

Genetic Characterization of *Campylobacter Jejuni* and *C. coli* Isolated From Broilers Using *flaA* PCR-Restriction Fragment Length Polymorphism Method in Shiraz, Southern Iran

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Background: Thermophilic campylobacters, particularly *Campylobacter jejuni* and *C. coli* are the main agents of human campylobacteriosis. *Campylobacter* contaminated chicken products is the most important source of foodborne gastroenteritis. Evaluation of genetic diversity among *Campylobacter* population is critical for understanding the epidemiology of this bacterium and developing effective control strategies against *Campylobacter* infections and other related disorders.

Objectives: The aim of this study was to investigate the polymorphism of thermophilic *Campylobacter* isolated from broiler fecal samples in Shiraz, southern Iran.

Materials and Methods: Ninety *Campylobacter* isolates were recovered from broiler feces using enrichment process followed by cultivation method. The isolates were species typing on the basis of polymerase chain reaction (PCR) detection of 16S rRNA and multiplex PCR for determining two thermophilic species. To evaluate strain diversity of thermophilic *Campylobacter* isolates, *flaA* PCR-Restriction Fragment Length Polymorphism (RFLP) was performed using DdeI restriction enzyme.

Results: All 90 *Campylobacter* isolates confirmed by m-PCR were successfully typed using *flaA*-PCR-RFLP. Eleven different types were defined according to *flaA*-typing method and the RFLP patterns were located at three separate clusters in RFLP image analysis dendrogram.

Conclusions: *Campylobacter jejuni* isolates significantly showed more variety than *C. coli* isolates. A relatively low genetic diversity existed among *C. jejuni* and *C. coli* isolated from broilers in Shiraz, southern Iran. In our knowledge, this was the first report of genetic diversity among broiler originated human pathogen thermophilic campylobacters in Shiraz, southern Iran.

Keywords: *Campylobacter Jejuni*; *C. coli*; Restriction Fragment Length Polymorphism; Iran

1. Backgrounds

Thermophilic *Campylobacter* species, particularly *Campylobacter jejuni* and *C. coli* are zoonotic bacteria frequently associated with human diarrhea in developing and industrialized countries (1). *Campylobacter* infections are acquired by ingestion of contaminated food, water, or milk, but the sources and transmission routes of campylobacteriosis in humans remain debatable. Numerous vehicles for transmission of *Campylobacter* infection have been described; however, chicken appears to be the most significant agent for sporadic cases (2-4). To reduce the occurrence of campylobacteriosis in humans, it is essential to improve general understanding of the epidemiology of infection.

The use of conventional bacteriological tests for differentiation and species identification of campylobacters is often hampered by the fact that these bacteria are fastidi-

ous and possess few distinguishing biochemical characteristics (5). In the past decades, various methods have been developed for discrimination of *Campylobacter* at the DNA levels to trace various sources of infection and genotyping replaced the traditional typing methods such as serotype classifications (6-8). Among various genotypic methods, PCR-Restriction Fragment Length Polymorphism analysis of the flagellin-A gene (*flaA*) is widely used because of its rapidity, ease and cost-effectiveness (9). The existence of extremely conserved and inconsistent regions in *flaA* gene makes the locus proper for scrutiny of the effects of different restriction enzymes (10).

2. Objectives

The aim of this study was to determine genetic diversity

of random collection of *C. jejuni* and *C. coli* strains isolated from broiler feces in Shiraz, southern Iran, using PCR-RFLP and investigate the association of *Campylobacter* species and genetic polymorphism.

3. Materials and Methods

Totally, 42 *C. jejuni* and 48 *C. coli* were recovered from fecal samples of the broilers in 10 visits of sampling from Shiraz slaughterhouse in January 2012. The fecal samples were collected in Tryptic Soy Broth (TSB) broth tubes by sterile gloves and brought to the laboratory on ice packs in less than six hours. To eliminate other bacteria, 0.8 µm membrane filter was used and 250 µL of filtered samples was 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), Amphotericin-B (10 mg/L)], incubated in a microaerophilic atmosphere (Merck, Germany) at 37°C for 4 hours, followed by incubation at 42°C for 44 hours. Thereafter, 50 µL of enriched samples in TSB was cultured on selective agar [brucella agar base (41 g/L), and above antibiotics with identical dose] (11). All culture media were from Merck, Germany. 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 (2). The type strains *Campylobacter jejuni* (ATCC 33291) and *Campylobacter coli* (RTCC 2541) were included as positive controls for culture identification of isolates.

