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Antimicrobial Resistance and Virulence-Associated Genes of *Campylobacter* spp. Isolated from Raw Milk, Fish, Poultry, and Red Meat

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This study was designed and conducted to evaluate the frequency, antimicrobial resistance, and presence of six virulence-associated genes among thermophilic Campylobacters isolated from raw milk, poultry (chicken, turkey, and duck), fish, cattle, and sheep meat. Out of 590 samples, which were recovered from different origins, 141 (23.9%) samples were positive for Campylobacters. *Campylobacter* spp. was isolated in 40.8% (106/260), 14% (28/200), and 8.7% (7/80) of poultry meat, red meat, and milk samples, respectively. Antimicrobial susceptibility test indicated a high frequency of resistance to ciprofloxacin, tetracycline, and nalidixic acid among the isolates. Furthermore, prevalence of *waaC*, *ciaB*, and *pldA* genes were 91.7%, 86.7%, and 80.8%, respectively; and, none of the isolates harbored both *wlaN* and *cgtB* genes, simultaneously. Moreover, there was a weak correlation between antibiotics resistance and presence of the pathogen genes. However, the existence of *Campylobacter* spp. isolates in food animal products, with high resistance to antibiotics and several virulence gene possessions, is alarming and increases the attention to the widespread use of antibiotics.

Keywords: thermophilic Campylobacters, prevalence, antimicrobial resistance, virulence-associated genes, milk, meat

Introduction

Members of genus *Campylobacter*, a group of gram-negative, spiral-shaped bacteria, are a number of pathogens that can cause gastroenteritis and traveler's diarrhea in humans.[1,2] This group of bacteria, particularly thermophilic *Campylobacter* spp., is one of the most important microorganisms causing foodborne illnesses in developed and developing countries. Among these thermophilic Campylobacters, *Campylobacter jejuni* and *C. coli* are more virulent and can cause different diseases in humans.[3–5] As a zoonotic pathogen, *Campylobacter* spp. can be transmitted to humans through consumption and/or handling of contaminated food, mainly fresh and atmosphere-modified packed meat, raw milk, water, seafood, and vegetables.[1,2,6] Poultry products have been suggested as the most significant sources of *Campylobacter* spp. infections;[1,7,8] on the other hand, cattle and sheep can also carry *C. jejuni* and *C. coli*.[9] Campylobacters can naturally colonize the gastrointestinal tracts of food animals and can contaminate the skin, meat, and meat products of these animals during slaughter and other processing stages in the slaughterhouse.[10] Therefore, implementation and monitoring of control programs at the production level are necessary to avoid a high level of contamination.[1,2]

It is well known that the rise of antimicrobial resistance and multidrug-resistant phenotypes in bacteria is a worldwide concern. The excessive use of antimicrobial drugs in veterinary medicine for controlling diseases or increasing the growth in food animals, and misuse of antibiotics in medicine are related to these resistances.[11,12] Furthermore, due to the use of similar antibiotics in veterinary and human medicine, the multidrug-resistant microorganisms develop and disseminate in humans after the extensive use of antibiotics.[5,13] In addition, resistance to one antimicrobial agent may provide cross-protection against another.[13]

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Selective drugs for human severe campylobacteriosis are erythromycin and ciprofloxacin, which are the members of the macrolide and fluoroquinolone families, respectively. In addition, gentamicin, tetracycline, and ampicillin can be used in medicine for the treatment of systemic *Campylobacter* spp. infections.[14,15] Although five antibiotics (ciprofloxacin, erythromycin, gentamicin, ampicillin, and tetracycline) are used against campylobacteriosis as the most important drugs, other antibiotics like amikacin can be used as an alternative treatment or a synergist drug for the mentioned antibiotics.[2,14]

Resistance to two antibiotics, belonging to a certain family, may indicate genetic characteristics of resistance, which can also affect the rest of those family members in a specific bacteria.[13] So, a study is needed to evaluate the antibiotic resistance of the main *Campylobacter* spp. infection's drugs and other similar antibiotics, which can belong to the same family. The increasing number of antibiotic-resistant *Campylobacter* spp. isolates is a serious public health issue over the last few years.[2,16,17] Therefore, previous research need to be updated annually to obtain new data about these antimicrobial resistances.

