Occurrence of virulence genes and strain diversity of thermophilic campylobacters isolated from cattle and sheep faecal samples

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Summary

The objective of this study was to investigate the genotypic characteristics of *Campylobacter* isolates in Shiraz, Iran. A total of 40 *Campylobacter* isolates including 20 *C. jejuni* and 20 *C. coli* were recovered from both cattle and sheep faeces by cultivation methods. The isolates were identified on the basis of polymerase chain reaction (PCR) detection of *16SrRNA* and multiplex PCR to determine two species. For confirmed isolates, PCR was carried out for the presence of virulence genes using specific primers. Other than verifying the genetic diversity of thermophilic *Campylobacter* isolates, *flaA* PCR-RFLP was performed. Results showed the high prevalence (100%) of the *cadF* gene and three genes associated with cytolethal distending toxin (CDT). Plasmid *virB11* gene was not found in any *Campylobacter* isolate, and dissimilarities and discrepancies occurred in *pldA*, *iamA*, *wlaN*, *waaC* and *cgtB* genes. Among the 40 *Campylobacter* isolates studied, nine different types were defined by *flaA*-typing. Results indicated genetic diversity among *Campylobacter* isolates recovered from cattle and sheep faecal samples. Findings showed the potential ability of *C. jejuni* and *C. coli* with cattle and sheep origins to cause infection in humans.

Key words: Thermophilic *Campylobacter* spp., Cattle, Sheep, Virulence genes, *flaA*-typing

Introduction

In humans, infections caused by thermophilic campylobacters, mainly *Campylobacter jejuni* and *C. coli* are well recognized, with symptoms ranging from mild diarrhea to more serious neuropathies (Scallan *et al*., 2011). Campylobacters are usually transmitted through water, milk, and food animals (Litrup *et al*., 2007; Huang *et al*., 2009). These bacteria are present in the intestinal tract of a wide range of warm blooded mammals and have been isolated from the faeces of farm animals including beef cattle, dairy cows and sheep, risking the contamination of food products in the case of dairy animals (Fitzgerald *et al*., 2001; Jamshidi *et al*., 2008). Molecular genetics of *Campylobacter* have not been comprehensively studied and the pathogenesis of infections is not clearly understood; nevertheless, several virulence-associated genes have been described in *Campylobacter*, most of which associated with pathogenicity (Zilbauer *et al*., 2008). As *Campylobacter* can be transferred from animals and their food products to man, it is necessary to formulate strategies for proper control and prevention of the infections it causes. To achieve this goal, it is imperative to characterize sources of contamination and to distinguish the *Campylobacter* isolates obtained from varied animal sources which are more virulent. Therefore, the first aim of the present study was to determine the occurrence of 10 virulence genes among sheep and cattle isolates. In this study, *cadF* (Ziprin *et al*., 2001), was selected as the pathogenic gene responsible for the expression of adherence and colonization, *virB11* (Bacon *et al*., 2000) and *pldA* (Ziprin *et al*., 2001) were selected as pathogenic genes responsible for the expression of invasion, *cdtA*, *cdtB*, and *cdtC* (Lara-Tejero and Galan, 2001) were selected as pathogenic genes responsible for the expression of toxin production, and *waaC*, *wlaN* and *cgtB* were selected as genes that are presumably involved in the expression of ganglioside mimics in Guillian-Barré syndrome and heptosyltransfrase and β-1,3-galactosyltransfrase production (Linton *et al*., 2000; Datta *et al*., 2003). Another virulence gene linked with *Campylobacter* invasiveness is the invasion-associated marker (*iam*) gene whose prevalence was examined in the present study (Carvalho *et al*., 2001).