3.1. DNA Extraction

DNA extraction was performed using the phenol-chloroform extraction technique previously described by Khoshbakht et al. (12). Briefly, a loopful colony of each isolates on agar plate was picked and suspended in 200 µL distilled water. After vortexing, samples were centrifuged at 10000 × g, the supernatants were discarded before adding 250 µL of buffer 1 (resuspension solution contained 100 µg/mL RNase) and 250 µL buffer 2 (Lysis buffer solution contained Tris-HCl and EDTA), a 550 µL saturated phenol was then added, mixed thoroughly and centrifuged at 8000 × g. The supernatant was collected into a new tube; 550 µL of the phenol was then added and centrifuged at 8000 × g again. The supernatant aqueous phase was collected into a fresh tube, previous to adding sodium acetate (2 M, pH 5.2, 0.1 × volume of each aliquot). Then 1.5 mL 100% ethanol were added to the aliquots and mixed, kept at -20°C for 1 hour, centrifuged at 12,000 × g, the supernatant was then discarded and DNA pellet was washed by 80% ethanol, before being dried and suspended in 30 µL of 1 × TE buffer until use.

3.2. Simple and Multiplex PCR Assay

Simple and multiplex PCR reactions were performed for identification of *Campylobacter* genus, *C. jejuni* and *C. coli* species, respectively. PCR amplifications were performed in a final volume of 25 µL. The reaction mixtures consisted of 2 µL DNA template, 2.5 µL 10 × 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 (1U Ampli Taq DNA polymerase), (CinnaGen, Iran), 1 µL (25 pmol) from the forward and reverse primers (CinnaGen, Iran), shown in Table 1 and the volumes of the reaction mixtures were reached to 25 µL using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing as shown in Table 1 for 1 minute and extension at 72°C for 1 minute. Final extension was performed at 72°C for 10 minute and the PCR products were remained in the thermal cycler at 4°C until they were collected. Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. 100 bp DNA ladder was used as molecular size markers. Visualization was undertaken using a UV transilluminator (BTS-20, Japan). The type strains *C. jejuni* (ATCC 33291) and *C. coli* (RTCC 2541) were included as positive controls for PCR identification of the isolates and the master mix without sample DNA used as negative control.

3.3. FlaA PCR-Restriction Fragment Length Polymorphism

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 (Table 1) of the *flaA* gene was amplified in a PCR reaction using a pair of specific primers listed in Table 1 previously described by Nachamkin et al. (13). Seven microliters of all the amplicons were restricted with 4 U DdeI (Thermo scientific, Germany) in 1.5 µL 10 × recommended restriction buffer in the final volume of 20 µL and then were incubated at 37°C for 15 hours. The digested PCR products (15 µL) were immediately separated on 2.5% agarose gel stained with ethidium bromide. Bands were photographed under UV transilluminator and the results were evaluated manually. The 50 bp DNA ladder (CinnaGen, Iran) was used as molecular marker to estimate the size of bands. Dendrogram was constructed based on the obtained DNA fragment patterns using Phoretix 1D Version 10 software.

3.4. Statistical Analysis

Statistical analysis was performed using SPSS version 12.0.1. Discrete variables were expressed as percentages and proportions were compared using Chi-squared test with the significance level defined at P < 0.05.

Table 1. Nucleotide Sequences used as Primers in the PCR Reaction for Identification of *Campylobacter* Genus and *C. jejuni* and *C. coli* Species^a

Name of Primer	Sequence (5´ - 3´)	Target Gene	Annealing Temperature, °C	Product Size, bp	Reference
MapA		mapA (<i>C. jejuni</i>)	52	589	(14)
F	CTATTTATTTTGGAGTGCTTGTC				
R	GCTTTATTGCCAATTTGTTTATTA				
Coli		ceuE (<i>C. coli</i>)	52	462	(14)
F	AATGAAAATGCTCCAACATG				
R	TGATTTATTATTGTAGCAGCG				
PLO6	GGTTAAGTCCCACAACGAGCCGC	16SrRNA (genus specific)	50	283	(15)
CAMPC5	GGCTGATCTACGATTACTAGCGAT	16SrRNA (genus specific)	50	283	(15)
Fla		flaA (RFLP)	45	1725	(13)
1	GGATTCGTATTAACACAAATGGTGC				
2	CTGTAGTAATCTTAAAACATTTG				

^a Abbreviations: F, forward; R, reverse; RFLP, restriction fragment length polymorphism.

