Prevalence and antibiotic resistance profile of thermophilic *Campylobacter* spp. of slaughtered cattle and sheep in Shiraz, Iran

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Article Info	Abstract
Article history: Received: 05 May 2015 Accepted: 08 March 2016 Available online: 15 September 2016 **Key words:** Antibiotic resistance Cattle PCR Sheep Thermophilic *Campylobacter* spp.	Although poultry meat is considered as the main source for human *Campylobacter* infections, there is limited information about non-poultry sources. The present study was aimed to investigate the prevalence and the antibiotic resistance of thermophilic *Campylobacter* spp. in fecal samples of the cattle and sheep in Shiraz, Iran. A total of 302 fecal samples were obtained from clinically healthy, slaughtered cattle and sheep from Shiraz slaughterhouse. The animals were clinically healthy before being slaughtered. The samples were cultured according to the specific cultivation method under thermophilic conditions. The susceptibility of *Campylobacter* isolates were determined for 13 antimicrobial agents. All enriched samples and cultured isolates were targeted for polymerase chain reaction (PCR) detection of *16S rRNA* and multiplex PCR for determining their species. Among 302 fecal samples, 65 (21.5%) and 205 (67.8%) samples were positive for the presence of *Campylobacter* species with the cultivation and PCR techniques, respectively. All 65 distinct isolates were susceptible to neomycin and colistin and the isolates showed high resistance to cephalotin (83.0%) and ciprofloxacin (67.7%). After the multiplex PCR, 78.5% of total positive samples showed the simultaneous presence of *Campylobacter jejuni* and *Campylobacter coli*. In conclusion, the results emphasized that non-poultry farms are important as a possible source of *Campylobacter* infections.

میزان شیوع و الگوی مقاومت آنتی بیوتیکی گونه‌های کمپیلوباکتر گرمادوست جداشده از گاوها و گوسفندان کشتارشده سالم در شیراز، ایران

چکیده

اگرچه گوشت طیور به عنوان منبع اصلی عفونت‌های کمپیلوباکتر در انسان تعیین شده است، اطلاعات محدودی در مورد منابع غیر طیور وجود دارد. مطالعه حاضر، به منظور بررسی شیوع و مقاومت آنتی بیوتیکی گونه‌های کمپیلوباکتر گرمادوست جدا شده از نمونه‌های مدفوع گاو و گوسفند در شیراز، ایران انجام شد. در کل، ۳۰۲ نمونه مدفوع از گاوها و گوسفندان کشتار شده به ظاهر سالم، از کشتارگاه صنعتی شیراز جمع آوری و مطابق با روش کشت اختصاصی تحت شرایط گرمادوست کشت داده شدند. میزان حساسیت جدایه‌های کمپیلوباکتر برای ۱۳ داروی ضد میکروبی تعیین شد. همه نمونه‌های غنی‌شده و جدایه‌های کشت شده، برای انجام واکنش زنجیره‌ای پلیمراز (PCR) از ژن *16S rRNA* و PCR- چندگانه برای تعیین دو گونه، مورد هدف قرار گرفتند. از مجموع ۳۰۲ نمونه، به ترتیب ۶۵ (۲۱/۵ درصد) و ۲۰۵ (۶۷/۸ درصد) نمونه با استفاده از کشت و PCR برای حضور کمپیلوباکتر مثبت بودند. همه ۶۵ جدایه کمپیلوباکتر، نسبت به نئومایسین و کلیستین حساس بودند و جدایه‌ها مقاومت بالایی را به سفالوتین (۸۳/۰ درصد) و سیپروفلوکساسین (۶۷/۷ درصد) نشان دادند. بعد از PCR-چندگانه، ۷۸/۵ درصد از کل نمونه‌های مثبت حضور همزمان هر دو گونه کمپیلوباکتر ژژونی و کمپیلوباکتر کولای را نشان دادند. در مجموع، نتایج تاکید کردند که مزارع غیر طیور به دلیل داشتن یک نقش احتمالی در منابع عفونت‌های کمپیلوباکتری، مهم هستند.