Strain discrimination is required to recognize sources of contamination and transmission routes. Despite being a significant aspect of campylobacteriosis risk assessment, the heterogeneity of *Campylobacter* strains isolated from cattle and sheep faecal samples has not been identified or studied in Iran yet. Restriction fragment length polymorphism (RFLP) analysis of the *flaA* gene has been shown to be a useful epidemiological tool (Wassenaar and Newell, 2000; Fitzgerald *et al*., 2001). Therefore, the second objective of this study was to determine the genetic diversity of cattle and sheep

feacal associated with *C. jejuni* and *C. coli* to better understand the molecular epidemiology of the isolates in Shiraz, southern Iran.

Materials and Methods

Bacterial isolates and culture method

A total of 40 *Campylobacter* isolates including 20 *C. jejuni* and 20 *C. coli* were recovered from sheep and cattle faeces during January 2012, from Shiraz slaughterhouse, Iran. Briefly, faecal samples were collected in Tryptic Soy Broth (TSB) tubes using sterile gloves, cooled and brought to the laboratory in less than 6 h. To eliminate other bacteria, a 0.8 µm membrane filter was used and 250 µl of the filtered samples were cultured in an enriched broth media [TSB (30 g/L), dextrose (2.5 g/L), sodium thioglycolate (0.5 g/L), Rifampicin (10 mg/L), Trimethoprim (10 mg/L), Vancomycin (10 mg/L), Ceftriaxone (10 mg/L), and Amphotracin-B (10 mg/L)], incubated in a microaerophilic atmosphere (Anaerocult C, Merck) at 37°C for 4 h, followed by incubation at 42°C for 44 h. Thereafter, 50 µl of the enriched samples in TSB were cultured on a selective agar [brucella agar base (41 g/L), and the above mentioned antibiotics with identical doses] (Ansari-Lari *et al*., 2011). The growth of thermophilic campylobacters was detected by their typical appearance on culture media, i.e. the presence of flat grayish colonies like droplets of water sprayed on the medium. Preliminary identification of *Campylobacter* species was based on phenotypic characteristics such as colony appearance, Gram staining, microscopic morphology, oxidase and catalase reaction, fermentation of glucose and nitrate reduction. *C. jejuni* (ATCC 33291) and *C. coli* (RTCC 2541) type strains were included as positive controls for both culture and PCR identification of isolates.

DNA preparation

DNA extraction was carried out using phenol-chloroform extraction techniques. Briefly, a loopful colony of each isolate on an agar plate was picked and suspended in 200 µl distilled water. After vortexing, the samples were centrifuged at 10,000 × g, and the supernatants were discarded before adding 250 µL buffer 1 (the resuspension solution contained 100 µg/ml RNase) and 250 µL buffer 2 (Lysis buffer). 550 µL saturated phenol was then added, mixed thoroughly and centrifuged at 8000 × g. The supernatant was then collected into a new tube and the same volume of phenol was added and centrifuged as above. The clear phase was collected into a new tube before adding sodium acetate (2 M, pH = 5.2, 0.1 × volume of each aliquot). The aliquots were mixed with 1.5 ml 100% ethanol, kept at -20°C for 1 h and centrifuged at 12,000 × g. The supernatant was then discarded and the DNA pellet washed by 80% ethanol before being dried and suspended in 30 µL of 1 x TE buffer until further use (Ansari-Lari *et al*., 2011).

PCR assay

Simple and multiplex PCR reactions were carried out to identify of *Campylobacter* genus and *C. jejuni* and *C. coli* species. PCR was then carried out to detect the 10 virulence factors listed in Table 1 for each confirmed isolate. PCR amplifications were performed in a final volume of 25 µL. The reaction mixtures consisted of 2 µL of the DNA template, 2.5 µL 10 x PCR buffer [75 mM Tris-HCl, pH = 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$], (CinnaGen, Iran), 1 µL dNTPs (50 µM), (CinnaGen, Iran), 1 µL (1 U Ampli Taq DNA polymerase), (CinnaGen, Iran), and 1 µL (25 pmol) from the forward and reverse primers (CinnaGen, Iran) shown in Tables 1 and 2. The volume of the reaction mixtures were received to 25 µL using distilled deionized water.

