Isolation, Antimicrobial Resistance, and Virulence Genes of Thermophilic Campylobacter Species from Backyard Ducks in Amol, Northern Iran

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ABSTRACT

Domestic poultry are considered natural reservoirs for the transmission of *Campylobacter* spp., mainly *C. jejun*i and *C. coli*, to other birds and humans. This study aimed to determine the Campylobacter infection status in backyard ducks in Iran. A total of 100 cloacal swabs were obtained from apparently healthy backyard ducks in different rural areas of Amol, a city in northern Iran. Bacterial isolation was based on traditional culture procedures, and genus and species identification were performed using an mPCR. All isolates were examined for antimicrobial resistance to seven antibiotics by Kirby Bauer's disk diffusion test. The virulence-associated genes *cadF, iamA, pldA, cdtA, cdtB, cdtC,* and *wlaN* were detected as well. Out of the 27 *Campylobacter* isolates recovered, 19 (70.4%) were *C. coli*, and 3 (11.1%) were C. jejuni. The remaining five isolates (18.5%) were not identified. All (100%) isolates showed resistance to ciprofloxacin. Most isolates were resistant to ampicillin, tetracycline, and nalidixic acid. The resistance rate to amoxicillin-clavulanic acid and erythromycin was moderate but was relatively low to gentamicin. Moreover, over two-thirds of the isolates were MDR. All virulence genes, except *iamA*, were variably detected. The *cadF* and *pldA* genes had the highest (92.6%) and lowest (7.4%) positivity rates, respectively. In addition, a statistically significant association was observed between *Campylobacter* spp. and most of the critical virulence genes ($p < 0.05$). Our findings imply that backyard ducks should be paid attention to as a major source of human campylobacteriosis.

Abbreviations

FQs: fluoroquinolones
CDT: cytolethal distending toxin
LOSSIAL: sialylated lipo-oligosaccharide
AMR: antimicrobial resistance

Introduction

Campylobacter species are generally considered a component of the normal gut flora of poultry [1]. Many species of domestic poultry and wild birds may be infected with thermophilic *Campylobacter* spp., mainly *C. jejuni* followed by *C. coli* and rarely *C. lari* [1, 2]. Ducks could be also a reservoir of Campylobacter spp. [3]. Recently, high rates of *Campylobacter* infection have been reported in domestic duck flocks in South Korea and Malaysia [4, 5]. In commercial poultry flocks, *Campylobacter* is not found in the first 2-3 weeks of age. This initial lag phase is probably related to maternal immunity [2]. Horizontal transmission from the environment, including contaminated water, feed, fomites, wild birds, other farm animals, rodents, and insects, is the major source of *Campylobacter* colonization. Vertical transmission of *Campylobacter* is unlikely, and the eggs are not contaminated [1, 2]. Despite extensive colonization in the cecum, colon, and cloaca (up to 109 colony-forming units/g feces), Campylobacter infections produce mild or no clinical diseases in poultry [1-3].

Food-borne bacterial pathogens are the most important etiologic agents of human gastroenteritis in the United States of America and worldwide [6]. *Campylobacter* causes more than 800 000 food-borne illnesses and 8000 hospitalizations in the USA each year [2]. The majority (50%-80%) of human campylobacteriosis cases occur through the ingestion of contaminated poultry products [7]. In a study in the United Kingdom, 50.7% of duck meat samples were infected with *Campylobacter*, which was comparable to chicken meat contamination (60.9%) [8]. Human infections are usually recognized by fever, diarrhea (watery/bloody), nausea, and abdominal pain after an incubation period of 2-5 days [1, 2]. In addition, GBS/ acute neuromuscular paralysis may occur as a post-infection disease in 0.1% of the infected individuals and eventually causes respiratory compromise and death [1]. Usually, *Campylobacter* enteritis is a self-limiting infection, but antimicrobial therapy is needed in severe cases or immune-compromised patients [9]. Fluoroquinolones (e.g., ciprofloxacin) and macrolide antibiotics (e.g., azithromycin and erythromycin) are the appropriate medications. Tetracycline and gentamicin are occasionally used as alternative agents to treat systemic infection in humans [6]. Today, these antibiotics are utilized in food animals as a growth promotor or a therapeutic medicine. In recent years, an increase in drug-resistant *Campylobacter* isolates, particularly to FQs, has been observed in poultry, which poses a threat to public health [9]. Moreover, the Centers for Disease Control and Prevention classified antimicrobial-resistant *Campylobacter* strains under "microorganisms with a threat level of serious" and estimated that the resistance rate to FQs, macrolides, and tetracyclines among *Campylobacter* isolates is 22%, 2%, and 49%, respectively, in the U.S. annually [6, 9].