Table 2. flaA Typing Results of *C. jejuni* and *C. coli* Isolates Recovered From Broiler Fecal Samples^a

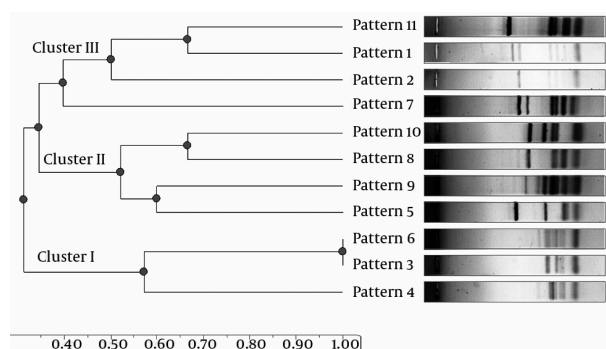
Species	No.	Occurrence of Different flaA-RFLP Patterns										
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
<i>C. jejuni</i>	42	8 (19)	4 (9.5)	4 (9.5)	0 (0)	3 (7.1)	2 (4.7)	7 (16.6)	2 (4.7)	4 (9.5)	6 (14.2)	2 (4.7)
<i>C. coli</i>	48	3 (6.2)	0 (0)	20 (41.6)	3 (6.2)	2 (4.1)	12 (25)	0 (0)	6 (12.5)	0 (0)	2 (4.1)	0 (0)
Total	90	11 (12.2)	4 (4.4)	24 (26.6)	3 (3.3)	5 (5.5)	14 (15.5)	7 (7.7)	8 (8.8)	4 (4.4)	8 (8.8)	2 (2.2)

^a Data are presented as No. (%)

4. Results

In total, 90 *Campylobacter* isolates including 48 *C. coli* and 42 *C. jejuni* were recovered from fecal samples and identified based on simple and multiplex PCR reactions. All 90 isolates were typed successfully and 11 different genotypes (Table 2) were defined by flaA-PCR-RFLP method. The most prevalent flaA-typing pattern of the isolates was F3 (26.6%). Among 42 *C. jejuni* originating from broiler fecal samples, 10 different types (all types except F4) were defined (Table 2) and the most common type was F1 (19%). Among 48 *C. coli* isolates, seven types (F1, F3, F4, F5, F6, F8 and F10) were defined and the most common type was type F3 (41.6%). Between 11 different types, four patterns (F2, F7, F9 and F11) were specific for *C. jejuni* and one pattern (F4) was specific for *C. coli* isolates. Statistical analysis of flaA-typing data showed that RFLP patterns F2 and F10 were significantly ($P < 0.05$) predominant among *C. jejuni* and RFLP patterns F3 and F6 were significantly ($P < 0.05$) predominant among *C. coli* isolates. The phylogenetic analysis based on the dendrogram generated from RFLP-PCR demonstrated that *C. jejuni* and *C. coli* isolates belong to three clusters (I, II and III in Figure 1). 45.5% (41/90) of isolates were found in cluster I and 27.7% (25/90) and 26.6% (24/90) of isolates belonged to clusters II and III, respectively. The dominant cluster of *C. jejuni* and

Figure 1. Dendrogram Based on Restriction Profiles of flaA Gene



Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with a dice coefficient distance tree was generated with Phoretix 1D Version 10 software. The numbers (0.40 to 1.00) show the similarity scale. Circles define the point of the similarity level.

C. coli isolates was significantly ($P < 0.05$) difficult, so that 72.9% (35/48) of *C. coli* belonged to cluster I, while 50% (21/42) and 35.7% (15/42) of *C. jejuni* belonged to clusters III and II, respectively (Figure 1).

5. Discussion

Typing of *Campylobacter* isolates from different sources provides epidemiological information needed for infection control and contributes to risk evaluation of transmission. Genotype-based identification methods have been developed to avoid the problems caused by biochemical inertness of *Campylobacter* species. These methods are now more frequently used, but have yet to be standardized. The application of molecular typing methods can offer a constant and highly discriminatory investigation of bacterial isolates. The present study considered the heterogeneity among *C. jejuni* and *C. coli* isolates obtained from broiler feces by molecular methods for the first time in Iran.