Table 1: Nucleotide sequences used as primers in the PCR reaction to identify 10 virulence genes

Name of primer	Sequence (5′ to 3′)	Target gene	Annealing temperature	Product size (bp)	Reference
DS-18 DS-15	CCTTGTGATGCAAGCAATC ACACTCCATTTGCTTTCTG	*cdtA*	49°C	370	(Hickey *et al*., 2000)
cdtB-113 cdtB-713	CAGAAAGCAAATGGAGTGTT AGCTAAAAGCGGTGGAGTAT	*cdtB*	51°C	620	(Datta *et al*., 2003)
cdtC-192 cdtC-351	CGATGAGTTAAAACAAAAGATA TTGGCATTATAGAAAATACAGTT	*cdtC*	48°C	182	(Datta *et al*., 2003)
6virB-232 virB-701	TCTTGTGAGTTGCCTTACCCCTTTT CCTGCGTGTCCTGTGTTATTTACCC	*virB11*	53°C	494	(Datta *et al*., 2003)
iamA F iamA R	GCGCAAAATATTATCACCC TTCACGACTACTATGCGG	*iamA*	52°C	518	(Carvalho *et al*., 2001)
wlaN F wlaN R	TGCTGGGTATACAAAGGTTGTG AATTTTGGATATGGGTGGGG	*wlaN*	56°C	330	(Wassenar *et al*., 2002)
waaC1 waaC2	TAATGAAAATAGCAATTGTTCGT GATACAAAAATCACTTTTATCGA	*waaC*	42°C	1029	(Godschalk *et al*., 2007)
cgtB F cgtB R	TTAAGAGCAAGATATGAAGGTG GCACATAGAGAACGCTACAA	*cgtB*	56°C	562	(Linton *et al*., 2000)
pldA-84 pld-981	AAGCTTATGCGTTTTT TATAAGGCTTTCTTCA	*pldA*	45°C	913	(Datta *et al*., 2003)
cadF F cadF R	TTGAAGGTAATTTAGATAT CTAATACCTAAAGTTGAAAC	*cadF*	42°C	400	(Konkel *et al*., 1999)
Fla1 Fla2	GGATlTCGTATTAACACAAATGGTGC CTGTAGTAATCTTAAAACATTTG	*flaA* (RFLP)	45°C	1725	(Nachamkin *et al*., 1993)

Table 2: Nucleotide sequences used as primers in the PCR reaction to identify *Campylobacter* genus and *C. jujeni* and *C. coli* species

Name of primer	Sequence (5´ to 3´)	Target gene	Annealing temperature	Product size (bp)	Reference
MapAF MapAR	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	*mapA*	52°C	589	(Denis *et al*., 1999)
Coli F Coli R	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG	*ceuE*	52°C	462	(Denis *et al*., 1999)
PLO6 CAMPC5	GGTTAAGTCCCGCAACGAGCCGC GGCTGATCTACGATTACTAGCGAT	*16SrRNA*	50°C	283	(Cardarelli-Leite *et al*., 1996)

Table 3: Occurrence of 10 virulence genes and *flaA*-typing results of thermophilic *Campylobacter* isolated from cattle faecal samples

Species	No.	Number of *flaA*-types (most prevalent pattern)	No. of bovine isolates positive for virulence genes (%)									
			cdtA	*cdtB*	*cdtC*	*cadF*	*virB11*	*iamA*	*wlaN*	*cgtB*	*waaC*	*pldA*
C. jejuni	10	5 (2, 4)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	0 (0%)	0 (0%)	10 (100%)	4 (40%)	10 (100%)	0 (0%)
C. coli	10	8 (4, 8)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	0 (0%)	5 (50%)	5 (50%)	0 (0%)	6 (60%)	0 (0%)
Total	20	9 (4)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	0 (0%)	5 (25%)	15 (75%)	4 (20%)	16 (80%)	0 (0%)

Table 4: Occurrence of 10 virulence genes and *flaA*-typing results of thermophilic *Campylobacter* isolated from sheep faecal samples