The pathogenesis of campylobacteriosis is not well understood. However, some of the putative virulence genes of Campylobacter which are associated with adhesion, colonization, invasion, and toxin production and are needed to induce infection have been investigated [7]. The *cadF* (*Campylobacter* adhesion to fibronectin) gene is responsible for adhesion and colonization. The pldA (Phospholipase A) and invasion-associated marker iam genes are involved in invasion [1, 7]. Among several different cytotoxins in *Campylobacter*, CDT, a tripartite toxin, which is encoded by three related genes, namely *cdtA*, *cdtB,* and *cdtC*, has been characterized in detail. Two heterodimeric subunits CdtA and CdtC are responsible for holotoxin binding to the cell membrane, and *CdtB* is an enzymatically active subunit [7]. The *wlaN* gene encodes the β-1,3-galactosyltransferase enzyme that is responsible for sialylated lipo-oligosaccharide (LOSSIAL) production, an essential pathogenic factor of GBS [10].

Duck rearing is a main part of poultry production in some countries of the world, such as China, France, South Korea, and Malaysia [4, 11, 12]. As a result, the highest consumption of duck meat (over 80%) has been reported in Asian countries [12]. In Iran, this industry has also been thriving and providing a part of human needs. As mentioned above, extensive research has been conducted on *Campylobacter* spp. infection in chickens, but the relationship between ducks and food-borne pathogens has been poorly investigated [11]. Therefore, the current study was performed to determine the infection status of Iranian backyard ducks to thermophilic campylobacters, and also the antibiotic resistance and virulence genes of the obtained isolates.

Result

Infection rate

The results of mPCR are presented in Figure 1. In addition, the geographic distribution of thermophilic *Campylobacter* spp. in different backyard flocks is shown in Table 1. Out of the 28 backyard duck flocks examined, 17 (60.7%) were positive for thermophilic

Abbreviations-Cont'd

MDR: multidrug-resistant
rs: Spearman's rank correlation coefficient
mPCR: multiplex polymerase chain reaction
ATCC: American-type culture collection
CLSI: Clinical and Laboratory Standards Institute
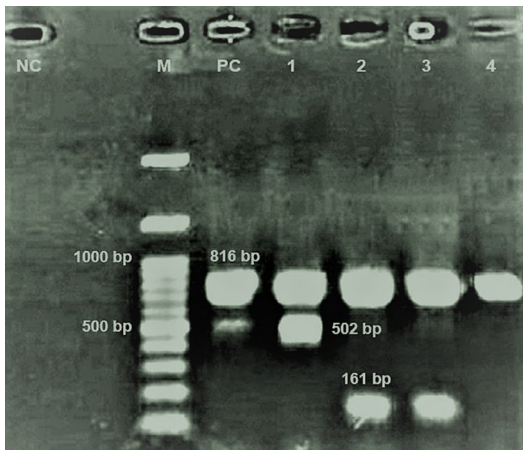GBS: Guillain-Barre syndrome

Thermophilic *Campylobacter* **species in backyard ducks**

Figure 1.
Multiplex PCR assay for the identity of the *16S rRNA* gene (816 bp) for *Campylobacter* genus, the cj0414 gene (161 bp) for *C. jejuni,* and the ask gene (502 bp) for *C. coli*. Lane NC: Negative control (deionized water), Lane M: 100-bp DNA ladder, Lane PC: Positive control (*Campylobacter coli* ATCC 43478), Lane 1: *C. coli* isolate, Lane 2: a 161-bp amplified fragment of the *cj0414* gene was sequenced and confirmed as *C. jejuni* isolate, Lane 3: *C. jejuni* isolate, and Lane 4: *Campylobacter* spp. isolate (unidentified).

Table 1.
Regional distribution of thermophilic *Campylobacter* species isolated from backyard ducks in Amol villages.

Rural region	No. of flocks sampled	No. of positive flocks (%)	Isolated Campylobacter
North	11	2 (18.2)	*Campylobacter coli*
West	7	5 (71.4)	*C. coli and* other spp.
South	4	4 (100.0)	*C. coli and* other spp.
East	6	6 (100.0)	*C. coli and C. jejuni*
Total	28	17 (60.7)	*C. coli, C. jejuni, and* other spp.

Campylobacter species. Moreover, out of 100 cloacal samples tested for *Campylobacter* spp., 27 (27%) were infected. The majority of the isolates (19/27, 70.4%) were *C. coli*, while only three isolates (11.1%) were *C. jejuni*. The remaining five (18.5%) isolates were thermophilic *Campylobacter* spp., but have not been identified. None of the swab samples had mixed *Campylobacter* infection. In the present study, *C. lari* was not detected.