Certain virulence-associated genes play an important role in the pathogenesis of *Campylobacter* spp. infections.[18,19] *ciaB* (*Campylobacter* spp.-invasive antigen B),[20] and *pldA* (a gene that encodes an outer membrane phospholipase A)[21] are involved in the invasion and colonization of the host cells. *VirB11*, a plasmid-associated gene, is also associated with host cell invasion.[22] Genes *cgtB*, *wlaN*, and *waaC* are involved in the β-1,3 galactosyltransferase production and biosynthesis of lipooligosaccharide (LOS). Presumably, these later genes are connected to the expression of ganglioside mimics in Guillain-Barre' syndrome, an acute peripheral polyneuropathy, after *C. jejuni* infection.[18,23,24]

In addition, along with the association between virulence and clinical infection, the virulence factors may also be associated with the antimicrobial resistance.[25] Furthermore, it is proven that antimicrobial-resistant *Campylobacter* species can cause severe diarrhea in comparison to antimicrobial-susceptible strains.[26] Thus, it is necessary to investigate the relationships between these two characteristics, resistance and virulence. Although, to the author's knowledge, few, if any, studies have focused on this issue.[25,27] Hence, the occurrences of six virulence-associated genes were studied in this research; then, the association between the virulence factor of the pathogen and antimicrobial resistance was evaluated through statistical analysis.

The microbiological safety of food is an important issue for consumers and industry. In addition, thermophilic Campylobacters, especially *C. jejuni* and *C. coli*, play important roles in most of the human *Campylobacter* spp. infections. So, this study was designed to evaluate the prevalence of thermophilic Campylobacters in raw milk, poultry, fish, and red meat. Furthermore, as it was mentioned above, the level of antimicrobial resistance, virulence-associated genes, and the relationship between them were analyzed in these isolates.

Materials and Methods

Study area and sample collection

A total of 590 samples were collected between July of 2014 and June of 2015 to determine the prevalence of *C. jejuni* and *C. coli* in seven different types of food. The following types of samples were used in this research: (i) chicken meat, including samples from two sites along the processing line in the slaughterhouse, after evisceration (50) and 24-hr postchilling steps (50), 30 breast package samples, and 30 samples from traditional markets; (ii) turkey meat, including breast package (40) and 30 samples from traditional markets; (iii) duck meat, including 30 samples from traditional markets; (iv) 50 samples from freshwater fish (*Salmo trutta*) meat obtained from a traditional fish market; (v) 200 cattle and sheep meat samples obtained from after final wash and 24-hr chilling steps in the slaughterhouse (50 samples per each animal/step); and (vi) 80 samples of milk, including 40 samples from bulk tank milk and 40 samples from filters.

The samples of the chicken, cattle, and sheep carcasses were collected during 20 visits to the main commercial poultry and/or large animal processing plants of northern Iran, located in Mazandaran province, Iran. In addition, the chicken, turkey, duck, and fish (*S. trutta*) meat samples were purchased from supermarkets or traditional markets located in Mazandaran and Golestan provinces, Iran. Furthermore, the raw milk samples (bulk tank milk and the filters) were collected during 20 visits to a farm located in Mazandaran province, Iran. Each milk sample (20 ml) was collected from bulk tank milk using a sterile syringe. The raw milk filters were taken aseptically from the milking lines directly after milking process and placed into sterile whirl-pack bags. Approximately, 25 g of meat samples (with skin except for cattle and sheep) were collected from the breast or thigh of the carcass by using sterile forceps and scalpel. Each sample was tightly sealed in the sterile plastic wrap and carried to the microbiology laboratory in special ice-filled container within 4 hr of sampling.

Isolation and identification of thermophilic Campylobacter

Isolation of *Campylobacter* species was done according to the EN/ISO 10272-1 (2006) standard methodology[28] employing slight modification.[6] Briefly, the meat (25 g) and the filter milk samples were homogenized for 2 min in a stomacher with 225 ml buffered peptone water (Difco) in the sterile plastic bags. Then, 10 ml of the homogenate solution was added to 90 ml of Preston's enrichment broth base (HiMedia Laboratories; M899) supplemented with *Campylobacter* selective supplement IV (HiMedia Laboratories; FD042) and 5% (v/v) defibrinated sheep blood. In addition, each milk sample (20 ml) was added to 80 ml of the same Preston's enrichment broth. Subsequently, all enrichment broths were incubated for 4 h at 37°C followed by 44 h at 42°C in a jar under a microaerophilic atmosphere (Anaerocult C; 5% O_2, 10% CO_2, and 85% N_2).

