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Prevalence, seasonality and antibiotic susceptibility of *Campylobacter* spp. isolates of retail broiler meat in Iran

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ABSTRACT

Campylobacter species, are the most common cause of bacterial gastroenteritis worldwide. The main route of transmission is generally believed to be via undercooked meat and meat products. This study was conducted to determine the prevalence, seasonality and antibiotic susceptibility of Campylobacter spp. isolates of retail broiler meat in Mashhad, Iran. From January 2013 through December 2013, 360 broiler meat samples were purchased in Mashhad, Iran. Identification of a presumptive Campylobacter species was performed using the cultural method and a polymerase chain reaction (PCR) assay. Antimicrobial susceptibility testing was performed using the disc diffusion method. Overall, 227 samples (63.1%) were positive for *Campylobacter*. The most prevalent *Campylobacter* spp. isolated was *Campylobacter jejuni* (88.1%). There was a significant seasonal prevalence of *Campylobacter* spp. in broiler meat in Mashhad, Iran ($P < 0.0001$). The highest isolation rate was also in summer (78.9%). The antimicrobial susceptibility test showed that 93.4% of the isolates were resistant to one or more antimicrobial agents. Resistances to tetracycline (87.2%) and ciprofloxacin (79.3%) were the most common resistances. The findings of this study showed a relatively high prevalence of *Campylobacter* contamination and antimicrobial resistance in broiler meats in Mashhad, Iran. To the authors' knowledge this is the first study on the seasonal prevalence and antibiotic susceptibility of *Campylobacter* spp. isolated from broiler meat in Iran.

1. Introduction

Campylobacter spp., especially C. jejuni and C. coli, are the main cause of acute gastroenteritis and traveler's diarrhea in people throughout the world (Jorgensen et al., 2011). Campylobacter spp. is commonly found in the intestinal tract of chickens and is transferred to the skin during slaughter and processing (Willis & Murray, 1997). Campylobacter spp. contamination commences at the farm level. It has been proposed that human infections follow increased Campylobacter in animals (Munot, Hanagal, Shouche, & Kapadnis, 2013). Campylobacter colonization in poultry has been reported to follow a seasonal pattern, peaking in the warmer months (Boysen, Vigre, & Rosenquist, 2011; Munot et al., 2013). Campylobacter jejuni infection in humans has shown peak isolation rates during the summer (Willis & Murray, 1997). Thus, reduction of the prevalence of positive flocks should contribute substantially to the reduction of this disease in people (Newell & Fearnley, 2003). The use of antimicrobial agents to promote growth and control diseases in food animals has resulted in the emergence and dissemination of antimicrobial-resistant bacteria, including antimicrobial-resistant Campylobacter (Abdollahpour, Zendehbad, Alipour, & Khayatzadeh, 2015; Chen et al., 2010). The patterns of the use of antimicrobial agents in one part of the world affect people's health in other parts of the world as a result of international travel and trade (World Health Organization, 2013). In countries with limited resources, Campylobacter spp. infection can be prevented by improving and targeting control measures in the food production chain. The objective of this study was to determine the prevalence, seasonality, and antimicrobial resistant pattern of *Campylobacter* spp. isolates in retail broiler meat in Mashhad, Iran.

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2. Materials and methods

2.1. Sample collection

Three hundred and sixty retail broiler meat samples were randomly purchased from 20 retail outlets in Mashhad, Iran from January 2013 through December 2013. Samples purchased in this study included legs and wings. The samples were packed individually and transported on ice to the laboratory within five hours of collection.