واژه های کلیدی: گاو، گوسفند، گونه های گرمادوست کمپیلوباکتر، مقاومت آنتی بیوتیکی، PCR

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Introduction

Campylobacter species, especially thermophilic Campylobacters like *campylobacter jejuni* and *coli,* are one of the important causes of diarrheal diseases in human. *Campylobacter* enteritis is the most frequently infection observed before the development of Guillain-Barré and Miller-Fisher syndromes, making the *Campylobacter* infection as a major public health issue.[1] These organisms widely discriminated in multitude of animal reservoirs showing varying degrees of resistance to different antibiotics.[2,3] In *Campylobacter* enteritis, the macrolides and fluoroquinolones are considered the drugs of choice.[4,5] However, in the past two decades, the antimicrobial resistance of *Campylobacter* spp. to the fluoroquinolones and macrolides has increased, mainly as a result of the approval of this group of antimicrobial for the use in food producing animals.[3,6] Among the campylobacters, the thermophilic species particularly *C. jejuni* are the most frequently isolated bacteria from human infections.[5] While poultry meat is considered as the main source of human *Campylobacter* infection, there is growing evidence suggesting that the non-poultry sources can be equally important.[7] Cattle, sheep and other food animals frequently carry *C. jejuni* and *C. coli,*[8] as commensals in their rumen and small intestine;[9] and carcasses may be contaminated at slaughtering process by direct or indirect fecal contamination.[10] In this context, it is necessary to estimate the distribution and antimicrobial susceptibility of the bacteria associated with food animals. Currently, there is limited information on the prevalence of human pathogen *Campylobacter* spp. and their properties against antimicrobials in slaughtered cattle and sheep in Iran. The thermophilic campylobacters are important in diarrheal diseases in human and food animals can play a carrier role.[1,5] the present study was conducted to determine the occurrence and antimicrobial resistance of thermophilic *Campylobacter* spp. isolated from the feces of slaughtered cattle and sheep in Shiraz, Iran. In addition, the identification of the microorganism using PCR method was compared with microbiological culture as a conventional strategy.

Materials and Methods

Sample collection and *Campylobacter* Culture. From September 2011 to January 2013, a total of 302 fecal samples from cattle (n = 182) and sheep (n =120) were collected, at a slaughterhouse in Shiraz, Iran. The feces were taken from rectum of randomly chosen clinically healthy animals before slaughter, according to the method that was previously described.[11] Briefly, fecal samples were collected in tryptic soy broth (TSB; Merck, Darmstadt, Germany) tubes and taken to the laboratory at 4 ˚C in less than 6 hr.

For eliminating the other bacteria, 0.8 µM membrane filter (Sigma-Aldrich, Hamburg, Germany) was used and filtered samples were cultured in an enriched broth media, (TSB; 30 g L^{-1}), dextrose (2.5 g L^{-1}), sodium thioglycolate (0.5 g L^{-1}), rifampicin (10 mg L^{-1}), trimethoprim (10 mg L^{-1}), vancomycin (10 mg L^{-1}), ceftriaxone (10 mg L^{-1}), amphotericin-B (10 mg L^{-1}). Cultures then were incubated in a microaerophilic atmosphere (Anaerocult C; Merck, Whitehouse Station, USA) at 37 ˚C for 4 hr, followed by incubation at 42 ˚C for 44 hr. Thereafter, 50 µL of enriched samples in the TSB were cultured on selective agar, brucella agar base (41 g L^{-1}) with 5.0% sheep blood and above antibiotics with identical dose.[11] The preliminary identification of *Campylobacter* species was done according to the phenotypic characteristics; such as colony appearance, Gram staining, microscopic morphology, oxidase and catalase reactions.[6] The strains *C. jejuni* (ATCC 33291) and *C. coli* (RTCC 2541) were included as positive controls in both culture and consequent PCR reactions. All above mentioned chemicals were obtained from HiMedia Laboratories Ltd. (Tarnaka, India) unless otherwise mentioned.

Antimicrobial susceptibility test. Susceptibility of *Campylobacter* isolates to 13 antibiotics were examined by the disk diffusion (Kirby Bauer's) technique using Mueller-Hinton agar (Merck, Hamburg, Germany) supplemented with 5.0% de-fibrinated sheep blood, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[12] The antibiotic discs and their concentrations were cefotaxime (30 µg, Polfa Tarchomin, Warszawa, Poland), cephalotin (30 µg, Polfa Tarchomin), chloramphenicol (30 µg, Bayer, Wuppertal, Germany), nalidixic acid (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (10 µg), tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (15 µg), enrofloxacin (5 µg), colistin (10 µg) and tylosin (30 µg). The susceptibility of the *C. jejuni* and *C. coli* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI.[12]

DNA preparation and PCR assay. Each enriched sample in the TSB was used for DNA extraction. Moreover, after preliminary identification of *Campylobacter* spp., each campylobacter colony on the selective agar was used for DNA extraction. The bacterial DNA was extracted and purified by the procedure described by Sambrook *et al*. using phenol-chloroform and CTAB/NaCl technique.[13] The purity and concentration of the DNA were estimated by spectro-photometry at 260 and 280 nm (Nanodrop 1000; Thermo Fisher Scientific, Waltham, USA).