Thereafter, 100 µl of each enriched sample was streaked onto the Columbia Blood Agar (HiMedia Laboratories), supplemented with the same antibiotics and sheep blood amount. Each plate was incubated at 42°C for 48 hr under the same condition. The suspected colonies with small, gray, drop-like, and shiny properties were picked from each plate and cultured on chocolate agar plates with 5% sheep blood for further investigation. The *Campylobacter* spp. isolates were confirmed by genus-specific polymerase chain reaction (PCR) assay as developed by Linton *et al.*[29]

DNA extraction and PCR analysis

Bacterial DNA was extracted from fresh *Campylobacter* spp. isolates using the phenol–chloroform technique as previously described by Khoshbakht *et al.*[8] Spectrophotometry was used at 260 and 280 nm to determine the purity and concentration of the DNA (Nanodrop 1000; Thermo Scientific). Genus and multiplex species-specific PCR reactions were performed for identification of the genus of *Campylobacter*, *C. jejuni*, and *C. coli* species, using primers targeted on *16SrRNA*, *mapA*, and *ceuE* genes, respectively,[29–31] which are shown in Table 1. After the confirmation of isolates, the PCR assay was done to detect the six virulence factors using the primers listed in Table 1.

The PCR amplifications were performed in a final volume of 25 μl. Each PCR reaction mixture consisted of 2 μl of the DNA template, 2.5 μl 10× PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, and 20 mM $[NH_4]_2SO_4$), 1 μl dNTPs (50 mM), 1 μl (1 U) Ampli *Taq* DNA polymerase, and 1 μl (25 pmol) from the forward and reverse primers, which are shown in Table 1. All materials were purchased from CinnaGen. The final volume of each reaction mixture was increased to 25 μl using distilled deionized water. The thermal cycler (MJ mini; BioRad) was adjusted under the following conditions: the initial denaturation step at 94°C for 5 min, followed by 35 amplification cycles of the denaturation step at 94°C for 1 min, the annealing step as shown in Table 1 for 1 min, and the extension step at 72°C for 1 min. The final extension step was carried out at 72°C for 10 min.

The amplified products were separated using electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml; CinnaGen). The DNA bands were photographed using an ultraviolet transilluminator (BTS-20), and the 100-bp DNA ladder (CinnaGen) was used as a molecular size marker. The *C. jejuni* RTCC 1097 and *C. coli* RTCC 1113 strains were included as PCR-positive controls. The sterile PCR water was used as a negative control.

Antimicrobial susceptibility testing

The *Campylobacter* spp. isolates were examined for antimicrobial susceptibility by employing the Kirby–Bauer disk diffusion method. The inhibition zone was determined with calipers and interpreted according to the Clinical and Laboratory Standards Institute (CLSI). In the cases when CLSI recommendations were not available for Campylobacters, the CLSI guidelines for Enterobacteriaceae were followed.[32] Ten antibiotic discs (Paramedical) were used in this research. The discs and their concentrations consisted of ciprofloxacin (5 μg), enrofloxacin (5 μg), ampicillin (10 μg), erythromycin (15 μg), gentamicin (10 μg), streptomycin (25 μg), tetracycline (30 μg), nalidixic acid (30 μg), amikacin (30 μg), and amoxicillin (20 μg). Each overnight culture of *Campylobacter* spp. isolate was suspended in sterile normal saline and adjusted to a turbidity of 0.5 McFarland standard. Each suspension was inoculated with a sterile swab on the entire surface of a 150 mm diameter Mueller-Hinton agar plate (Oxoid Ltd.) supplemented with 5% sheep blood. The agar surface of each plate was allowed to dry for 3 min. The antimicrobial discs were aseptically applied on the plates. After incubation at 42°C for 48 hr under the microaerophilic atmosphere, the inhibition zones were measured. *Staphylococcus aureus* ATCC 12600, *Escherichia coli* RTCC 1161, and *C. jejuni* RTCC 1097 were used as quality control strains.

Statistical analysis

The results were analyzed using the SPSS software, version 16.1 (SPSS, Inc.). The Pearson chi-square and Fisher's exact two-tailed tests were used to assess the following items: the association between the different isolation rates of two *Campylobacter* species and type of food samples, the proportions of isolates resistant to different antimicrobial agents, and the prevalence of the virulence factors in isolates with various origins. p Value was lower than 0.05

Table 1. Nucleotide Sequences Used as Primers in the Polymerase Chain Reactions for Identification of *Campylobacter* Genus, Species, and Their Virulence Genes