Antimicrobial resistance

All 27 *Campylobacter* isolates were examined for resistance to seven antibiotics belonging to five antibiotic classes. As shown in Table 2, all Campylobacter isolates were resistant to ciprofloxacin (100%). Moreover, most strains exhibited resistance to ampicillin (81.5%), tetracycline (77.8%), and nalidixic acid (74.1%). Resistance to amoxicillin/clavulanic acid and erythromycin was moderate at 51.9% and 44.4%, respectively, whereas resistance to gentamicin was relatively low (25.9%). Moreover, no statistically significant association was found between *Campylobacter* spp. and resistance to tested antibiotics (Table 2). Thirteen AMR patterns were observed in Campylobacter isolates, eight of which were MDR, and 19 out of 27 Campylobacter isolates (70.4%) were found to be MDR (Table 3).

Virulence genes

The results of PCR are presented in Figure 2. In

Table 2.
Antimicrobial resistance rate of *Campylobacter spp*. in backyard ducks.

Antimicrobial class	Anti microbial	No. of resistant Campylobacter isolates (%)			Total (n=27)	*p*-Value
		C. coli (n=19)	*C. jejuni* (n=3)	Other spp. (n=5)	No. (%)	
Fluoroquinolones	CIP	19 (100.0)	3 (100.0)	5 (100.0)	27 (100.0)	*NC*
	NAL	15 (78.9)	1 (33.3)	4 (80.0)	20 (74.1)	0.297[ns]
Macrolides	ERY	7 (36.8)	1 (33.3)	4 (80.0)	12 (44.4)	0.227[ns]
Tetracyclines	TET	15 (78.9)	1 (33.3)	5 (100.0)	21 (77.8)	0.119[ns]
β-Lactams	AMP	17 (89.5)	1 (33.3)	4 (80.0)	22 (81.5)	0.072[ns]
	AMC	10 (52.6)	1 (33.3)	3 (60.0)	14 (51.9)	1.000[ns]
Aminoglycosides	GEN	3 (15.8)	1 (33.3)	3 (60.0)	7 (25.9)	0.092[ns]

Abbreviations: CIP: Ciprofloxacin, NAL: Nalidixic acid, ERY: Erythromycin, TET: Tetracycline, AMP: Ampicillin, AMC: Amoxicillin/clavulanic acid, GEN: Gentamicin, *NC*: Not calculated, and ns: Not statistically significant (represents no significant association between *Campylobacter* spp. and AMR; *p* > 0.05)
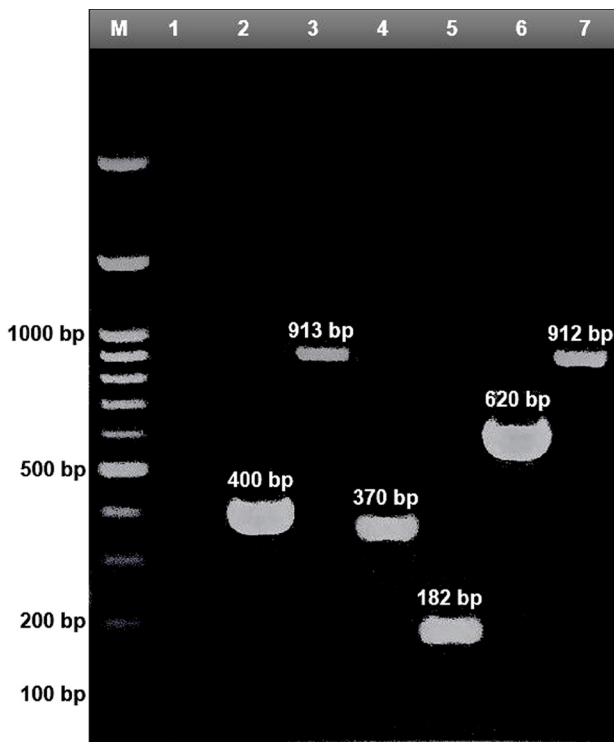
Figure 2.
Amplified PCR products of virulence genes (except the *iamA* gene) among *Campylobacter* isolates from backyard ducks. Lane M: 100-bp DNA ladder, Lane 1: negative control (deionized water), Lane 2: *cadF* gene positive, Lane 3: *pldA* gene positive, Lane 4: *cdtA* gene positive, Lane 5: *cdtC* gene positive, Lane 6: *cdtB* gene positive, and Lane 7: *wlaN* gene positive.

total, 27 *Campylobacter* isolates were screened for the presence of seven putative virulence and toxin genes, and the details of our findings are summarized in Table 4. The *cadF* (adhesion) gene with a positivity rate of 92.6% was the most prevalent gene. All C. coli and *C. jejuni* isolates were positive for this gene. Regarding invasion-related genes, 2 (7.4%) of the isolates carried *pldA*, while *iamA* was not detected in any of the isolates. Among the genes encoding *CDT, cdtA, cdtB,* and *cdtC* were present in 25.9%, 85.2%, and 29.6% of the isolates, respectively. Moreover, 25.9% of stains possessed the *cdtABC* gene cluster. The *wlaN* gene associated with LOSSIAL production was found in 14 (51.9%) of the isolates. A statistically significant association was observed between *Campylobacter* spp. and the majority of virulence-related genes (*cadF, cdtA, cdtB, cdtC, cdtABC,* and *wlaN*) ($p < 0.05$). Six virulence gene patterns (genotypes) were found in 25 of 27 *Campylobacter* isolates (92.5%) (Table 5).