Simple and multiplex PCR reactions were done for identification of *Campylobacter* isolates at genus and species (*C. jejuni* and *C. coli*) level, respectively, using specific primers (Table 1). The PCR amplifications were performed in 25 µL final volume. The reaction mixtures consisted of 2.0 µL of the DNA template (50 ng), 2.5 µL

Table 1. Primers used in PCR reactions for identification of *Campylobacter* genus and species.

Name of primer	Sequence (5` to 3`)	Target gene	Annealing temperature	Product size (bp)	Reference
MapAF	CTATTTTATTTTTGAGTGCTTGTG	*mapA*	52 °C	589	14
MapAR	GCTTTATTTGCCATTTGTTTTATTA	(*C. jejuni*)			
Coli F	AATTGAAAATTGCTCCAACTATG	*ceuE*	52 °C	462	14
Coli R	TGATTTTATTATTTGTAGCAGCG	(*C. coli*)			
PLO6	GGTTAAGTCCCGCAACGAGCCGC	*16S rRNA*	50 °C	283	15
CAMPC5	GGCTGATCTACGATTACTAGCGAT	(Genus)			

10X PCR buffer, 1.0 μL dNTPs (50 μM), 0.2 μL (1 U) Taq DNA polymerase, (CinnaGen, Tehran, Iran), 1.0 μL (25 pmol) of each forward and reverse primers for simple and multiplex PCR reactions (Table 1). The volume of the reaction mixture was reached to 25.0 μL using distilled deionized water. The thermal cycler (MJ Mini, BioRad, Hercules, USA) was adjusted under the following conditions: Initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing (as shown in Table 1) for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 5 min and the PCR products were remained in the thermal cycler at 4 °C until they were collected.

Amplified products were separated by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 μg mL^{-1}, CinnaGen, Tehrran, Iran), and visualized in an ultraviolet light transluminator (BTS-20M; Uvitec, Cambridge, UK). The 100-bp DNA (Vivantis, Subang Jaya, Malaysia) and 100-bp plus DNA (CinnaGen) ladders were used as molecular size marker (Fig. 1).

Statistical Analysis. Data were analyzed using SPSS version 16.1 (SPSS Inc., Chicago, USA). Discrete variables were expressed as percentages and proportions were compared using the Chi-square test. Statistical significant difference was considered at value of $p ≤ 0.05$.

Results

From a total number of 302 fecal samples, 65 (21.5%) and 205 (67.8%) samples were positive for the presence of thermophilic *Campylobacter* species with cultivation and PCR procedures, respectively. When the cultivation method was compared with the PCR method, The PCR method had better specificity and sensitivity than cultivation methods with an overall agreement of 53.6%. Furthermore, a higher level of detection power was observed using the PCR method for the detection of *campylobacter* isolates. All samples with positive culture were also positive for the genus specific simple PCR. The PCR results showed that the prevalence of thermophilic

Campylobacter in the cattle and sheep fecal samples were 130/182 (71.4%) and 75/120 (62.5%), respectively. Totally, from 205 PCR positive specimens, 161 (78.5%) samples showed positive results for both the *C. jejuni* and *C. coli* specific primers in the multiplex PCR reaction. In these PCR positive samples, 6 (2.9%) and 26 (12.6%) samples were positive for the *C. coli* and *C. jejuni*, respectively. Moreover, 12 (5.8%) samples were negative in the multiplex PCR, which were considered as other thermophilic *Campylobacter* species. The PCR method showed higher level of the specificity than the culture method. The multiplex PCR results showed the simultaneous presence of two thermophilic *campylobacter* species in positive samples, but the culture method could only detect one specie in each positive sample. The comprehensive results of distribution of thermophilic *Campylobacter* species among cattle and sheep fecal
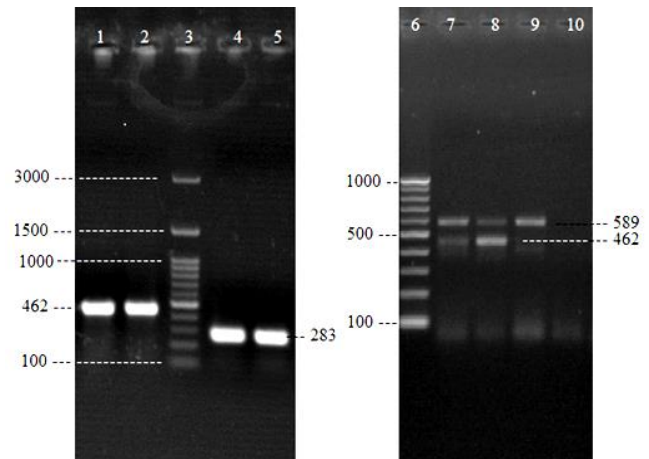

