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. Others 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, tamA, 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 neuropa(thies (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 pathogens 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 gangloside 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
faecal 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 Amphotericin-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 dose] (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 MgCl2, 50 mM KCl, 20 mM (NH4)2SO4], (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

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence (5’ to 3’ )</th>
<th>Target gene</th>
<th>Annealing temperature</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS-18</td>
<td>CTTTTGGATCAGGAAGCATTC</td>
<td>cdtA</td>
<td>49°C</td>
<td>370</td>
<td>(Hickey et al., 2000)</td>
</tr>
<tr>
<td>DS-15</td>
<td>ACATCCATTTGCTCTCTTG</td>
<td>cdtB</td>
<td>51°C</td>
<td>620</td>
<td>(Datta et al., 2003)</td>
</tr>
<tr>
<td>cdtB-113</td>
<td>CGAAACGCAAATGAGGTTT</td>
<td>cdtC</td>
<td>48°C</td>
<td>182</td>
<td>(Datta et al., 2003)</td>
</tr>
<tr>
<td>cdtB-713</td>
<td>AAGCTAAGGCGGGCGATAT</td>
<td>virB11</td>
<td>53°C</td>
<td>494</td>
<td>(Datta et al., 2003)</td>
</tr>
<tr>
<td>cdtC-192</td>
<td>CGATGAGGTTAAGAAATTTT</td>
<td>iamA</td>
<td>52°C</td>
<td>518</td>
<td>(Carvalho et al., 2001)</td>
</tr>
<tr>
<td>cdtC-351</td>
<td>TCTCTGGTCTGTTACCTACCTTT</td>
<td>wlaN</td>
<td>56°C</td>
<td>330</td>
<td>(Wassenar et al., 2002)</td>
</tr>
<tr>
<td>virB-701</td>
<td>CCTTCCTGTTTCTGTTTCTTCC</td>
<td>waaC</td>
<td>42°C</td>
<td>1029</td>
<td>(Godschalk et al., 2007)</td>
</tr>
<tr>
<td>iamA F</td>
<td>GCCAAGAAATATATCCAACC</td>
<td>cdtB</td>
<td>56°C</td>
<td>562</td>
<td>(Linton et al., 2000)</td>
</tr>
<tr>
<td>iamA R</td>
<td>TTACCAAGCATAGTACC</td>
<td>cdtB</td>
<td>42°C</td>
<td>913</td>
<td>(Datta et al., 2003)</td>
</tr>
<tr>
<td>wlaN F</td>
<td>TCGCGTGTATACAAAGGTGTTG</td>
<td>pldA</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>wlaN R</td>
<td>ATTTTTGGTATGGGTTG</td>
<td>pldA</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>waaC C1</td>
<td>TTAAGGAAGGATAGTATGGT</td>
<td>pldB</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>waaC C2</td>
<td>GATCAAAAAAATGCGGATTCA</td>
<td>pldC</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>cdtB R</td>
<td>GTTCTGGTCTGTTTCTTCA</td>
<td>pldD</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>pldA-84</td>
<td>AACCTTGATGCTTTTTTTTTT</td>
<td>pldA</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>pld-981</td>
<td>TATTTTTTTTTTTTTTTTTTTTT</td>
<td>pldB</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>cadF F</td>
<td>TTAGGAATATGATAGTATAT</td>
<td>cadF</td>
<td>42°C</td>
<td>400</td>
<td>(Konkel et al., 1999)</td>
</tr>
<tr>
<td>cadF R</td>
<td>CTATATCTAAAGGGCGAC</td>
<td>cadF</td>
<td>42°C</td>
<td>400</td>
<td>(Konkel et al., 1999)</td>
</tr>
<tr>
<td>Fia1</td>
<td>GGAAGTTCCATTTAACCGAAGC</td>
<td>fiaA</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
<tr>
<td>Fia2</td>
<td>CTCTGGATTTTCAAACCAATCA</td>
<td>fiaA</td>
<td>45°C</td>
<td>1725</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
</tbody>
</table>
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 DdeI (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 *pilA* 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*, *cgdB* 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.
isolates were (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 was 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 cytotoxic 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, DdeI 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 Etkinaya, 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 findings should be taken into account in future investigations towards developing effective control strategies against C. jejuni and C. coli infections.

Acknowledgement

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