Target gene	*Sequence (5′ to 3′)*	*Annealing temperature (°C)*	*Product size (bp)*	*Reference*
16SrRNA	F: ATCTAATGGCTTAACCATTAAAC R: GGACGGTAACTAGTTTAGTATT	59	857	29
mapA	F: CTATTTTATTTTTGAGTGCTTGTG R: GCTTTATTTGCCATTTGTTTTATTA	52	589	31
ceuE	F: AATTGAAAATTGCTCCAACTATG R: TGATTTTATTATTTGTAGCAGCG	52	462	30
pldA	F: AAGCTTATGCGTTTTT R: TATAAGGCTTTCTTCA	45	913	18
ciaB	F: CAGAAGGAGAAATTTGTGAGC R: ATATCCCATTCTAATGCCACC	58	355	44
cgtB	F: TTAAGAGCAAGATATGAAGGTG R: GCACATAGAGAACGCTACAA	56	562	24
wlaN	F: TGCTGGGTATACAAAGGTTGTG R: AATTTTGGATATGGGTGGGG	56	330	47
waaC	F: TAATGAAAATAGCAATTGTTCGT R: GATACAAAAATCACTTTTATCGA	42	1,029	48
VirB11	F: TCTTGTGAGTTGCCTTACCCCTTTT R: CCTGCGTGTCCTGTGTTATTTACCC	53	494	18

F, forward; R, reverse.

indicating the significant statistic. The logistic regression analysis was also used to study the effect of an antibiotic resistance on another antibiotic resistance and/or the effect of the existence of a virulence gene on other genes. The relationships between antibiotic resistance and virulence genes were also analyzed using the Cramer's V method.

Results

Distribution of Campylobacter *spp.*

Overall, 141 (23.9%) *Campylobacter* spp. were isolated from 590 samples. All types of food origins revealed *Campylobacter* spp. contamination, except for the fish samples. The isolates of *Campylobacter* spp. were detected in 40.8% (106/260) of the poultry meat, which was statistically higher than the isolation rate of *Campylobacter* spp. in the red meat (14%) and milk (8.7%) samples ($p < 0.001$). A summary of the *Campylobacter* spp. prevalence is shown in Table 2. Overall, among 141 isolates of *Campylobacter* spp., the detection rate of *C. jejuni* (56%) was higher than *C. coli* (29.1%), with regard to PCR analysis. In addition, 14.9% of the isolates were other species. Although the isolation rate of *C. coli* was higher than the isolation rate of *C. jejuni* in the sheep and turkey samples, the *C. jejuni* was significantly detected more than the *C. coli* ($p = 0.001$) in others.

In the chicken samples, those which were obtained from the traditional market had the highest prevalence of *Campylobacter* spp. (70%), followed by the after evisceration step in the slaughterhouse (62%), the breast package (40%), and the 24-hr after chilling step in the slaughterhouse (24%) samples. Furthermore, in the turkey samples, the isolation rate of the *Campylobacter* spp. was higher in the traditional market samples (36.6%) than in the breast package samples (22.5%). Among the red meat samples, the contamination of meat with *Campylobacter* spp. in the samples of after 24-hr chilling at 4°C step was lower than the samples of after final wash step obtained from the slaughterhouse. Pearson chi-square test highlighted the efficacy of the chilling step in decreasing the rate of *Campylobacter* spp. infection in all the slaughterhouse samples ($p < 0.001$). In addition, the samples that originated from the traditional market had more *Campylobacter* spp. contamination.

Antimicrobial susceptibility testing

C. jejuni and *C. coli* isolates were only tested through the Kirby–Bauer disk diffusion assay and all of these isolates (79 *C. jejuni* and 41 *C. coli*) were resistant to one or more than one antimicrobial agent. The result of the antimicrobial resistance of the isolates is summarized in Table 3. Overall, five isolates (4.2%) were resistant to only one of the antimicrobial agents, and 17 isolates (14.2%) showed resistance to two antimicrobial agents. Multidrug-resistant (MDR) isolates, which were defined as a resistance to three or more than three drugs, were found in 98 *Campylobacter* spp. isolates (81.6%). On the other hand, the occurrence of MDR isolates in *C. coli* isolates (90.2%) was generally more than *C. jejuni* isolates (77.2%). Resistance to ciprofloxacin was the most common of all (82.5%), followed by tetracycline (79.2%) and nalidixic acid (75.8%). The lowest antimicrobial resistance (2.5%) was observed for gentamicin.