Species	No.	Number of *flaA*-types (most prevalent pattern)	No. of sheep isolates positive for virulence genes (%)									
			cdtA	*cdtB*	*cdtC*	*cadF*	*virB11*	*iamA*	*wlaN*	*cgtB*	*waaC*	*pldA*
C. jejuni	10	5 (2)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	0 (0%)	9 (90%)	3 (30%)	7 (70%)	10 (100%)	2 (20%)
C. coli	10	7 (8)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	0 (0%)	10 (100%)	3 (30%)	6 (60%)	7 (70%)	3 (30%)
Total	20	8 (2)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	0 (0%)	19 (95%)	6 (30%)	13 (65%)	17 (85%)	0 (25%)

The thermal cycler (MJ mini, BioRad, USA) was adjusted under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing as shown in Tables 1 and 2 for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min and the PCR products were left in the thermal cycler at 4°C until collected. Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Visualization was undertaken using a UV transilluminator (BTS-20, Japan), and the 100 bp DNA ladders were used as molecular size markers.

The *flaA*-typing of *C. jejuni* and *C. coli* isolates

Isolates identified as *C. jejuni* and *C. coli* by m-PCR were typed by PCR-RFLP for the *flaA* gene. A fragment of 1725 bp of the *flaA* gene was amplified in a PCR reaction using a pair of specific primers listed in Table 2 (previously described by Nachamkin *et al*., 1993). All amplicons were restricted with 4 U *Dde*I (Thermo Scientific, Germany) in a 1.5 µl 10x recommended restriction buffer and were then incubated at 37°C for 15 h. The digested PCR products (15 µl) were immediately separated on 2.5% agarose gel stained with ethidium bromide. Bands were photographed under a UV transilluminator and the results were evaluated manually. A 50 bp DNA ladder (CinnaGen, Iran) was used as a molecular marker to estimate the size of the bands.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1. Discrete variables were expressed as percentages and proportions were compared using a Chi-squared test with the significance level of $P<0.05$. The significance of the association between the *flaA* PCR-RFLP pattern and *Campylobacter* species was evaluated by Fisher's exact test. P-values less than 0.05 were considered as significant.

Results

Detection of virulence genes by PCR

Among 40 *Campylobacter* isolates including 10 *C. jejuni* and 10 *C. coli* recovered from sheep samples and 10 *C. jejuni* and 10 *C. coli* recovered from cattle samples, the prevalence of *cdtA*, *cdtB*, *cdtC* and *cadF* virulence genes were 100% (40/40). The plasmid associated virulence marker, *virB11* gene, was not present in any of the isolates. The frequency of the *pldA* gene among *C. jejuni* and *C. coli* strains was 2/20 (10%) and 3/20 (15%), respectively, which included only sheep isolates; cattle isolates did not show the presence of the gene. Furthermore, for all 40 *Campylobacter* isolates, the presence of *iamA*, *wlaN*, *cgtB* and *waaC* genes were 60, 52.5, 42.5 and 82.5%, respectively. Detailed results of the PCR detection of the 10 virulence genes in the two thermophilic *Campylobacter* species obtained from cattle and sheep faeces are shown in Tables 3 and 4.

The *flaA*-typing results

In total, nine different types (p1-p9) were defined by *flaA*-typing (Fig. 1). Among 20 *C. jejuni* originating from both cattle and sheep faecal samples, six different types (p1-p6) were defined (Tables 3 and 4), the most common type being type p2 (35%). The occurrence of p1, p3, p4, p5, and p6 types among *C. jejuni* isolates were 15% (3/20), 5% (1/20), 25% (5/20), 15% (3/20) and 5% (1/20), respectively. Among 20 *C. coli* originating from both cattle and sheep faecal samples, nine types were defined, and the most common type being type p8