Statistical analysis of phenotypic antimicrobial resistance with virulence genes

There was no significant correlation between phenotypic resistance to antibiotics and genotype (virulence genes) in *Campylobacter* spp. isolated from backyard ducks ($rs = -0.35$, $p = 0.08$).

Table 3.
Antimicrobial resistance (AMR) patterns in *Campylobacter* isolates from backyard ducks.

Antibiotic resistance pattern	No. of *Campylobacter* isolates in a given AMR pattern (%)			
	C. coli (n=19)	C. jejuni (n=3)	Other spp. (n=5)	Total (n=27)
CIP	---	2 (66.7)	---	2 (7.4)
CIP-NAL	1 (5.3)	---	---	1 (3.7)
CIP-TET	1 (5.3)	---	---	1 (3.7)
CIP-NAL-TET	---	---	1 (20.0)	1 (3.7)
CIP-NAL-AMP-AMC	3 (15.8)	---	---	3 (11.1)
CIP-NAL-TET-AMP*	3 (15.8)	---	---	3 (11.1)
CIP-ERY-TET-AMP*	1 (5.3)	---	---	1 (3.7)
CIP-NAL-TET-AMP-AMC*	4 (21.1)	---	---	4 (14.8)
CIP-ERY-TET-AMP-AMC*	1 (5.3)	---	---	1 (3.7)
CIP-NAL-ERY-TET-AMP*	2 (10.5)	---	---	2 (7.4)
CIP-ERY-TET-AMP-GEN*	1 (5.3)	---	1 (20.0)	2 (7.4)
CIP-NAL-ERY-TET-AMP-AMC*	---	---	1 (20.0)	1 (3.7)
CIP-NAL-ERY-TET-AMP-AMC-GEN*	2 (10.5)	1 (33.3)	2 (40.0)	5 (18.6)
Total	19 (100.0)	3 (100.0)	5 (100.0)	27 (100.0)
No. of MDR* isolates (%)	14 (73.7)	1 (33.3)	4 (80.0)	19 (70.4)

* MDR pattern: resistance of *Campylobacter* isolates to at least three antimicrobial classes

Table 4.

Frequency of virulence genes in *Campylobacter* isolates from backyard ducks.

Campylobacter Species	No. of isolates	No. of positive isolates for a specific gene (%)							
		cadF	*iamA*	*pldA*	*cdtA*	*cdtB*	*cdtC*	*cdtABC*	*wlaN*
C. coli	19	19 (100.0)	0	1 (5.3)	2 (10.5)	18 (94.7)	3 (15.8)	2 (10.5)	13 (68.4)
C. jejuni	3	3 (100.0)	0	1 (33.3)	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	1 (33.3)
Other spp.	5	3 (60.0)	0	0	2 (40.0)	2 (40.0)	2 (40.0)	2 (40.0)	0
Total	27	25 (92.6)	0	2 (7.4)	7 (25.9)	23 (85.2)	8 (29.6)	7 (25.9)	14 (51.9)
p-Value		0.037*	NC	0.242 ns	0.003*	0.016*	0.007*	0.003*	0.010*

Abbreviations: *NC*: Not calculated, and ns: Not statistically significant

* indicates a statistically significant association between *Campylobacter* spp. and virulence genes ($p < 0.05$)

Table 5.

Virulence gene patterns (genotypes) in *Campylobacter* isolates from backyard ducks.

Virulence gene pattern	No. of Campylobacter isolates in a given genotype (%)			
	C. coli (n=19)	*C. jejuni* (n=3)	Other spp. (n=5)	Total (n=27)
cadF	1 (5.3)	---	1 (20.0)	2 (7.4)
cadF-cdtB	4 (22.0)	---	---	4 (14.8)
cadF-cdtB-wlaN	11 (57.9)	---	---	11 (40.7)
cadF-cdtB-cdtC-wlaN	1 (5.3)	---	---	1 (3.7)
cadF-cdtA-cdtB-cdtC	1 (5.3)	2 (66.7)	2 (40.0)	5 (18.5)
cadF-pldA-cdtA-cdtB-cdtC-wlaN	1 (5.3)	1 (33.3)	---	2 (7.4)
Total	19 (100.0)	3 (100.0)	3 (60.0)	25 (92.5)

Discussion

Backyard poultry can serve as a transmission source for a variety of food-borne pathogens, including thermophilic *Campylobacter* spp., for other bird and human populations because biosecurity practices in backyard flocks are commonly not monitored and executed [13]. There is little data on the on-farm prevalence of *Campylobacter* in domestically reared duck flocks. Therefore, this research aimed to estimate the infection rate, AMR, and genes associated with the virulence of *Campylobacter* spp. among backyard ducks in Iran.