A separate evaluation of the differences in resistance to an antibiotic in different isolation sources revealed no statistically significant difference ($p > 0.05$) except for the isolates obtained from sheep. Resistance to streptomycin in these sheep isolates was significantly higher than resistance to streptomycin in other isolates ($p < 0.001$). There was no significant difference in the rate of antimicrobial resistance between *C. coli* and *C. jejuni* isolates ($p > 0.05$). The logistic regression showed a statistical correlation between

Table 2. Prevalence of *Campylobacter* Species in the Various Food Samples

Biological origin	*Source/number of samples*	Campylobacter *species (%)*	Campylobacter jejuni *(%)*	Campylobacter coli *(%)*	*Other* Campylobacter *species (%)*
Chicken	After evisceration/50	31 (62)	19 (61.3)	7 (22.5)	5 (16.2)
	24 hr after chilling/50	12 (24)	6 (50)	3 (25)	3 (25)
	Breast package/30	12 (40)	8 (66.6)	1 (8.4)	3 (25)
	Traditional market/30	21 (70)	12 (57.1)	5 (23.9)	4 (19)
	Total sample of chicken/160	76 (47.5)	45 (59.2)	16 (21.1)	15 (19.7)
Turkey	Breast package/40	9 (22.5)	4 (44.4)	4 (44.4)	1 (11.2)
	Traditional market/30	11 (36.6)	5 (45.4)	6 (54.6)	0 (0)
	Total sample of turkey/70	20 (28.6)	9 (45)	10 (50)	1 (5)
Duck	Traditional market/30	10 (33.3)	7 (70)	2 (20)	1 (10)
Fish	Traditional market/30	0 (0)	—	—	—
Cattle	After final wash/50	8 (16)	4 (50)	3 (37.5)	1 (12.5)
	After 24-hr chilling at 4°C/50	3 (6)	2 (66.6)	0 (0)	1 (33.4)
	Total sample of cattle/100	11 (11)	6 (54.6)	3 (27.3)	2 (18.1)
Sheep	After final wash/50	11 (22)	5 (45.4)	6 (54.6)	0 (0)
	After 24-hr chilling at 4°C/50	6 (12)	2 (33.3)	2 (33.3)	2 (33.3)
	Total sample of sheep/100	17 (17)	7 (41.2)	8 (47.1)	2 (11.7)
Milk	Bulk tank/40	2 (5)	2 (100)	0 (0)	0 (0)
	Filter/40	5 (12.5)	3 (60)	2 (40)	0 (0)
	Total sample of raw milk/80	7 (8.8)	5 (71.4)	2 (28.6)	0 (0)
Total	Total samples/590	141 (23.9)	79 (56)	41 (29.1)	21 (14.9)

TABLE 3. PERCENTAGE OF *CAMPYLOBACTER* SPP. ISOLATES RESISTANT TO VARIOUS ANTIMICROBIAL AGENTS

Species	*Biological origin*	*No. of isolates*	*Prevalence of resistance to antimicrobial agents (%)*								
			Ciprofloxacin	*Enrofloxacin*	*Ampicillin*	*Erythromycin*	*Gentamicin*	*Streptomycin*	*Tetracycline*	*Nalidixic acid*	*Amikacin*	*Amoxicillin*
C. jejuni	Chicken	45	35 (77.8)	14 (31.1)	12 (26.7)	3 (6.7)	1 (2.2)	6 (13.3)	34 (75.5)	33 (73.3)	2 (4.4)	15 (33.3)
	Turkey	9	8 (88.9)	4 (44.4)	4 (44.4)	0 (0)	0 (0)	0 (0)	7 (77.8)	6 (66.7)	0 (0)	3 (33.3)
	Duck	7	6 (85.7)	3 (42.8)	2 (28.5)	1 (14.2)	0 (0)	1 (14.2)	5 (71.4)	5 (71.4)	1 (14.2)	3 (42.8)
	Cattle	6	5 (83.3)	3 (50)	3 (50)	0 (0)	0 (0)	0 (0)	4 (66.7)	6 (100)	0 (0)	1 (16.7)
	Sheep	7	6 (85.7)	3 (42.8)	2 (28.6)	1 (14.3)	0 (0)	5 (71.4)	6 (85.7)	6 (85.7)	1 (14.3)	1 (14.3)
	Milk	5	3 (60)	1 (20)	3 (60)	0 (0)	0 (0)	3 (60)	3 (60)	3 (60)	0 (0)	2 (40)
	Total	79	63 (79.7)	28 (35.4)	26 (32.9)	5 (6.3)	1 (1.3)	15 (19)	59 (74.7)	59 (74.7)	4 (5.1)	40 (33.3)
C. coli	Chicken	16	13 (81.2)	5 (31.2)	7 (43.7)	1 (6.2)	1 (6.2)	3 (18.7)	14 (87.5)	13 (81.2)	2 (12.5)	7 (43.7)
	Turkey	10	10 (100)	4 (40)	4 (40)	0 (0)	0 (0)	2 (20)	9 (90)	7 (70)	0 (0)	3 (30)
	Duck	2	2 (100)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	2 (100)	2 (100)	0 (0)	1 (50)
	Cattle	3	3 (100)	2 (66.7)	1 (33.3)	0 (0)	0 (0)	0 (0)	2 (66.7)	2 (66.7)	0 (0)	1 (33.3)
	Sheep	8	6 (75)	3 (37.5)	4 (50)	1 (12.5)	0 (0)	7 (87.5)	8 (100)	6 (75)	0 (0)	3 (37.5)
	Milk	2	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)	2 (100)	1 (50)	1 (50)	1 (50)	0 (0)
	Total	41	36 (87.8)	16 (39)	17 (41.5)	2 (4.9)	2 (4.9)	15 (36.6)	36 (87.9)	32 (78)	3 (7.3)	15 (36.6)
Total		120	99 (82.5)	44 (36.7)	43 (35.8)	7 (5.8)	3 (2.5)	30 (25)	95 (76.2)	91 (75.8)	7 (5.8)	40 (33.3)

