Original Article

Prevalence, antimicrobial resistance, and virulence-associated genes of *Campylobacter* isolates from raw chicken meat in Shiraz, Iran

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Abstract

Background: *Campylobacter* is recognized as a major cause of foodborne gastroenteritis in humans in many countries and may be transferred from animals to humans. The consumption of chicken meat is identified as a major cause of *Campylobacter* infection in humans. **Aims:** To find out the contamination rate of chicken meat with *Campylobacter*, the antimicrobial resistance (AMR) pattern, and the virulence-associated genes of the isolates. **Methods:** Ninety packed chicken meat from 7 main poultry slaughterhouses in Shiraz were analyzed for *Campylobacter* spp. isolation through microbiological methods. Specific primers were used for the identification of the *Campylobacter* isolates on species level by polymerase chain reaction (PCR). Antibiotic resistant profiles were determined using the disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) standards. All the isolates were screened for 7 virulence-associated genes, namely *cdt*A, *cdt*B, *cdt*C, *cad*F, *pld*A, *cgt*B, and *vir*B11 by PCR. **Results:** Out of 90 chicken meats, 26 (28.9%) *Campylobacter* spp. have been isolated. Resistance to ciprofloxacin (CIP), nalidixic acid (NA), and cefixime (CFM) was observed in all the isolates. Resistance to trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), ampicillin (AMP), and chloramphenicol (CHO) was 80.8%, 88.5%, 76.9%, and 30.8%, respectively. Multidrug resistance (MDR) phenotype was observed in 80.8% of the *Campylobacter* isolates. All the isolates were positive for *cdt*A, *cdt*B, *cdt*C, and *cad*F genes. *pld*A and *cgt*B were detected in 65.4% and 15.4% of the isolates, respectively. **Conclusion:** In this study, the presence of several virulence genes and an alarming level of MDR in *Campylobacter* spp. isolates were reported. Particularly, resistance to CIP and TET should be highlighted, since both are key drugs for the treatment of human campylobacteriosis.

Key words: *Campylobacter*, Chickens, Meat, Resistance, Virulence

Introduction

Campylobacter species are part of the gut microbiota of livestock, domestic animals, and birds. However, they can cause gastroenteritis in infected people and are identified as one of the main causes of foodborne diseases (Barletta *et al*., 2013; Changkwanyeun *et al*., 2015; Cha *et al*., 2016; Facciola *et al*., 2017; Martin *et al*., 2018). In humans, handling and/or consumption of contaminated meat especially poultry was recognized as the primary source of infection. While *Campylobacter jejuni* is responsible for 85% of the infections in humans, *Campylobacter coli* accounts for most of the remainder (Giacomelli *et al*., 2014). Clinical presentation of *Campylobacter* infection in humans varies in spectrum from mild to severe diarrhea, and inflammatory bloody diarrhea (Al-Mahmeed *et al*., 2006). Although such infections are usually self-limiting, several complications can arise, the most important of which are bacteraemia, Guillain-Barré syndrome (GBS), reactive arthritis (RA), and inflammatory bowel syndrome (IBS) (Wagenaar, 2018). The clinical presentations of *Campylobacter* gastroenteritis may be modulated by several virulence factors (Zilbauer *et al*., 2008). The first and essential stage of pathogenesis is intestinal epithelium adherence mediated by various bacterial surface adhesins such as *cad*F, which encodes the outer membrane protein that interacts with fibronectin (Facciola *et al*., 2017). Subsequent cell damage is mediated by various cytotoxins, the most studied of which is cytolethal distending toxin (CDT) which consists of three subunits (*cdt*A, -B, -C) (Zilbauer *et al*., 2008; Facciola *et al*., 2017). *pld*A, phospholipase A, and *vir*B11 are among the genes responsible for the expression of invasion, whereas genes *wla*N and *cgt*B are related to the expression of GBS (Datta *et al*., 2003).