In the present study, thermophilic *campylobacters* were confirmed using both the standard culture methods and mPCR in 27% (27/100) of the cloacal samples of backyard ducks. This finding was in agreement with the *Campylobacter* infection rate in chicken meat samples in Iran, which was reported at 28.9% (26/90) [14], while lower isolation rates of *Campylobacter* spp. were identified in the urban duck fecal samples in Iran (17.3%) [15] and in turkey, game bird (pheasant and quail), and duck cecal samples in Canada (11.9%, 4.5%, and 3.4%, respectively) [16]. On the other hand, a higher level of *Campylobacter* infection was found among the duck and goose intestinal samples in Iran (34.2%) [17] and in mallard duck and white-fronted goose cloacal samples in Poland (32.8% and 45.5%, respectively) [18]. Although *C. jejuni* was reported as the prevailing species in most studies, the most prevalent species identified in the current study was *C. coli* (70.4%). This finding was in accordance with the research completed in Spain and Germany [19, 20]. The results of this study showed that *Campylobacter* infection is highly prevalent in ducks and these hosts can be considered the main source of *Campylobacter* spp. (both *C. jejuni* and *C. coli*) and a possible risk of human campylobacteriosis.

In this research, the resistance rate to ciprofloxacin was 100%, and high resistance to nalidixic acid was observed among *Campylobacter* isolates (74.1%), while *C. coli* (78.9%) was more resistant to nalidixic acid than *C. jejuni* (33.3%). Similarly, in a study by Wysok *et al.* (2020), most of the *Campylobacter* strains

from domestic goose cecal samples revealed resistance to ciprofloxacin (92%) and nalidixic acid (88%) [21]. In another study, high resistance to ciprofloxacin was found among human isolates in Europe, China, and Korea [22]. Contrarily, previous studies reported relatively low resistance to ciprofloxacin and nalidixic acid among *Campylobacter* strains [23, 24]. Overall, the very high resistance rate to FQs in this study may result from the expansive usage of this class of antimicrobials, such as enrofloxacin and sarafloxacin, to treat certain infections (e.g. *Escherichia coli*) in the poultry industry [25].

In the present study, 44.4% of *Campylobacter* isolates (36.8% *C. coli* and 33.3% *C. jejuni*) indicated moderate resistance to erythromycin. Our result was similar to those of Wei et al. (2014) [4] and Ghoneim et al. (2020) [26]. On the other hand, all *Campylobacter* strains isolated from layer hen cloacal swabs and farm environment samples in Tunisia were resistant to erythromycin [27]. Overall, the findings of this research showed that the use of macrolides (e.g., spiramycin and tylosin) for therapeutic purposes and growth promotion in commercial poultry has caused the high prevalence of macrolide-resistant *Campylobacter* isolates in backyard flocks [6].

High resistance to tetracycline was shown in *Campylobacter* strains (77.8%) in this research, while *C. coli* was more resistant to this antibiotic compared to *C. jejuni* (78.9% *vs*. 33.3%). Similarly, high resistance to tetracycline was reported in Iran (70.6%) [28] and China (nearly 100%) [29]. Conversely, a study in Belgium showed relatively low or moderate resistance to tetracycline (48.3%) among *Campylobacter* strains isolated from the intestinal samples of international travelers [30]. Our findings indicated that tetracyclines should be used cautiously to treat animals and humans.

In the current research, the rate of gentamicin-resistant *Campylobacter* strains was relatively low (25.9%) and C. jejuni (33.3%) exhibited higher resistance to gentamicin than *C. coli* (15.8%). Similarly, Qin et al. (2012) reported a relatively low rate of resistance against gentamicin (>20%) among *Campylobacter* isolates from broiler chickens [31]. In another study, relatively low resistance to gentamicin was estimated among Campylobacter strains obtained from human and chicken sources in the U.S. [25]. Consequently, aminoglycosides (e.g., gentamicin) can be utilized to treat acute and systemic *Campylobacter* infections in humans and to prevent bacterial infections in various avian species [6, 25].

In our study, 81.5% of *Campylobacter* isolates (89.5% of *C. coli vs*. 33.3% of *C. jejuni)* were resistant to ampicillin. These results were similar to those of Giacomelli *et al.* (2014) [32] and Casagrande Proietti et al. (2020) [33]. However, beta-lactams, such as pen-

icillin, are the most commonly used antibiotics for turkeys in Germany [34]. On the other hand, 51.9% of Campylobacter strains (52.6% *C. coli* and 33.3% *C. jejuni*) demonstrated moderate resistance to amoxicillin/clavulanic acid (co-amoxiclav) in this investigation, which was similar to previous estimates by Jehanne et al. (2021) in France [35] and Hadiyan et al. (2022) in Iran [36]. As a result, oral beta-lactams, such as co-amoxiclav, can be an appropriate choice to treat human *Campylobacter* infection due to the rising resistance of *C. jejuni* and *C. coli* to FQs, erythromycin, and tetracycline [25].