resistances to some antibiotics. Increase in resistance to amoxicillin, tetracycline, streptomycin, and gentamicin lead to an increase in resistance to ampicillin ($p = 0.0001$), nalidixic acid ($p = 0.007$), amikacin ($p = 0.024$), and amikacin ($p = 0.036$), respectively.

Detection of the virulence genes

The prevalence of the six putative virulence genes in two thermophilic *Campylobacter* species is shown in Table 4. Among 120 *Campylobacter* spp. isolates, including 79 *C. jejuni* and 41 *C. coli*, the prevalence of *waaC*, *ciaB*, and *pldA* genes was 91.7%, 86.7%, and 80.8%, respectively. In addition, lower rates of occurrence were found for *cgtB* (45%) and *wlaN* (26.7%) markers. The plasmid-associated virulence marker, *virB11* gene, was not detected in isolates. *waaC* was the most prevalent gene in *C. jejuni* (100%) followed by *ciaB* gene (91.1%), whereas in *C. coli*, the most prevalent gene was *ciaB* gene (78%) followed by *waaC* gene (75.6%). The occurrence of *wlaN* and *cgtB* genes, involved in LOS synthesis, varied in two thermophilic *Campylobacter* isolates. *wlaN* and *cgtB* genes were not observed in the same isolate, simultaneously. The logistic regression showed that the presence of the *pldA* gene can decrease the presence of the *cgtB* gene ($p = 0.034$).

No strong correlation was observed between resistance to antibiotics and the presence of the virulence genes surveyed in this research, using the Cramer's V analysis. Only, weak relationships were observed between the tetracycline and *pldA* gene (0.18), streptomycin and *pldA* gene (0.2), ampicillin and *cgtB* gene (0.19), and enrofloxacin and *wlaN* gene (0.17).

Discussion

The results of this study indicated that the poultry meat, especially the chicken meat, is the main source of *Campylobacter* species followed by red meat and raw milk samples. In general, these results were consistent with the findings of previous studies on poultry meat, red meat, and raw milk. Panzenhagen *et al.* isolated *Campylobacter* spp. in 45% of the poultry carcasses in Brazil.[33] In addition, Kashoma *et al.* showed the contamination of *Campylobacter* spp. in 9.5% of beef carcasses in Tanzania.[15] Furthermore, a low rate of contamination (2.91%) was also reported in milk samples.[34] However, many studies suggested that there are variations in the *Campylobacter* spp. contamination rates among countries. Economou *et al.* isolated *Campylobacter* spp. in 29% of chicken meat samples in Greece,[35] whereas Wei *et al.* observed a high frequency of *Campylobacter* spp. contamination (80.1%) in South Korea.[5] Jamali *et al.*, and Zendehbad *et al.* suggested that these differences could be due to the variations in geographical and seasonal factors, type and number of the samples, isolation methods, transport conditions, and different sanitary conditions on farms and slaughterhouses[36,37]; therefore, finding a direct correlation among various studies might be difficult. No *Campylobacter* spp. contamination was detected in the fish samples in this study. This result might be due to the temperature of the water that is not favorable for thermophilic Campylobacters.

The results showed that the poultry meat obtained from the traditional markets frequently was much contaminated

Table 4. Prevalence of the Virulence-Associated Genes in the *Campylobacter* spp. Isolates