2.2. Isolation and identification of Campylobacter

The meat samples were processed immediately upon arrival using aseptic techniques. Of each meat sample, 25 g from each was homogenized and transferred to 225 mL of Preston enrichment broth base (HiMedia Laboratories, Mumbai, India, M899) containing *Campylobacter* selective supplement IV (HiMedia Laboratories, Mumbai, India, FD042) and 5% (v/v) defibrinated sheep blood. After inoculation at 42 °C for 24 h in a microaerophilic condition (85% N2, 10% CO2 and 5% O2), 0.1 mL of the enrichment broth was then streaked onto the *Campylobacter* selective agar base (HiMedia Laboratories, Mumbai, India), supplemented with an antibiotic for the selective isolation of *Campylobacter* species (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood, and incubated at 42 °C for 48 h under the same condition. One presumptive *Campylobacter* colony from each selective agar plate was subcultured, and identification of a presumptive *Campylobacter* species was performed using standard microbiological and biochemical procedures (Zendehbad, Arian, & Alipour, 2013).

2.3. Identification of Campylobacter by DNA extraction and PCR conditions

From Preston's broth, we extracted DNA from 360 samples after the enrichment step using a Genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol. In this study, we used the PCR procedures described previously (Denis et al., 1999). Three genes selected for the identification of the *Campylobacter* spp., *Campylobacter jejuni*, and *Campylobacter coli* were the 16S rRNA gene (Linton, Lawson, Owen, & Stanley, 1997), the mapA gene (Stucki, Frey, Nicolet, & Burnens, 1995), and the ceuE gene (Gonzalez, Grant, Richardson, Park, & Collins, 1997), respectively. The sequences of the primers used for gene amplification are presented in Table 1. Amplification reactions were performed in a 30-mL mixture that contained 0.6 U Taq polymerase (Fermentas, GmbH, Germany), 100 μmoL l^{-1} each of dNTP, 0.11 μmoL l^{-1} of MD16S1, and MD16S2 primers. It also contained 0.42 μmoL l^{-1} of MDmapAl, MDmapA2, COL3, and MDCOL2 primers in the Fermentas buffer (Fermentas, GmbH, Germany). Amplification reactions were conducted using a DNA thermal cycler (Master Cycle Gradient, Eppendorf, Germany). There was one cycle of 10 min at 95 °C, followed by 35 cycles each of which consisted of 30 s at 95 °C. Also, they were cycled for 1.5 min at 59 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. The amplification generated 857, 589, and 462 bp DNA fragments corresponding to the *Campylobacter* genus, *C. jejuni* and *C. coli*, respectively. We used *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560) as the positive controls and DNAase free *Campylobacter* spp. as the negative control. The PCR products were stained with 1% solution of ethidium bromide and viewed under UV light after gel electrophoresis on 1.5% agarose (Rahimi & Ameri, 2011).

Table 1
Primers for polymerase chain reaction (PCR) amplification of campylobacterial DNA for identification DNA.

Organism	Primer	PCR product (bp)	Sequence
Campylobacter spp.	*16SrRNA*	857	F[a]: 5′ ATC TAA TGG CTT AAC CAT TAA AC 3′ R[a]: 5′ GGA CGG TAA CTA GTT TAG TAT T3′
Campylobacter jejuni	*mapA*	589	F: 5′ CTA TTT TAT TTT TGA GTG CTT GTG 3′ R: 5′ GCT TTA TTT GCC ATT TGT TTT ATT A3′
Campylobacter coli	*ceuE*	462	F: 5′ AAT TGA AAA TTG CTC CAA CTA TG 3′ R: 5′ TGA TTT TAT TAT TTG TAG CAG CG 3′

[a] F = forward; R = reverse.

2.4. Antimicrobial susceptibility testing

One strain from each *Campylobacter*-positive sample was selected for susceptibility tests. Antimicrobial susceptibility testing for 280 isolated strains of C. *jejuni* and 29 isolated strains of *C. coli* was performed by the Kirby–Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) supplemented with 5% defibrinated sheep blood, according to the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2006). The following antimicrobial impregnated disks (HiMedia Laboratories, Mumbai, India) were used: amoxicillin (30 μg), ampicillin (10 μg), azithromycin (15 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), enrofloxacin (10 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), neomycin (30 μg), streptomycin (300 μg) and tetracycline (15 μg). After incubation at 42 °C for 48 h in a microaerophilic atmosphere, the susceptibility of the *Campylobacter* spp. to each antimicrobial agent was measured, and the results were interpreted in accordance with interpretive criteria provided by CLSI (CLSI, 2006). *Staphylococcus aureus* and *Escherichia coli* were used as quality control organisms in the determination of antimicrobial susceptibility (Rahimi & Ameri, 2011).