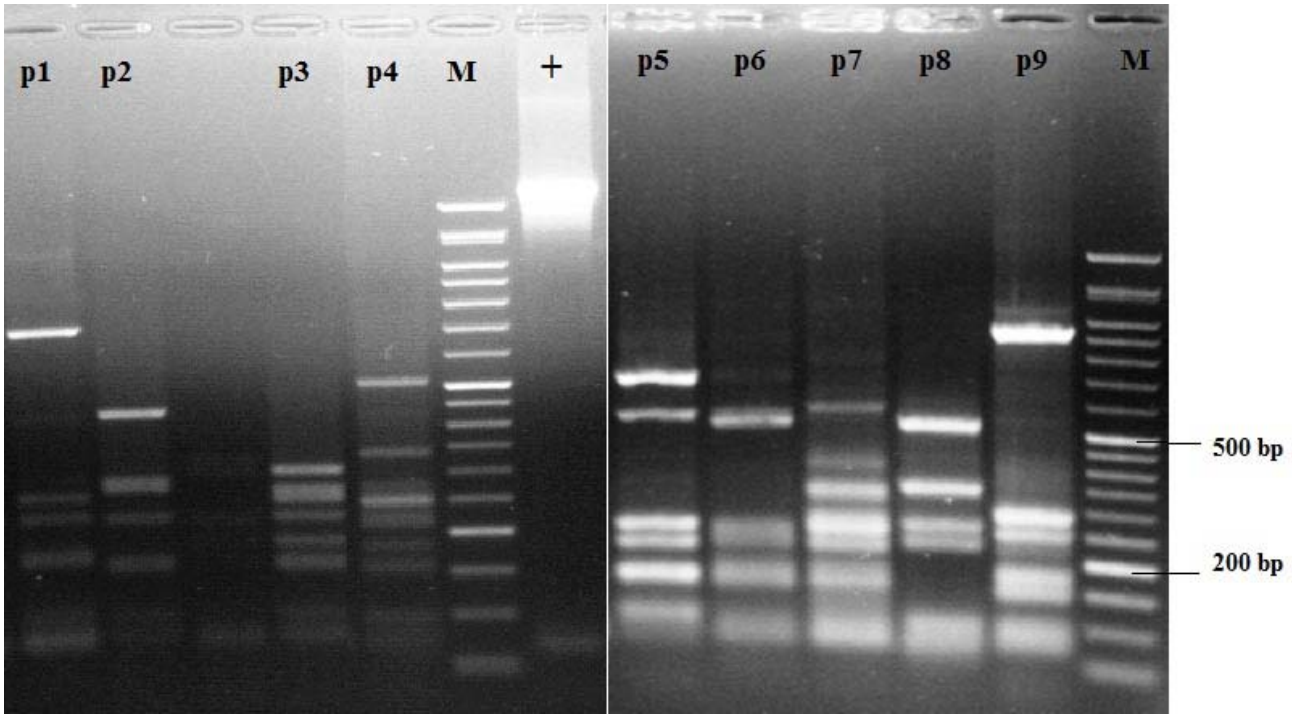

Fig. 1: Different *flaA*-typing patterns of *Campylobacter* isolates obtained from faecal samples of cattle and sheep using restriction endonuclease *DdeI*. Lanes p1-p9: Different restriction types of flagellin A gene, Lane +: Un cut *flaA* gene PCR product, and Lane M: Molecular weight marker (CinnaGen, Iran)

(35%). The occurrence of p1, p2, p3, p4, p5, p6, p7 and p9 types among *C. coli* isolates were 15% (3/20), 5% (1/20), 5% (1/20), 10% (2/20), 10% (2/20), 5% (1/20), 10% (2/20), and 15% (3/20), respectively. Between the nine different types, three patterns (p7, p8 and p9) were specific for *C. coli* strains. The most prevalent types among sheep and cattle isolates were p2 and p4, respectively. The p6 and p7 types were not detected among sheep and cattle isolates. The frequencies of p1 to p9 types among 20 sheep isolates were 1, 3, 5, 2, 2, 2, 0, 1 and 4, respectively. The frequencies of p1 to p9 types among 20 cattle isolates were 1, 2, 3, 1, 5, 3, 2, 0 and 3, respectively.