Species	*Biological origin*	*No. of isolates (%)*	*Prevalence of virulence genes (%)*					
			virB11	pldA	ciaB	cgtB	wlaN	waaC
C. jejuni	Chicken	45	0 (0)	44 (97.8)	45 (100)	16 (35.6)	14 (31.1)	45 (100)
	Turkey	9	0 (0)	7 (77.8)	7 (77.8)	5 (55.6)	2 (22.2)	9 (100)
	Duck	7	0 (0)	7 (100)	7 (100)	2 (28.6)	3 (42.8)	7 (100)
	Cattle	6	0 (0)	3 (50)	4 (66.7)	5 (83.3)	1 (16.7)	6 (100)
	Sheep	7	0 (0)	5 (71.4)	5 (71.4)	6 (85.7)	1 (14.3)	7 (100)
	Milk	5	0 (0)	2 (40)	4 (80)	3 (60)	0 (0)	5 (100)
	Total	79	0 (0)	68 (86.1)	72 (91.1)	35 (44.3)	21 (26.6)	79 (100)
C. coli	Chicken	16	0 (0)	14 (87.5)	14 (87.5)	6 (37.5)	6 (37.5)	12 (75)
	Turkey	10	0 (0)	9 (90)	9 (90)	3 (30)	1 (10)	8 (80)
	Duck	2	0 (0)	1 (50)	2 (100)	1 (50)	1 (50)	1 (50)
	Cattle	3	0 (0)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)	3 (100)
	Sheep	8	0 (0)	3 (37.5)	4 (50)	6 (75)	2 (25)	6 (75)
	Milk	2	0 (0)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)
	Total	41	0 (0)	29 (70.7)	32 (78)	19 (46.3)	11 (26.8)	31 (75.6)
Total		120	0 (0)	97 (80.8)	104 (86.7)	54 (45)	32 (26.7)	110 (91.7)

with Campylobacters than the meat package samples. It should also be noted that poultry meat in the traditional market can become more contaminated during the manual slaughter of poultry–more commonly during the evisceration stage–as the carcasses of the poultry are polluted with intestinal microorganisms. In addition, the water, which is used in these types of markets, is almost unhygienic water and Campylobacters can easily be circulated in this environment and spread on the surface of carcasses.[10,38] Using the chlorinated water, increasing health condition, and decreasing the temperature (freezing and chilling) can significantly reduce carcass contamination. These factors are more observed in the large-scale slaughterhouse.[38,39]

In addition, as it was expected, the *C. jejuni* was the predominant species in this research and this was consistent with several studies.[33,37,39] However, an increased occurrence of the *C. coli* was observed in the sheep isolates in this research. This difference could be due to different husbandry systems. Most sheep herds are grazing in the pasture and this type of husbandry system increases exposure to multiple sources of contamination.[40] In this case, the environmental sources like natural water ponds can be polluted with swine and boar wastes and slurries. *C. coli* is one of the most common *Campylobacter* species that was found in these animals,[40] hence, it can be concluded that pasture grazing increases the *C. coli* contamination in sheep. Further ecosystem studies are required to determine the reason for this result.

These results revealed a high frequency of *Campylobacter* spp. resistance to ciprofloxacin, tetracycline, and nalidixic acid. These high levels of resistances have also been reported in other studies, especially over the last few years.[1,33,36,37] Because of an extensive use of these antibiotics for treatment, control and prevention of diseases at the farm, the selection, and development of antimicrobial-resistant Campylobacters can occur.[5,36] Furthermore, some studies have suggested that the mutation in *gyrA*, a gene that encodes the DNA gyrase subunit A, is a reason for fluoroquinolones and nalidixic acid resistance. The widespread use of these antibiotics can also increase the rate of *gyrA* mutation in the Campylobacters.[41]

Campylobacter spp. can produce beta-lactamases by $bla_{OXA\text{-}61}$, $bla_{OXA\text{-}184}$, and $bla_{OXA\text{-}193}$. In addition, they can use two major efflux systems, known as the CmeABC and CmeDEF efflux pump, so they can be resistant to penicillin, amoxicillin, and ampicillin.[15,42,43] The moderate resistance to amoxicillin and ampicillin was observed in this research, which reflects this point.

The results showed a higher rate of resistance to streptomycin in isolates obtained from sheep compared to other isolates ($p < 0.001$). Streptomycin in combination with penicillin (like Pen-strep) is one of the most important drug choices for treatment of pneumonia and other diseases in animals and it is widely used In Iran, especially in the sheep industry. So, the Campylobacters with the property of resistance to penicillin can also be resistant to streptomycin and, thereby, can be selected and spread in the sheep isolates.

