycline hydrochloride and resistant to nalidixic acid. However, 13 antibiotics including ampicillin, azithromycin, cefdinir, cefotetan, cefoxitin, cefpodoxime, chloramphenicol, ciprofloxacin, clarithromycin, gentamicin, norfloxacin, piperacillin/tazobactam, tetracycline exhibited variable results. Ampicillin, cefdinir, cefotetan, cefoxitin, cefpodoxime, ciprofloxacin and tetracycline showed a sensitivity of less than 60% against all the isolates tested (Table 2). High rate of resistance level to different antibiotics was observed including nalidixic acid (100%) ampicillin (85.72%), cefpodoxime (85.72%), cefdinir (78.57%), cefoxitin (42.86%), ciprofloxacin (42.86%), and tetracycline (42.86%). Similar results of high resistance level in *Campylobacter* isolates from different sources have been reported in several studies (Zhao *et al.,* 2010; Mukherjee *et al*., 2014; Nguyen *et al.*, 2016).

During the present investigation, all the isolates were 100% resistant to nalidixic acid which is in agreement to the finding of Ghosh *et al.* (2013). Varying degree of

Table 2
Sensitivity of individual antibiotic against *Campylobacter* isolates

Antibiotic tested	Percentage sensitivity of antibiotic
Amoxyclav (AMC)	100.00%
Ampicillin (AMP)	14.28%
Azithromycin (AZM)	85.71%
Cholramphenicol (C)	85.71%
Cefdinir (CDR)	21.43%
Ciprofloxacin (CIP)	57.14%
Cefpodoxime (CPD)	14.28%
Cefotetan (CTN)	57.14%
Cefoxitin (CX)	57.14%
Clarithromycin (CLR)	92.85%
Doxycycline hydrochloride	100.00%
Gentamicin (GEN)	92.85%
Nalidixic acid (NA)	00.00%
Norfloxacin (NX)	87.71%
Piperacillin/Tazobactam (PIT)	78.57%
Tetracycline (TE)	57.14%

Table 3
Multidrug resistance profiles of *C. jejuni* isolates

Antibiotic resistance profile	No. of resistant isolates (%)
AMP, AZM, C, CDR, CIP, GEN, NA, NX, TE	1 (7.15)
AMP, C, CDR, CIP, CPD, NA, NX, TE	1 (7.15)
AMP, CDR, CPD, CTN, CX, NA, PIT	2 (14.30)
AZM, CDR, CIP, CPD, CTN, CX, NA	1 (7.15)
AMP, CDR, CIP, CPD, NA, PIT	1 (7.15)
AMP, CDR, CPD, CTN, CLR, NA	1 (7.15)
AMP, CDR, CPD, CX, NA	2 (14.30)
AMP, CIP, CPD, CTN, NA	1 (7.15)
AMP, CDR, CPD, NA, TE	1 (7.15)
CPD, CTN, CX, NA, TE	1 (7.15)
AMP, CDR, CPD, NA	1 (7.15)
AMP, CIP, NA	1 (7.15)

AMP=Ampicillin; AZM=Azithromycin; C=Chloramphenicol; CDR=Cefdinir; CIP=Ciprofloxacin; GEN=Gentamicin; NA=Nalidixic acid; NX=Norfloxacin; TE=Tetracycline; CPD=Cefpodoxime; CTN=Cefotetan; CX=Cefoxitin; PIT=Piperacillin/Tazobactam

resistance to other antibiotics viz ampicillin (85.72%), ciprofloxacin (42.86%), norfloxacin (12.29%) reported in the present study confirms the finding as reported in other studies (Rajagunalan *et al.* 2012; Nobile *et al*., 2013). Further five *Campylobacter* isolates showed multiple drug resistance to more than seven antibiotics against all the 16antibiotics tested. However, all 14 isolates showed resistance to three or more antibiotics (Table 3). Multiple drug resistance in *Campylobacter* spp has been reported by other workers (Tambur *et al*., 2010; Nguyen *et al*., 2016).

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