PCR-RFLP genotyping method was used to explain genetic diversity among these isolates. All 90 isolates confirmed based on m-PCR were typed successfully using fla typing method. Harrington et al. (16) in their study compared three different methods of flaA-RFLP technique and showed that, full flaA gene and DdeI digestion are proper for fla-typing of campylobacters. Other similar studies indicated the benefits of full length flaA gene and DdeI (17, 18), which we used in this study. In the current study, 11 distinct flaA-types were obtained, using DdeI, which did not show high diversity among isolates compared with other studies on *Campylobacter* isolates from some other sources (19-22). These differences in genetic variation among thermophilic *Campylobacter* strains can be explained by geographical diversity, different number of isolates compared with other studies and difference in origin. Compared with other poultry-originated *Campylobacter* isolates in some studies (9), the isolates in the present study showed lower diversity, but some other investigations showed lower diversity (1).

Results showed that among 11 RFLP patterns, six (54.5%) patterns were similar between *C. jejuni* and *C. coli* strains. Ertas et al. (1) showed seven different fla-types among *C. jejuni* and *C. coli* isolates, which all were similar among two species. Nevertheless, Oporto et al. (22) in analysis of thermophilic campylobacters from cattle, sheep and swine farms obtained 46 different patterns, which only five (10.8%) were similar between *C. jejuni* and *C. coli* isolates. These data indicate low genetic diversity among our study broiler originated thermophilic campylobacters. More studies are necessary to evaluate genetic diversity differences between two thermophilic species. Isolation of *Campylobacter* from each cloacal swab, without resorting to pooling, made it possible to perform an epidemiological study using a molecular method such as flaA-typing to evaluate the relatedness of *Campylobacter* isolates of each farm.

Our isolates were not recovered from a single commercially reared flock and samples were collected randomly from separate flocks in slaughtering line at the end of the rearing period. Genetic differences between poultry-originated isolates may be due to variety in age

of chickens, site of sampling and category of rearing (23). According to the results, *C. jejuni* isolates showed significantly ($P < 0.05$) higher diversity (10 patterns) than *C. coli* isolates (7 patterns). In addition, predominant flaA-typing patterns of *C. jejuni* strains were significantly ($P < 0.05$) different from *C. coli* isolates. Acik and Cetinkaya (21) showed that among 209 isolates, 28 different flaA types were found. Twenty-three flaA types were identified among 179 *C. jejuni* isolates and the remaining five from *C. coli* isolates. Using RFLP image analysis, dendrogram was drawn and we examined the genomic association between the *Campylobacter* isolates tested in the study. *C. jejuni* and *C. coli* were randomly distributed in three clusters, but most isolates were found in cluster I (Figure 1). The results showed that *C. jejuni* and *C. coli* isolates significantly ($P < 0.05$) belonged to different clusters in phylogenic dendrogram drawn according to flaA-RFLP patterns. Clusters I and III had significant ($P < 0.05$) relatedness with *C. coli* and *C. jejuni*, respectively. This can show the differences between fla-typing features of these two species, which can be observed by sequencing and comparison of all flaA PCR products. Three of four patterns, which were not present in *C. coli* isolates, were in cluster III.

In conclusion, the presence of campylobacters in broiler feces can contaminate the environmental and human food chain. Therefore, detection of *Campylobacter* spp. in broiler originated samples is important to identify possible sources of infection and to have a better understanding of the epidemiology of infection subtyping of isolates is considered essential. Results indicated that Fla-typing is a simple and low-cost typing method characterized by a high level of discrimination power and it is an efficient tool for identifying any outbreak-related strains in short-term investigations. The results of this study revealed a comparatively low heterogeneity among *C. jejuni* and *C. coli* isolated from the broilers in southern Iran. In addition, results showed that the predominant RFLP patterns of *C. coli* and *C. jejuni* isolates can be significantly different and belong to various distinct phylogenic clusters. These molecular epidemiologic findings should be taken into account in the investigations towards developing effective control strategies against *C. jejuni* and *C. coli* infections.

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Authors' Contributions

All Authors had equal contribution in the manuscript.

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