Results of the statistical analysis

The occurrence of *iamA*, *cgtB* and *pldA* genes in *Campylobacter* isolated from sheep faecal samples were significantly ($P<0.05$) more than those of cattle faecal isolates. The incidence of the *wlaN* gene in *Campylobacter* isolated from cattle faecal samples was significantly ($P<0.05$) more than that of sheep faecal isolates. The occurrence of other virulence markers did not differ significantly between cattle and sheep samples. Furthermore, the presence of the *waaC* gene in *C. jejuni* isolates was significantly more than that of *C. coli* strains. Statistical analysis of *flaA*-typing data showed that RFLP pattern 2 and RFLP pattern 8 were significantly predominant among *C. jejuni* and *C. coli* isolates, respectively. No correlation was observed between the presence of any virulence gene and the particular RFLP type.

Discussion

Research on the occurrence of potentially pathogenic *Campylobacter* in domestic animals and food with animal origins is essential to the consumers' safety. The primary objective of this study was to investigate the presence of 10 putative virulence markers among *C. jejuni* and *C. coli* isolated from cattle and sheep faecal samples. The first genes tested in this study were cytolethal distending toxin (CDT) associated genes. The CDT of *Campylobacter*, encoded by the *cdtA*, *cdtB*, and *cdtC* genes, damages host enterocytes and makes the penetration of the intestinal epithelium possible. The carrying of these genes can be variable, and can clarify virulence differences among strains (Rozynek *et al*., 2005). Another gene which was tested in the present study was the *cadF* gene, an adhesin and fibronectin-binding protein involved in the process of invasion, influencing microfilament organizations in host cells (Monteville *et al*., 2003). Analysis of the prevalence of the *cadF*, *cdtA*, *cdtB*, and *cdtC* genes revealed that all thermophilic *Campylobacter* isolates carried these markers, regardless of their origin and species. Similar observations have indicated the high prevalence of these genes in *Campylobacter* species isolated from various sources (Bang *et al*., 2003; Datta *et al*., 2003; Müller *et al*., 2006). The high percentage of these genes among isolates may indicate the important role of these virulence markers in *Campylobacter* pathogenesis. Another virulence gene linked with *Campylobacter* adherence and invasion and localized on the pVir plasmid, is the *virB11* gene (Bacon *et al*., 2000). Unlike