Overall, *C. coli* strains identified in the present research had more resistance to important antibiotics than *C. jejuni* isolates. Furthermore, 70.4% of *Campylobacter* isolates exhibited resistance against three or more antimicrobial classes. In this study, the prevalence rate of MDR was much higher for *C. coli* than for *C. jejuni* (73.7% *vs*. 33.3%), which was similar to the results of an investigation conducted in China [37]. Determining the virulence factors of *Campylobacter* is very important to better understand the infection rate [18]. Therefore, several critical virulence genes were identified in this research.

The prevalence of *cadF*, the most prevalent virulence gene, among Campylobacter isolates obtained from backyard ducks was 92.6% (25/27), which enhances the ability of *Campylobacter* to attach to host fibronectin and colonization of the intestine [7]. This result was similar to the research conducted by Kim et al. (2019) in South Korea (93.3%) [38] and Rossler et al. (2020) in Argentina (92%) [39], while the low prevalence rate of the cadF gene was detected in broiler chickens in South Africa (23.1%) [40]. In general, the prevalence of virulence genes responsible for adhesion in *Campylobacters* is very high regardless of the source and geographic area [18].

Both the invasion-associated marker (*iam*) and *pldA* genes encode pathogenic factors related to the *Campylobacter* invasion of intestinal epithelial cells. Moreover, the *pldA* gene encodes an outer membrane protein, phospholipase A, that is involved in hemolytic activity [7]. In this study, none of the *Campylobacter* isolates had the *iamA* gene, which was consistent with the results of a previous investigation in Brazil [41]. In addition, 7.4% (2/27) of the isolates possessed the *pldA* gene in our study. Similarly, a low frequency of the *pldA* gene was found among strains isolated from duck samples in South Korea (3.6%) [42] and individuals with diarrhea in Iran (15%) [43]. In contrast, the high prevalence rates of *iam* and *pldA* genes have been reported in earlier studies [44, 45]. The reasons for the considerable diversity of *iam* and *pldA* genes are not yet elucidated [41].

CDT is a key marker for *Campylobacter* pathoge-

nicity in humans, which is produced by three linked genes named *cdtA, cdtB*, and *cdtC*. The *CdtB* subunit has type I DNase activity and causes cell cycle arrest at the G2/M phase, while CdtA and CdtC subunits are responsible for the binding of *CDT* and its internalization into host cells. Ultimately, *CDT* leads to distention and cell death. However, the role of this toxin during Campylobacter colonization in the avian hosts is unclear [46]. The presence of three cdt genes is necessary for the function of CDT holotoxin [7, 46]. In this investigation, a relatively low frequency of the *cdtABC* gene cluster among backyard duck *Campylobacter* isolates was detected (25.9%), while all *C. jejuni* strains possessed the *cdtABC* genes. A similar rate of *cdtABC* was reported among all *Campylobacter* isolates from American crows in the U.S. and healthy pet birds in Iran, with a range of 20%-33% [47, 48], while the higher frequency of the *cdtABC* cluster was previously detected in Ireland (86%) [49] and Spain (100%) [50]. In general, the prevalence of CDT (*cdtABC*) genes in different research is highly variable, which may be due to heterogeneity in the genetic reservoir of *Campylobacter* strains [51].

The *wlaN* gene is responsible for the biosynthesis of sialylated lipo-oligosaccharide (LOSSIAL), which has a structure similar to human GM1 ganglioside. The LOSSIAL factor may cause autoimmune diseases, such as GBS polyneuropathy, following Campylobacter infection [10]. In this research, 51.9% of the *Campylobacter* strains had the *wlaN* gene, while this gene was more prevalent among *C. coli* isolates than *C. jejuni* (68.4% vs. 33.3%). The frequency of wlaN was 10% in wild birds in South Korea [52], 36% in human and broiler chicken sources in Egypt [53], and 44% in human stool samples in Hungary [54], which was lower than our result. Previous investigations have shown no association between the source of isolates and the presence of the *wlaN* gene [10].

Virulence gene patterns in the current study demonstrated that C. jejuni isolates carried more virulence factors than C. coli. In other words, *C. jejuni* strains are likely more pathogenic. Finally, we indicated a statistically significant association between *Campylobacter* spp. and the presence of virulence genes, especially genes related to the production of cytotoxins (CDT) and the occurrence of GBS. *Campylobacter* isolates obtained from backyard ducks can be a threat to food hygiene and human health.