Currently, macrolides (mainly erythromycin (ERY) and azithromycin (AZI)) and fluroquinolones are the

drugs of choice for the treatment of *Campylobacter* infection (Hao *et al*., 2016). Antimicrobial resistance (AMR) in *Campylobacter* spp. from human and animal samples has been reported in many countries including Iran (Kang *et al*., 2006; Taremi *et al*., 2006; Feizabadi *et al*., 2007; Rahimi *et al*., 2010; Mansouri-najand *et al*., 2012; Zendehbad *et al*., 2013; Giacomelli *et al*., 2014; Zendehbad *et al*., 2015; Cha *et al*., 2016). Antimicrobial resistance in food producing animals has serious implications for humans, as it may lead to the distribution of AMR through the food chain. It has been shown that the emergence of AMR in *Campylobacter* is strictly related to the use of antimicrobials in veterinary medicine (Aarestrup *et al*., 2008).

Multiple Iranian studies have reported the prevalence and AMR of *Campylobacter* spp. in livestock, broiler flocks, poultry, and red meat (Kang *et al*., 2006; Taremi *et al*., 2006; Rahimi *et al*., 2010; Ansari-Lari *et al*., 2011; Zendehbad *et al*., 2013; Zendehbad *et al*., 2015). Some studies have focused on the prevalence of virulence genes in *Campylobacter* spp. isolated from animals and/or humans (Hamidian *et al*., 2011; Khoshbakht *et al*., 2013; Ghorbanalizadgan *et al*., 2014; Raeisi *et al*., 2017). In Iran, *Campylobacter* spp. strains are reported as the cause of 5.4-10.8% of acute diarrhea (Feizabadi *et al*., 2007; Hassanzadeh and Motamedifar, 2007; Jafari *et al*., 2009; Ghorbanalizadgan *et al*., 2014).

An understanding of the *Campylobacter* epidemiology, including the contamination rate and AMR in animal source, is a key step in developing effective strategies for reducing both the *Campylobacter* infection and the likelihood of AMR in humans. The aims of this study were to find out the rate of *Campylobacter* spp. contamination in chicken meat, to investigate the AMR pattern and virulence-associated genes of *Campylobacter* isolates from chicken. Furthermore, we compared the *Campylobacter* isolates from chicken meat with the strains isolated from patient with diarrhea in our previous study (Aminshahidi *et al*., 2017) for any possible similarities of AMR and virulence associated genes. Results on the antibiotic resistance profiles of *Campylobacter* isolates may be used for the development of a database to be used for effective clinical treatment of the illness during outbreak of foodborne disease.

Materials and Methods

Samples

From September to November of 2016, 90 packed chickens ready for distribution in the market were purchased from 7 main poultry slaughterhouses in Shiraz, Iran. All samples were transported in cool box to Clinical Microbiology Research Center where all the experiments were carried out. We also included the *Campylobacter* strains of human source that have been isolated from children with acute diarrhea in our previous study (Aminshahidi *et al*., 2017), in order to determine and compare their virulence associated gene patterns.

Isolation and identification of *Campylobacter*

Sample processing was done within 2 h following the reception in our laboratory according to the International Standards Organization (ISO) standard method (EN/ISO10272-1, 2006). Briefly, 25 g of skinless chicken meat was homogenized in Stomacher 400 circulator (Seward, England) with 225 ml of Preston enrichment broth base (Himedia, India), supplemented with CAMP SELECTVIAL (MAST, UK) and 5% sheep blood in sterile plastic bags. After 24 h of incubation at 42°C under microaerophilic conditions (10% CO_2, 5% O_2, and 83% N_2) created in an Anoxomat (MART®, Microbiology, B.V., Netherlands), 100 µL of mixture was streaked on the selective medium (Skirrow *Campylobacter* selective agar, Oxoid, UK). The latter was made using Columbia agar (Biolife, Italy) as the base, to which the following was added: 7% of lysed horse blood, vancomycin, trimethoprim, and polymyxin B (Sigma, USA) with final concentrations of 0.01 g, 0.005 g, and 2500 IU per L, respectively. The plates were then incubated at 42°C for 2-3 days under the same condition.