2.5. Statistical analyses

The data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp. Redmond, WA, USA) for statistical analysis. Using SPSS version 16.0 (SPSS, Inc. Chicago, IL, USA), we performed chi-squared tests and Fisher's exact tests. A P value of less than 0.05 was considered significant.

Table 2
Seasonal occurrence of Campylobacter in Campylobacter strains isolated from broiler meat samples in Mashhad, Iran.

Season	Samples (n = 360)			Campylobacter spp. (n = 227)		
	Number of samples	Campylobacter spp. Isolated positive	P. value	C. jejuni	C. coli	P. value
Spring	90	58 (64.4%)		49 (84.5%)	9 (15.5%)	
Summer	90	71 (78.9%)	<0.0001	65 (91.5%)	6 (8.5%)	0.82
Autumn	90	57 (63.3%)		50 (87.7%)	7 (12.3%)	
Winter	90	41 (45.6%)		36 (87.8%)	5 (12.2%)	
Total	360	227 (63.1%)		200 (88.1%)	27 (11.9%)	

3. Results

3.1. Isolation and identification of Campylobacter

Overall, 227 samples (63.1%) were positive for *Campylobacter* spp. The most prevalent *Campylobacter* spp. was *C. jejuni* which was found in 200 of the contaminated samples (88.1%). *C. coli* was identified in the remaining 27 contaminated samples (11.9%). Table 2 and Fig. 1 show monthly occurrence of *Campylobacter* spp. isolated from the meat samples in Mashhad, Iran. A statistically significant seasonal pattern was also observed ($P < 0.0001$) and the prevalence of positive batches was higher in June and July (76.7% and 90%, respectively). The highest isolation rate was in summer (78.9%). There was not a statistically significant difference between the seasonality prevalence of *C. jejuni* and *C. coli*.

3.2. DNA extraction and PCR conditions

The PCR assay identified 8 *Campylobacter*-contaminated samples of broiler meat samples that were negative using the cultural method. The difference between the numbers of *Campylobacter* spp. detected by these two assays was not statistically significant.

3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility test was performed on 200 *C. jejuni* and 27 *C. coli* isolates. The results of antibiotic testing are summarized in Table 3. Overall, 205 of 227 *Campylobacter* isolates (90.3%) were resistant to one or more antimicrobial agents. As many as 42 (18.5%) isolates were resistant to only one of the antimicrobial agents that were tested, and 91 strains (43.2%) showed resistance to two of the antimicrobial agents. Multi-resistance, which was defined as resistance to three or more of the drugs that were tested was found in 72 of the Campylobacter strains (31.7%). Resistance to tetracycline was the most common finding (87.2%), followed by resistance to ciprofloxacin (79.3%) and nalidixic acid (63.9%). There was a significant difference in the resistance of Campylobacter spp. to different antimicrobial agents ($P \leq 0.0001$).