In conclusion, the current study highlights that backyard ducks harbor commensal thermophilic campylobacters and can be regarded as potential reservoirs of *Campylobacter* infection for other hosts. Awareness of owners' backyard poultry flocks about the risk of the transmission of zoonotic diseases, including campylobacteriosis, hygiene and biosecurity measures (e.g., cleaning and disinfection, daily water and food change, rodent and insect control, and keeping wild birds away from backyard flocks), veterinary care, and antibiotic monitoring are essential for improving husbandry practices and avian health in backyard flocks, and decrease the prevalence of zoonotic pathogens between commercial and backyard poultry farms, leading to reduced infection in humans.

Materials and Methods
Sample collection

This study was conducted on June 2021-July 2022 in different geographical regions of the rural area of Amol (a city in northern Iran). A total of 100 healthy ducks from 28 backyard flocks were tested for *Campylobacter* infection. A cloacal swab sample was taken from all birds. To do this, the vent was cleaned with disinfectant iodine solution (10%) and a swab was inserted into the cloaca and was rotated. The samples were stored in a Cary-Blair transport medium (12.6 g/991 ml; HiMedia, India) at 4°C and were directly transmitted to the laboratory. The swabs were examined 4 h after sampling.

Bacterial examination

The swab samples were cultured in enrichment Preston broth consisting of Preston broth base (25 g/945 ml; MilliporeSigma, USA), *Campylobacter* selective supplement IV, modified with polymyxin B [2500 IU/500 mL], rifampicin [5 mg/500 mL], trimethoprim lactate [5 mg/500 mL], and amphotericin B [5 mg/500 mL] (MilliporeSigma, USA), and 5% lysed sheep blood (Zistroyesh, Iran). The inoculated broths were incubated at 37°C for 4 h, followed by 44 ± 4 h at 42°C in a microaerobic chamber (85% nitrogen, 10% carbon dioxide, and 5% oxygen) (Anaerocult® C; MilliporeSigma, USA). One loop full of enriched sample (1 μl) was cultivated on Preston selective agar containing *Campylobacter* agar base (19.75 g/500 mL; HiMedia, India), 5% defibrinated sheep blood, and mentioned antibiotics at the same doses. Bacterial cultures were incubated in a microaerobic environment at 42°C for 48 h. Subsequently, suspected colonies of *Campylobacter* were purified on brain heart infusion agar (52 g/L; MilliporeSigma, USA) with 5% sheep blood. Plates were incubated for 24 h at 42°C in a similar atmosphere. Preliminary recognition of *Campylobacter* isolates was performed based on colony characteristics (greyish, round, flat, and shiny with a regular edge), examination of typical cellular shapes ("S" or "seagull-like"), rapid darting motility using the phase-contrast microscopy, and biochemical reactions consisting of oxidase test (tetramethyl-p-phenylene-diamine), catalase test (3% H_2O_2), and glucose fermentation. Finally, a molecular assay was performed to confirm presumptive colonies [1].

DNA extraction

A pure single colony of each *Campylobacter* isolate was suspended in 200 μl sterile deionized H_2O. Bacterial DNA was prepared by boiling at 95°C for 15 min. The samples were centrifuged at 11000 rpm for 2.5 min and the supernatants were stored at -20°C until utilization [55].

Genus and species identification

A mPCR was performed following the method described previously by Yamazaki-Matsune *et al.* (2007) [56]. The target genes of *16S rRNA* for the *Campylobacter* genus, *ask* (aspartokinase) for *C. coli*, *glyA* (serine hydroxymethyltransferase) for *C. lari*, and *cj0414*

Table 6.
Antimicrobial resistance (AMR) patterns in Campylobacter isolates from backyard ducks.

Target gene	Primer	Sequences (5'- 3')	Annealing tempera-ture (°C)	Size (bp)	Reference
16S rRNA (Campylobacter)	C412F	GGATGACACTTTTCGGAGC	58	816	[56]
	C1228R	CATTGTAGCACGTGTGTC			
ask (C. coli)	CC18F	GGTATGATTTCTACAAAGCGAG	58	502	[56]
	CC519R	ATAAAAGACTATCGTCGCGTG			
glyA (C. lari)	CLF	TAGAGAGATAGCAAAAGAGA	58	251	[56]
	CLR	TACACATAATAATCCCACCC			
cj0414 (C. jejuni)	C-1	CAAATAAAGTTAGAGGTAGAATGT	58	161	[56]
	C-3	CCATAAGCACTAGCTAGCTGAT			
cadF	cadF-F2B	TTGAAGGTAATTTAGATATG	45	400	[55]
	cadF-R1B	CTAATACCTAAAGTTGAAAC			
iamA	iamA F	GCGCAAAATATTATCACCC	52	518	[59]
	iamA R	TTCACGACTACTATGCGG			
pldA	pldA-84	AAGCTTATGCGTTTTT	45	913	[55]
	Pld-981	TATAAGGCTTTCTCCA			
cdtA	DS-18	CCTTGTGATGCAAGCAATC	49	370	[55]
	DS-15	ACACTCCATTTGCTTTCTG			
cdtB	cdtB-113	CAGAAAGCAAATGGAGTGTT	51	620	[55]
	cdtB-713	AGCTAAAAGCGGTGGAGTAT			
cdtC	cdtC-192	CGATGAGTTAAAACAAAAGATA	47	182	[55]
	cdtC-351	TTGGCATTATAGAAAATACAGTT			
wlaN	wlaN F	AGGGTTTTAATAGTTGCAATTTCTC	50	912	[60]
	wlaN R	ATGAAATTTTTAATATCTTTACG-GAATTAA			