All the *Campylobacter* suspected colonies after examination with Gram stain and wet smear were confirmed by 23S rRNA polymerase chain reaction (PCR), and species were identified using specific primer sets as presented in Table 1.

DNA extraction and PCR analysis for virulence-associated genes

NucleoSpin® Tissue kit (MACHEREY-NAGEL, Germany) kit was used for bacterial DNA extraction. Spectrophotometry was used at 260 and 280 nm to determine the purity and concentration of the extracted DNA (Nanodrop, nd1000, USA). All isolates were screened for 7 virulence-associated genes, including three cytolethal toxin production genes: *cdt*A, *cdt*B, *cdt*C; GBS associated gene: *cgt*B; adherence and colonization factor *cad*F; phospholipase *pld*A; and *vir*B11 invasion factors by PCR assays. In order to identify the virulence-associated genes, all 26 *Campylobacter* strains isolated in this study underwent PCR using the primers listed in Table 1. In addition to *Campylobacter* isolates from chicken meat in this study, we also investigated virulence associated genes of the 7 *Campylobacter* strains isolated from children with acute diarrhea from our previous study (Aminshahidi *et al*., 2017).

Polymerase chain reaction was performed in the final volume of 25 µL, including 2.5 µL PCR buffer (CinnaGen, Iran), 1.5 mM of $MgCl_2$ (CinnaGen, Iran), 1 µL of mixed dNTP 10 mM (CinnaGen, Iran), 1 µL of 10 picomol of each primer (Bioneer, South Korea), 1.25 units of *Taq* polymerase (CinnaGen, Iran), and 2 µL of template. The final volume of each reaction increased to 25 µL with distilled water. The solutions were then subjected to the following cycling conditions: 94°C for 5 min, 94°C for 30 s, annealing step for 30 s (the optimal annealing temperature varied depending on the gene mentioned in Table 1), 72°C for 30 s (35 cycles), and a

Table 1: List of primers used

Gene name	Primers	Annealing temperature	Amplicon size (bp)	Reference
*cdt*A	F: CCTTGTGATGCAAGCAATC R: ACACTCCATTTGCTTTCTG	49°C	370	Khoshbakht *et al*. (2013)
*cdt*B	F: CAGAAAGCAAATGGAGTGTT R: AGCTAAAAGCGGTGGAGTAT	51°C	620	Khoshbakht *et al*. (2013)
*cdt*C	F: CGATGAGTTAAAACAAAAAGATA R: TTGGCATTATAGAAAATACAGTT	48°C	182	Khoshbakht *et al*. (2013)
*vir*B11	F: TCTTGTGAGTTGCCTTACCCCTTTT R: CCTGCGTGTCCTGTGTTATTTACCC	53°C	494	Khoshbakht *et al*. (2013)
*iam*A	F: GCGCAAAATATTATCACCC R: TTCACGACTACTATGCGG	52°C	518	Khoshbakht *et al*. (2013)
*wla*N	F: TGCTGGGTATACAAAGGTTGTG R: AATTTTGGATATGGGTGGGG	56°C	330	Khoshbakht *et al*. (2013)
*cgt*B	F: TTAAGAGCAAGATATGAAGGTG R: GCSCATAGAGAACGCTACAA	56°C	562	Khoshbakht *et al*. (2013)
*pld*A	F: AAGCTTATGCGTTTTT R: TATAAGGCTTTCTCCA	45°C	913	Khoshbakht *et al*. (2013)
*cad*F	F: TTGAAGGTAATTTAGATATG R: CTAATACCTAAAGTTGAAAC	42°C	400	Khoshbakht *et al*. (2013)
hipO	F: ACTTCTTTATTGCTTGCTGC R: GCCACAACAAGTAAAGAAGC	50°C	323	pubmlst.org/*Campylobacter*
C. coli	F: TCAAGGCGTTTATGCTGCAC R: CCATCACTTACAAGCTTATAC	50°C	323	pubmlst.org/*Campylobacter*
23s rRNA	F: TATACCGGTAAGGAGTGCTGGAG R: ATCAATTAACCTTCGAGCACCG	50°C	650	pubmlst.org/*Campylobacter*