Fig. 1. Agarose gel electrophoresis of *16S rRNA* genus specific (283 bp), *mapA* (589 bp) and *ceuE* (462 bp) gene, genus specific and multiplex PCR products, respectively. Lanes 1: Positive control for *ceuE* gene; 2: *ceuE* gene; 3: 100-bp plus DNA marker; 4: Positive control for *16S rRNA* genus specific; 5: *16S rRNA* PCR products of sample; 6: 100-bp DNA marker; 7 and 8: *mapA* and *ceuE* genes PCR products of samples; 9: Positive control for *mapA* gene; and 10: Negative control.

Table 2. Prevalence of thermophilic *Campylobacter* species in cattle and sheep fecal samples. The data within the parentheses are presented as percentage.

Animal source	Number of samples	Positive in culture method	Positive for *16S rRNA* PCR	Positive in multiplex PCR			
				C. coli	*C. jejuni*	*C. coli + C. jejuni*	Other spp.
Cattle	182	42 (23.0)	130 (71.4)	3 (2.3)	16 (12.3)	104 (80.0)	7 (5.3)
Sheep	120	23 (19.1)	75 (62.5)	3 (4.0)	10 (13.3)	57 (76.0)	5 (15.0)
Total	302	65 (21.5)	205 (67.8)	6 (2.9)	26 (12.6)	161 (78.5)	12 (5.8)

Table 3. Antimicrobials resistance of *Campylobacter* isolates. The data within the parentheses are presented as percentage.

Antimicrobial agent	Cattle		Sheep		Total
	C. jejuni (n = 30)	*C. coli* (n = 12)	*C. jejuni* (n = 18)	*C. coli* (n = 5)	
Ampicillin	13 (43.3)	3 (25.0)	9 (50.0)	2 (40.0)	27 (41.5)
Chloramphenicol	2 (6.6)	1 (8.3)	2 (11.1)	0 (100)	5 (7.6)
Enrofloxacin	6 (20.0)	3 (25.0)	2 (11.1)	0 (0.0)	11(16.9)
Ciprofloxacin	20 (66.6)	9 (75.0)	10 (55.5)	5 (100)	44 (67.6)
Tetracycline	9 (30.0)	2 (16.6)	4 (22.2)	0 (0.0)	15 (23.0)
Gentamicin	3 (10.0)	1 (8.3)	1 (5.5)	0 (0.0)	5 (7.6)
Neomycin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Erythromycin	2 (6.6)	3 (25.0)	1 (5.5)	2 (40.0)	8 (12.3)
Nalidixic acid	4 (13.3)	4 (33.3)	3 (16.6)	4(80.0)	15 (23.0)
Colistin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cephalotin	27 (90.0)	10 (83.3)	17 (94.4)	0 (0.0)	54 (83.0)
Cefotaxime	7 (23.3)	2 (16.6)	6 (33.3)	2 (40.0)	17 (26.1)
Tylosin	6 (20.0)	2(16.6)	9 (50.0)	0 (0.0)	17 (26.1)

samples with culture and PCR methods are shown in Table 2. Antibiotic susceptibility test showed high resistance to cephalotin (83.0%) and ciprofloxacin (67.7%) and low resistance to erythromycin (12.3%), neomycin and colistin (0.0%). Table 3 shows the resistance of the isolates to different antimicrobials. The results showed that *C. coli* was significantly more resistant than *C. jejuni* to nalidixic acid and erythromycin ($p \leq 0.05$). In addition, the data showed that *C. coli* isolated from sheep were more susceptible than other isolates to these antibiotics.

Discussion

Food animals have been incriminated as the main source for *Campylobacter* infection in humans.[3,16] The main source of carcass contamination is intestinal contents during manual skinning, evisceration, washing and processing in the slaughterhouse.[17] Therefore, determining its prevalence is the first step to assess the food safety continuum before setting targets and taking efficient measures to decrease animal pathogen carriage and finally reducing of the hazard of human infection.[18] Most of the previous studies have investigated *C. jejuni* and *C. coli* in the diarrheic animals such as cattle and sheep,[19,20] but studies related to healthy animals are limited.[2,18,21] The primary purpose of the present study was to investigate the prevalence of *C. jejuni* and *C. coli* in fecal samples of clinically healthy slaughtered sheep and cattle in Shiraz, Iran. The results of the present study showed 21.5% (65 of 302) of the examined animals were positive for Campylobacter spp. in routine cultivation method using the enrichment procedure and specific selective medium that was in accordance with other studies.[21,22] The frequency of *Campylobacter* spp. among sheep isolates (19.1%) using culture method was in accordance with other studies conducted in Portugal,[23] (15.0%), and Brazil,[24] (20.0%) and did not significantly differ from the presence of the organism in cattle. Nevertheless, unlike the study of Kassa *et al*. the occurrence of *Campylobacter* spp. in cattle was higher than sheep by means of cultivation