(oxidoreductase) for *C. jejuni* were amplified using specific primer sets (Table 6). Briefly, the amplification reaction was performed in a 25 µl final volume, including 2 µl DNA of bacteria, 12.5 µl PCR Master Mix 2X (Sinaclon, Iran), 0.5 µl forward and reverse primers (Sinaclon, Iran), and 6.5 µl distilled deionized H₂O. The mPCR was performed by a thermocycler (Bio-Rad, USA) according to the following program: an initial 15 min denaturation at 95°C, 35 cycles of denaturation (95°C, 0.5 min), annealing (58°C, 1.5 min), extension (72°C, 1 min), and a final step of 7 min at 72°C. Amplified products (10 µl each) were run on electrophoresis 1.5% agarose gel stained with DNA-safe stain (Sinaclon, Iran) in 1X tris-acetate-EDTA buffer and were seen under UV light. The 100-bp DNA ladder (Sinaclon, Iran) was employed as a molecular weight standard. *C. coli* strain ATCC 43478 and sterile deionized H₂O were utilized as positive and negative controls, respectively. Moreover, one of the *C. jejuni* isolates obtained in this study was subjected to sequencing of a 161-bp PCR amplicon of the *cj0414* gene by the Sanger sequencing method (Codon Genetic Group, Iran). Based on nucleotide BLAST analysis, the sequence data of the *cj0414* gene and *C. jejuni* strain 2016-IZSVE-19-111250 (Gen-Bank: CP053659.1) from Italy were 99.24% identical.

Antibiotic susceptibility testing

The antimicrobial sensitivity of identified *Campylobacter* strains was assessed using Kirby Bauer's disk diffusion test according to the CLSI guideline for fastidious organisms [57]. The used antibiotic disks (Padtanteb, Iran) consisted of ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), ampicillin (AM, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), and gentamicin (GM, 10 µg). Bacterial colonies were suspended in nutrient broth (25g/L; MilliporeSigma, USA) to acquire a McFarland turbidity of 0.5. The prepared suspensions were cultured on Mueller-Hinton agar media (38g/L; MilliporeSigma, USA) containing 5% defibrinated sheep blood, and were incubated microaerobically at 42°C for 24 h. The zone of bacterial growth inhibition was measured for each antibiotic and evaluated under interpretive criteria provided by CLSI. Acquired resistance to at least one drug in three or more antibiotic classes was considered MDR [58].

Detection of virulence genes

The genomic DNA was amplified by PCR to detect genes involved in adhesion (*cadF*), invasion (*iamA* and *pldA*), production of toxin (*cdtA, cdtB, cdtC*), and biosynthesis of sialylated lipooligosaccharide (wlaN) using the primer sets listed in Table 6. The reaction mixture (25 μl) consisted of 2 μl DNA of bacteria, 12.5 μl PCR Master Mix 2X (Sinaclon, Iran), 1 μl of each primer (Sinaclon, Iran), and 8.5 μl deionized H_2O. The PCR assays were completed according to the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation (95°C, 1 min), annealing at a temperature specific to the primer pair for 1 min, extension (72°C, 1 min), and a final extension step at 72°C for 6 min. The PCR product of each gene (10 μl each) was electrophoresed on 1.5% agarose gel stained with DNA-safe stain (Sinaclon, Iran) in 1X TAE buffer and was visualized under UV light. Sterile deionized H_2O was used as the negative control. The amplicon size was determined using the 100-bp DNA ladder.

Statistical analysis

Statistical analysis was performed by SPSS 23. In this study, the correlation between phenotypic resistance to antibiotics and genotype (virulence genes) was determined using Spearman's correlation coefficient. Furthermore, Fisher's exact test was used to evaluate the association between *Campylobacter* spp. and phenotypic resistance to antibiotics and virulence genes. *p*-value < 0.05 was considered statistically significant.

Authors' Contributions

H.G., and R.A.J. conceived and planned the experiments. H.G., R.A.J., D.G., and R.K. carried out the experiments. H.G. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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