F: Forward, and R: Reverse

final extension step (72°C for 8 min) in a thermal cycler (Applied Biosystem, USA; Veriti). Subsequently, 8 µL of the PCR product was subjected to gel electrophoresis (Biorad, Wide mini-sub® Cell GT, USA) employing 1.5% Agarose (Invitrogen, 16500, USA), stained by means of GelRed Nucleic Acid Gel Stain (Biotium, 41002, USA), and visualized by gel documentation (UVitec, DBT-08, UK).

Antimicrobial susceptibility testing

To determine the susceptibility of the *Campylobacter* isolates to antimicrobial agents, isolates were inoculated on Muller-Hinton agar with 5% horse blood. Following antibiotic discs have been used for antimicrobial susceptibility testing: ciprofloxacin (CIP, 5 µg), AZI (15 µg), ERY (15 µg), gentamicin (GEN, 10 µg), nalidixic acid (NA, 30 µg), ampicillin (AMP, 10 µg), meropenem (MRP, 10 µg), cephalothin (CEP, 30 µg), cefixime (CFM, 5 µg), cefuroxime (CXM, 30 µg), ceftriaxone (CTR, 30 µg), cefepime (FEP, 30 µg), amikacin (AMK, 30 µg), tetracycline (TET, 30 µg), chloramphenicol (CHO, 30 µg), and trimethoprim/sulfamethoxazole (SXT, 25 µg) (Rosco Neo-Sensitabs Denmark). The plates were then incubated overnight at 42°C in a microaerophilic condition. The results were interpreted according to the company's breakpoints which are in accordance to M100-S25 document of Clinical and Laboratory Standards Institute (CLSI) standards (2015). In this study isolates are classified as multidrug resistant (MDR) if they are resistant to minimum three different classes of antimicrobial agents (Tanwar *et al*., 2014).

Results

In total, out of 90 chicken meat samples, 26 (28.9%) *Campylobacter* spp. were isolated; 24 isolates (96%) were *C. jejuni* and the other 2 (4%) isolates were *C. coli*.

Virulence-associated genes

Irrespective of their source of isolation, all the isolates (26 isolates of chicken meat and 7 isolates of diarrheal stool from children) were positive for cdtA, *cdt*B, *cdt*C, and *cad*F (Fig. 1). The *cgt*B gene was observed in 15.4% and 100% of *Campylobacter* isolates from chicken and human sources, respectively. The gene *pld*A was positive in all human isolates, whereas only 65.4% of chicken *Campylobacter* isolates had this gene. All 33 *Campylobacter* isolates were negative for *vir*B11 gene.