4. Discussion

4.1. Isolation and identification of Campylobacter

In this study, *Campylobacter* spp. were isolated from 63.1% of broiler meat samples and most of the isolates were C. *jejuni* (88.1%). In general, these findings were consistent with the findings of previous studies on broiler meats reported from different regions of Iran (Rahimi & Ameri, 2011; Zendehbad et al., 2013). The prevalence of *Campylobacter* spp. in poultry has been found to vary from 50% to 98% in different countries (Bester & Essack, 2008; Garin et al., 2012; Kovalenko, Roasto, Liepinš, Mäesaar, & Hörman, 2013; Messad, Hamdi, Bouhamed, Ramdani-Bouguessa, & Tazir, 2014; Oyarzabal, Williams, Zhou, & Samadpour, 2013). A direct correlation of the various studies may be difficult because of the differences in the sampling procedures and isolation methods (Zendehbad et al., 2013), farm management practices, and geographical and seasonal variations (McDowell et al., 2008). Results of our study indicated that the incidence rate of *Campylobacter* spp. contamination of broiler meat in Mashhad, Iran, ranged from 78.9% in summer to 45.6% in winter. A seasonality of broiler flock colonization has been observed especially in developed countries, leading to a peak prevalence during the warm summer months. However, studies conducted in the United Kingdom, United States of America and Canada (Quebec) have not observed such seasonal influence on the prevalence rate of *Campylobacter* spp. contamination (McDowell et al., 2008). The influence of season may be associated with the increased ventilation of houses and the increased amount of insects during the warm summer and autumn months. If large volumes of air are introduced, it is conceivable that flies with *Campylobacter* from the outside are introduced into the flock (Hald, Sommer, & Skovgård, 2007). However, in developing countries, a seasonal variation does not seem to be observed, perhaps because the temperature variations are not as extreme as in many of the developed countries (McDowell et al., 2008).

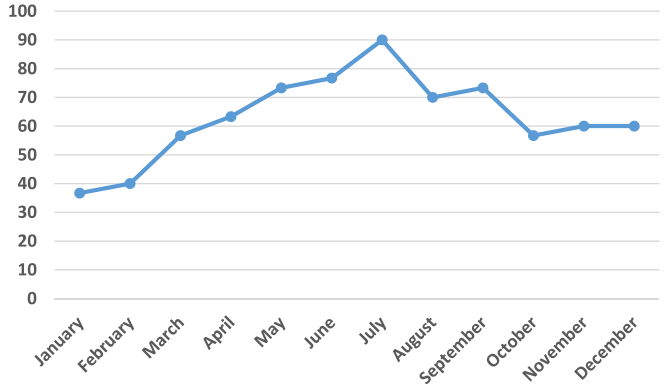

Fig. 1. Monthly occurrence of *Campylobacter* strains isolated from broiler meat samples in Mashhad, Iran from January 2013 through December 2013.

Table 3
Prevalence and Antimicrobial resistance profiles of Campylobacter strains isolated from broiler meat samples in Mashhad, Iran.

Antimicrobial agent	*Campylobacter* spp. (N = 227)	*Campylobacter jejuni* (N = 200)	*Campylobacter coli* (N = 27)
Amoxicillin	32 (14.1%)	26 (13%)	6 (22.2%)
Ampicillin	16 (7.0%)	13 (6.5%)	3 (11.1%)
Chloramphenicol	12 (5.3%)	9 (4.5%)	3 (11.1%)
Ciprofloxacin	180 (79.3%)	168 (84.0%)	12 (44.4%)
Enrofloxacin	64 (28.2%)	60 (30.0%)	4 (14.8%)
Erythromycin	85 (37.4%)	76 (38.0%)	9 (33.3%)
Gentamicin	8 (3.5%)	7 (3.5%)	1 (3.7%)
Nalidixic acid	145 (63.9%)	132 (66.0%)	13 (48.1%)
Neomycin	47 (20.7%)	42 (21%)	5 (18.5%)
Tetracycline	198 (87.2%)	181 (90.5%)	17 (63.0%)
Total			
Resistance to 1 antimicrobial	42 (18.5%)	38 (19.0%)	4 (14.8%)
Resistance to 2 antimicrobials	91 (43.2%)	82 (40.1%)	9 (33.3%)
Resistance to >2 antimicrobials	72 (31.7%)	65 (32.5%)	7 (25.9%)

4.2. DNA extraction and PCR conditions

Result of this study showed the PCR assay has a better analytical and diagnostic sensitivities to detect *Campylobacter* spp. than the cultural method. The PCR was designed for rapid and definitive identification to the species level of *C. jejuni* and *C. coli*. Isolation and identification of *Campylobacter* spp. have traditionally involved the use of selective culture media combined with biochemical tests. This method is expensive, laborious, and time consuming, whereas the PCR assay is fast and cost-effective (Rahimi & Ameri, 2011).