method in the present study.[16] Other reports indicated the high prevalence of campylobacters in cattle.[18,25,26] These dissimilarities of the prevalence of the campylobacter among different animals may be due to the physiological differences of gastrointestinal tract or various flora and consistency of the feces of these animals. Conventional culture method for isolation of *Campylobacter* generally requires 4 days to show a negative result and 6 to 7 days to confirm a positive result and this phenotypic distinction is not always accurate.[27] Faster identification of *Campylobacter* in feces would facilitate earlier implementation of proper strategies for treatment, control and prevention. In the present study, the occurrence of *Campylobacter* were 71.4% and 62.5% in the cattle and sheep fecal samples, respectively, using genus specific PCR; which indicates a high prevalence of campylobacters in these food animals. As a result, cultivation method does not supply a factual evaluation of the frequency of *Campylobacter* species in the sheep and cattle and other food animal samples. Furthermore, this method has lower sensitivity than PCR. The number of live microorganisms decreases during transport of the samples and many of the cells die and cannot show growth in cultivation methods. Accordingly, the true prevalence of *Campylobacter* in fecal samples obtained by PCR is indeed more than the prevalence obtained by culture. The PCR can show the presence of both dead and live cells in different samples. Multiplex PCR was carried out to determine the prevalence of *C. jejuni* and *C. coli* among genus specific PCR positive specimens and the isolates. Although other studies,[16,28] reported the isolation of each species separately using the culture method (which was in agreement with the present study) surprisingly, in the present study, multiplex PCR results showed the simultaneous presence of two thermophilic species in 78.5% of *Campylobacter* positive samples. This fact confirms that these two species are in combination and cooperation in natural environment and their hosts' milieu. Furthermore, the results showed that the specificity of the PCR method was better than conventional cultivation method. Totally, 12 specimens with positive PCR

were negative in multiplex PCR, which were considered as other non-pathogen *Campylobacter* species. According to a previous study, *C. coli* has been found to be common in humans and chickens but rare in sheep and cattle,[25] however the present study showed high prevalence of this micro-organism con-currently with *C. jejuni* in healthy cattle and sheep fecal samples. This high prevalence may be due to the age of animals which are often slaughtered at the end of the breeding period as Besser *et al*. previously described the increase in prevalence of *Campylobacter* during breeding period.[29]

Nowadays, there is limited data on the antibiotic susceptibility patterns of the *Campylobacter* spp. isolated from various sources. Erythromycin and ciprofloxacin are two of the recommended antibiotics for treatment of the *Campylobacter* enteritis in human.[4,5] According to the results, *Campylobacter* spp. isolated from cattle and sheep showed 12.3% resistance to erythromycin and high resistance to ciprofloxacin which can be a serious challenge for treatment of human campylobacteriosis associated with food animal origins. All 65 cultured isolates were susceptible to neomycin and colistin and showed low level of resistance to gentamicin and chloramphenicol. Gentamicin and chloramphenicol-resistant isolates were unusual and these results were similar to other studies.[2,3,6] Some other studies showed high resistance to cipro-floxacin[2,6] and erythromycin.[21,30] However, the results of a few studies showed the susceptibility to erythromycin[2,3] and ciprofloxacin.[3,31] Comparing between *C. jejuni* and *C. coli* strains, the statistical analysis did not show significant difference in antibiotic resistance against the majority antimicrobials. However, these data showed that *C. coli* significantly ($p \leq 0.05$) was more resistant than *C. jejuni* to nalidixic acid and erythromycin.

In conclusion, the results indicate the high prevalence of *C. jejuni* and *C. coli*, in healthy cattle and sheep as food animals, emphasizing the importance of non-poultry farms as possible sources of the *Campylobacter* infection. Resistance of *C. jejuni and C. coli* to the macrolides (e.g., erythromycin) and the fluoroquinolones (e.g., ciprofloxacin) was the most alarming finding in this study, which may be as a result of high consumption of these antibiotics in veterinary and human medicine. It seems that more control and prevention strategies are needed against thermophilic *Campylobacter* with animal origin. Moreover, we must have more vigilant usage of the antibiotics in food animals and establish a surveillance of developing resistance to antibiotics among animal isolates.

Acknowledgments

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