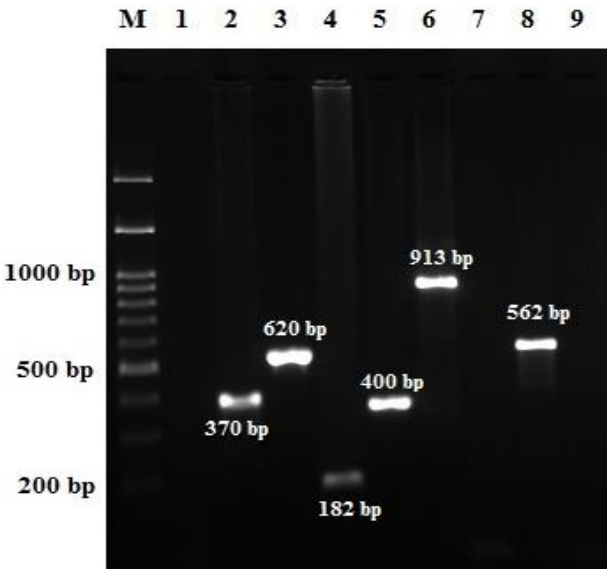

Fig. 1: PCR for identification of virulence-associated genes of *Campylobacter* strains. Lane M: Ladder, 100 bps to 1.5 kb DNA, and Lanes 1-9: *Campylobater jejuni* strains tested for virulence genes. Lanes 1, 7, 9: Negative controls, Lane 2: Positive *cdt*A gene (370 bp), Lane 3: Positive *cdt*B gene (620 bp), Lane 4: Positive *cdt*C gene (182 bp), Lane 5: Positive *cad*F gene (400 bp), Lane 6: Positive *pld*A gene (913 bp), and Lane 8: Positive *cgt*B gene (562 bp)

Antimicrobial susceptibility testing

A total of 24 *C. jejuni* and 2 *C. coli* isolates from the chicken meat samples underwent antimicrobial susceptibility testing for 16 antibiotics from 8 antimicrobial classes. Table 2 presents the AMR profile of the 26 *Campylobacter* strains isolated from the chicken meat in this study.

Table 2: Antimicrobial resistance profiles of *Campylobacter* strains isolated from chicken meat in Shiraz, Iran

Antimicrobial agent	*C. jejuni* (n=24), n (%)	*C. coli* (n=2), n (%)
Ampicillin (AMP)	19 (79.2)	1 (50)
Trimethoprim/sulfamethoxazole (SXT)	19 (79.2)	2 (100)
Chloramphenicol (CHO)	6 (25)	2 (100)
Erythromycin (ERY)	0 (0)	2 (100)
Azithromycin (AZI)	0 (0)	2 (100)
Meropenem (MRP)	0 (0)	0 (0)
Cephalothin (CEP)	23 (95.8)	2 (100)
Cefuroxime (CXM)	24 (100)	1 (50)
Ceftriaxone (CTR)	23 (95.8)	1 (50)
Cefixime (CFM)	24 (100)	2 (100)
Cefepime (FEP)	0 (0)	0 (0)
Nalidixic acid (NA)	24 (100)	2 (100)
Ciprofloxacin (CIP)	24 (100)	2 (100)
Amikacin (AMK)	0 (0)	1 (50)
Gentamicin (GEN)	0 (0)	1 (50)
Tetracycline (TET)	21 (87.5)	2 (100)
Multidrug resistance (MDR)	19 (79.2)	2 (100)

Resistance to CIP, NA, and CFM was observed in all isolates. High rates of resistance to CEP, CXM, and CTR were observed among the *Campylobacter* isolates, which were 96.2%, 96.2%, and 92.3%, respectively. High levels of resistance to SXT, AMP, and TET were observed among the *Campylobacter* isolates, which were 80.8%, 76.9%, and 88.5%, respectively. While all the *C. jejuni* strains were sensitive to ERY and AZI, both *C. coli* isolates were resistant. Evidence of resistance to MRP and FEP was obtained in *Campylobacter* isolates. In this study almost 80% of the isolates were MDR; *C. jejuni* was resistant to 4-5 classes of antimicrobials, while *C. coli* to 6-7 classes.

Discussion

The consumption of poultry, in particular chicken, is recognized as a major cause of *Campylobacter* infection in humans. Since, chicken meat is more frequently consumed by the Iranian population, the aims of this study was to determine the contamination rate of chicken meat with *Campylobacter*, the AMR pattern, and the virulence-associated genes profile of the isolates in Shiraz, Southwest of Iran. We have found that 28.9% of the chicken meat samples in Shiraz were contaminated with *Campylobacter* species.