4.3. Antimicrobial susceptibility testing

The resistance to tetracycline was highly prevalent in the *Campylobacter* isolates obtained in this study. This finding is

consistent with previously reported studies on tetracycline-resistant *Campylobacter* in Iran and other countries (Chen et al., 2010; Rahimi & Ameri, 2011; Zendehbad et al., 2013).

As previously reported (Rahimi & Ameri, 2011; Zendehbad et al., 2013) and similar to tetracycline, our findings revealed a high prevalence of fluoroquinolone-resistant *Campylobacter* in broiler meats in Iran. Overall, there was a significant increase in the *Campylobacter* resistance to ciprofloxacin and nalidixic acid. However the prevalence of fluoroquinolone-resistant *Campylobacter* varies greatly from 9.4% in Grenada to 99% in China and other countries (Hariharan, Sharma, Chikweto, Matthew, & DeAllie, 2009; Qin et al., 2011; Zendehbad et al., 2013). The relatively high fluorochinolone resistance rates among the *Campylobacter* isolates were probably caused by the broad use of this class of antibiotics in veterinary medicine (Norstrom, Johnsen, Hofshagen, Tharaldsen, & Kruse, 2007). This class of antibiotics is used for both prevention and control of poultry diseases. It is well known that the use of fluoroquinolones in poultry selects for fluoroquinolone-resistant mutants and leads to the emergence of fluoroquinolone-resistant *Campylobacter* in the treated birds (Chen et al., 2010). When fluoroquinolones and tetracycline were taken into use in animal production in Asia and in Europe, antimicrobial resistance started to increase among human isolates at the same time (Endtz et al., 1991). In the United Kingdom and the USA, the same phenomenon was observed after the approval of the use of fluoroquinolones in veterinary medicine (Nachamkin et al., 2002).

Overall, the gentamicin resistance rates identified in this study are comparable to the results reported in other studies (Chen et al., 2010; Rahimi & Ameri, 2011). The low rate of resistance to gentamicin may be attributed, in part, to the fact that gentamicin is rarely used in the poultry industry either prophylactically or therapeutically due to its intramuscular route of administration, which may be impractical for large-scale application on poultry farms (Zendehbad et al., 2013). Another interesting finding of this study is the low rate of resistance to the phenicols in *C. jejuni*, especially to chloramphenicol, which was inconsistent with the findings of previous studies in Iran and other countries (Hariharan et al., 2009; Zendehbad et al., 2013). This may be due to the fact that the use of this drug in poultry is banned in Iran.

Among the 227 *Campylobacter* isolates obtained in this study, multidrug-resistance was detected in 31.7%. A few studies have also reported a high frequency of multi-drug resistance in *Campylobacter* in Iran (Dallal et al., 2010; Zendehbad et al., 2013). However, this is a much higher rate than those reported in Poland (7.0%) (Maćkiw, Korsak, Rzewuska, Tomczuk, & Rożynek, 2012), Finland (18%) (Lyhs, Katzav, Isohanni, Heiska, & Maijala, 2010), and Ireland (0.8%) (Oza, McKenna, McDowell, Menzies, & Neill, 2003). Multi-resistance poses a threat to people by limiting the therapeutic choice of antibiotic. Because of the increased reporting of antimicrobial resistance in *Campylobacter* worldwide, attempts should be made to control the antimicrobial use in animal husbandry (Chen et al., 2010).

4.4. Limitations

Potential limitations of our results are due to the fact that the survey was performed in only one area. Therefore, no statements can be made whether the prevalence of Campylobacter spp. and the susceptibility of the isolates are the same in other regions of Iran. Further studies are required for a more in-depth understanding of the prevalence of this important foodborne pathogen in Iran. Another limitation of this is study was because of some logistics issues, preparation of new antibiotic discs could not be performed according to the CLSI guidelines. However, we believe this limitation did not have an effect on the results of the antimicrobial susceptibility in this study.

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