Because these isolates can circulate throughout different food animals and finally is transferred to humans, high resistance to different groups of antibiotics in *Campylobacter* spp. isolates is alarming and important. In addition, these antibiotics, for example, ciprofloxacin, are considered the drugs of choice for treating human campylobacteriosis; hence, the increased resistance of such strains poses a public health problem.[15] Low resistances to gentamicin, amikacin, and erythromycin were observed in this study. The low resistance to these antibiotics could be due to the fact that these antibiotics are rarely used in Iran.[37]

This study indicated the high level of MDR in *Campylobacter* spp. isolates. Furthermore, the presence of MDR isolates was more detected in the *C. coli* isolates than the *C. jejuni* isolates. Other authors have also found the same finding and suggested that the *C. coli* strains could acquire horizontal resistance genes better than the *C. jejuni* isolates and/or those target genes could mutate faster in the *C. coli* isolates than in the *C. jejuni* strains.[4,36,37] However, there are a number of reasons related to the mechanism of MDR in Campylobacters. The presence of a major efflux system, as mentioned above, is responsible for resistance to a broad range of antibiotics. Destruction or inactivation of antibiotics (by enzymes encoded by chromosomal or plasmid

genes) and low-level access of antimicrobial agents to their targets are the other reasons.[42]

The results of this research showed a correlation between isolates and resistance to two antibiotics. Whenever an isolate was resistant to an antibiotic (like amoxicillin), the chance of resistance to another antibiotic from the same family (like ampicillin) was higher. It is thus evident that more antibiotic resistance mechanisms are common in a family of antibiotics. For example, if one isolate of *C. jejuni* can produce class D β-lactamase, it can be resistant to amoxicillin and ampicillin, simultaneously.[40] It should be mentioned that further experimental evidence is needed to support this conclusion. Decreasing and selecting a better combination of antibiotics for treatment and growth factor in the food-producing animals can be key points for controlling the important issue of antibiotic resistance in the world.[1]

Concerning the safety of consumers and the importance of health control program of campylobacteriosis, it is necessary to investigate the virulence properties of *Campylobacter* spp. as potentially pathogenic bacteria.[8] The high occurrence of *pldA* and *ciaB* genes was observed in this study. Similar to the results of this research, Feodoroff *et al.* detected the *pldA* and *ciaB* genes in 61% and 98% of isolates.[44] Moreover, Datta *et al.* reported a high prevalence of *pldA* gene (88%–100%) in isolates obtained from the broilers.[45] In an earlier study in Iran, Khoshbakht *et al.* detected 91.7% of positive isolates for the *pldA* gene in isolates from poultry feces.[8] However, in contrast to this approach, a low frequency of *pldA* gene was observed in a study on sheep and cattle in Iran.[9] In agreement with the results of other studies,[8,9,46] the plasmid-associated virulence marker, *virB11* gene, was not present in any of the isolates in this research. In some other studies, this gene was identified in a very low rate of the tested isolates.[18,19] The nature of the virB11 plasmid and geographical differences may be the reason.[8]

The three LOS-associated genes, *wlaN*, *cgtB*, and *waaC*, were also examined in this study. The high frequency of *waaC* (91.7%) was found in *Campylobacter* isolates. In addition, a moderate prevalence of *wlaN* and *cgtB* genes was observed. *wlaN* and *cgtB* genes produce β-1, 3 galactosyltransferase and along with *waaC* gene are connected with the Guillain-Barre' and Miller-Fischer syndromes.[23,24] *wlaN* and *cgtB* genes were not observed in isolates, simultaneously. Müller *et al.* discussed the same results.[46] They inversely demonstrated that the presence of *cgtB* and *wlaN* in the *Campylobacter* spp. could be due to a specific function of β-1, 3 galactosyltransferases during colonization and invasion processes. However, further *in vitro* and *in vivo* investigations are required to prove this theory and elucidate the role of virulence genes in the pathogenesis of *Campylobacter* spp. infections.

The results obtained from this research showed no correlation between the prevalence of the virulence genes and the *Campylobacter* species. In addition, using the Cramer's V analysis, no strong correlation was observed between resistance to antibiotics and the presence of virulence genes, which was similar to the results of other studies. Ghunaim *et al.* detected some interactions between *ciaB* gene and the expression of antimicrobial resistance to ciprofloxacin and erythromycin.[25] However, they also observed no significant correlation between them. Anyway, further genetic investigations are needed to prove the relationship between virulence factors and resistance to antimicrobial agents.

In conclusion, the results indicated that the apparently healthy food animals, and especially poultry, are potential sources for human campylobacteriosis. In addition, the results showed that the *Campylobacter* spp. contamination in the fish products is rare. However, the majority of the *Campylobacter* spp. isolates obtained in this study were MDR and had putative virulence genes. These data are alarming and raise public health concern, and increase the attention to the widespread use of antibiotics. Hence, to decrease contamination of meat, it is necessary to apply good hygienic standards and food safety assurance programs in the entire slaughtering process and milk bulk centers. Also, effective criteria should be implemented to reduce using antibiotics in the veterinary industry.

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