The frequency of *Campylobacter*-contaminated chicken meats observed in the present study is lower than those reported in the previous studies (Taremi *et al*., 2006; Rahimi and Ameri, 2011) (45.5% and 43.5%, respectively). It should be noted that this variation in contamination rate between this and other studies in Iran could be related to the time of sampling, that is, during the production of chicken meat from slaughterhouses to consumers. The contamination rate presented in this study is relatively similar to a recent Canadian report where 23.5% of the retail chicken meat was contaminated with *Campylobacter* species (Narvaez-Bravo *et al*., 2017). Although *Campylobacter* was described as the most common cause of human bacterial gastroenteritis worldwide (Barletta *et al*., 2013; Changkwanyeun *et al*., 2015; Cha *et al*., 2016; Facciola *et al*., 2017; Martin *et al*., 2018), this is not the case in Iran where less than 11% of acute diarrhea was caused by *Campylobacter* species (Jafari *et al*., 2008; Jafari *et al*., 2009; Aminshahidi *et al*., 2017). One reason for such a disparity is that Iranian people usually consume completely cooked chicken in their regimen.

Research on the virulence factors of *Campylobacter* in animal source foods is essential for consumer safety. For this reason we have investigated the profile of 7 virulence-associated genes of *Campylobacter* isolates from chicken source, and their possible similarities with *C. jejuni* strains isolated from patients with acute diarrhea in Shiraz (Aminshahidi *et al*., 2017). The results of the present study revealed high prevalence of four virulence-associated genes, including three *cdt* genes and one *cad*F gene in *Campylobacter* isolates regardless of their origin and species. Furthermore, the *vir*B11 gene with plasmid origin was absent in all 33 *Campylobacter* isolates from both sources. The product of *cad*F gene is an adhesion-binding protein that is involved in the process of chicken gut colonization and the invasion process of host cells (Ziprin *et al*., 2001; Monteville *et al*., 2003; Rozynek *et al*., 2005). The combination of *cdt*A, B, C genes and *cad*F virulence-associated genes in all the *Campylobacter* isolates of chicken source indicates that many strains originating from chicken meat could be potentially pathogenic for consumers. Since *cgt*B and *pld*A genes were detected in 100% of the *Campylobacter* isolates from children with diarrhea, in comparison to only 12.5% and 62.5% of the isolates from the chicken samples, we believe that the *Campylobacter* infections in the patients with diarrhea may have sources other than contaminated chicken meat.

Antimicrobial resistance, in particular MDR, is a growing, global, public health problem. These results revealed a high frequency of *Campylobacter* isolates resistance to fluoroquinolones (100%), TETs (88.5%), and cephalosporins (92.3%-100%). The majority of the *Campylobacter* isolates of this study (80.2%) were MDR; this is threatening for the chicken consumers, since poultry is recognized as the primary source of human campylobacteriosis in many industrialized countries (Kittl *et al*., 2011). This resistance could be linked to the extensive use of these antimicrobial agents in poultry production with lower regulations. This hypothesis is supported by the low rate of CIP resistance (37.5%) in isolates from countries with strict antimicrobial controls in animals (Kittl *et al*., 2011). In our study, all *C. jejuni* isolates, which are the predominant *campylobacter* species in human campylobacteriosis, were susceptible to ERY and AZI. Therefore, ERY could still be the drug of choice for empirical therapy before obtaining the results of

antimicrobial susceptibility testing as many other countries (Wieczorek and Osek, 2013; Zhang *et al*., 2016). A similar pattern of AMR to cephalosporins, co-trimoxazole, CIP, NA, TET, macrolides, and aminoglycosides found among human (Aminshahidi *et al*., 2017) and chicken isolates, indicates a possible link between resistance in *Campylobacter* isolates from chicken and human sources.

In conclusion, the results showed that the majority of *Campylobacter* isolates obtained in this study were MDR. Particularly, resistance to CIP and TET should be highlighted, since both are key drugs for the treatment of human campylobacteriosis; therefore, resistance to them presents a potential risk for public health. In addition, the similarities observed between the isolates from chicken and human sources in terms of their AMR and virulence genes in our region could indicate that many *Campylobacter* strains originating from chicken meat could be considered as a potential pathogenic source for consumers.

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Conflict of interest

The authors have no conflicts of interest to declare.

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