the above mentioned genes, *virB11* gene was not detected in *Campylobacter* isolates. Some investigations have reported similar results (Müller *et al*., 2006; Feng *et al*., 2009), while in other studies, this gene was identified in 7-20% of the tested isolates (Bang *et al*., 2003; Datta *et al*., 2003; Wieczorek and Osek, 2008). These differences may be due to genetic variations of the isolates from diverse geographical areas and the plasmid nature of *virB11*. Carvalho *et al*. (2001) showed the presence of the genetic marker of *Campylobacter* strains, isolated from children with diarrhea and symptom-free children, to be associated with adherence and invasion and named it "invasion-associated marker" (IAM). However, Al-Mahmeed *et al*. (2006) discussed the absence of this marker in isolates from clinical cases in children but not from older patients, and suggested more research on its prevalence. Similar to the *iamA* gene, the *pldA* gene is responsible for the expression of invasion (Ziprin *et al*., 2001). The results of the present study showed dissimilar occurrences of *iamA* and *pldA* genes in cattle and sheep isolates. The *pldA* gene was not present in cattle isolates and the presence of the *iamA* gene in *Campylobacter* isolated from sheep samples was more than that of cattle isolates, causing them to be potentially more virulent and invasive than cattle isolates. The next virulence markers examined in the present study were the three lipooligosaccharide (LOS)-associated genes, *wlaN*, *cgtB* and *waaC*. The results showed differences in the occurrence of LOS-associated genes among cattle and sheep isolates, where the occurrence of the *cgtB* gene was higher in sheep isolates and that of the *wlaN* gene higher in cattle isolates. Molecular mimicry between LOS in the *Campylobacter* cell wall and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of the Guillain-Barré Syndrome (GBS), an acute paralytic human neuropathy (Ang *et al*., 2004). Several genetic mechanisms responsible for producing variation in LOS have been described as variation in homopolymeric tracts, single-base deletions, insertions, and mutations that can lead to gene inactivation or glycosyltransferases with different acceptor specificities, resulting in the expression of different LOS structures (Gilbert *et al*., 2002). In addition, previous studies show that a *C. jejuni* strain without GM1-like molecules acquires large DNA fragments, including lipooligosaccharide synthesis genes, from a strain expressing GM1-like molecules, and transforms consequently into a number of potential GBS-inducible transformants, which exhibit a high degree of genetic and phenotypic diversity (Phongsiasay *et al*., 2006). Therefore, the recognition of the genes involved in LOS synthesis and the study of their regulation is of great interest to obtain better understanding of the pathogenesis mechanisms used by these bacteria (Gilbert *et al*., 2000). In the presence of different LOS-associated genes, these diversities may lead to variations in LOS antigenic structure of *Campylobacter* strains and change antigenic properties of campylobacters originating from different sources (Nakari *et al*., 2005). Results showed that cattle and sheep *Campylobacter* isolates have different LOS-associated genetic properties in this geographical area. Moreover, this result shows that *C. jejuni* was not identifiable from *C. coli* based on the distribution of these virulence genes. Aiming at assessing strain diversity, isolates were characterized by *flaA* PCR-RFLP. Comparisons of molecular types of *Campylobacter* spp. originating from different origins will help obtain a better understanding of the epidemiology of campylobacteriosis in humans. The presence of highly conserved and variable regions in the *flaA* gene makes this locus suitable for PCR-RFLP analysis (Shi *et al*., 2002); furthermore, among different restriction enzymes, *Dde*I has been reported to provide the best discrimination for veterinary isolates (Ayling *et al*., 1996). According to the results, *C. coli* isolates showed higher diversity compared with *C. jejuni* strains. In addition, the predominant *flaA*-typing pattern of *C. jejuni* strains was significantly different from that of *C. coli* isolates (Tables 3 and 4). In a total of 20 *Campylobacters* isolated from cattle, the predominant *flaA*-typing pattern was pattern p4 (20%) and among 20 *Campylobacters* isolated from sheep, the predominant *flaA*-typing pattern was pattern p2 (20%). There were no significant differences in the numbers of distinct RFLP patterns (p8 and p9) among sheep and cattle isolates (Tables 3 and 4). In the current study, a total number of nine distinct *flaA*-types were obtained not showing high diversity in isolates compared with other similar studies on cattle and sheep *Campylobacter* isolates (Fitzgerald *et al*., 2001; Bang *et al*., 2003; Açik and Etinkaya, 2005; Oporto *et al*., 2007). These differences in genetic variation among thermophilic *Campylobacter* strains can be explained by the geographical diversity of strains. Although it is known that chickens are major reservoirs for *Campylobacter* spp., it is also essential to establish the importance of other reservoirs, particularly cattle and sheep, to evaluate their relative contribution to human infection. In the present study, a high prevalence of different putative virulence-associated genes among *C. jejuni* and *C. coli* isolates was determined. These markers and determinants show a potential *Campylobacter* pathogenicity for people; however, they do not imply direct bacterial virulence. The results did not show any correlation between profiles generated by molecular techniques and the presence of virulence markers.

In conclusion, the role of cattle and sheep as reservoirs of these pathogens might be important for understanding the epidemiology of *Campylobacter* infections. However, *Campylobacter* characteristics and pathogenic properties and diversities have not been extensively described in the peer reviewed literature, particularly in Iran. These finding should be taken into account in future investigations towards developing effective control strategies against *C. jejuni* and *C. coli* infections.

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