



Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis of Three Lipooligosaccharide-Associated Genes of *Campylobacter jejuni* and *Campylobacter coli* Isolated From Animal Samples

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Abstract

Background: Several genetic mechanisms are used by *Campylobacter* spp. to achieve pathogenesis. One of the involved virulence factors is lipooligosaccharide-associated genes, which are related to ganglioside mimicry by *Campylobacter* species.

Objectives: The current study was conducted to determine the genetic diversity of 3 LOS-associated genes among *Campylobacter jejuni* and *C. coli* isolated from animal fecal samples.

Methods: One hundred broiler, cattle, and sheep fecal *C. jejuni* and *C. coli* isolates, which had been collected previously from Shiraz slaughterhouses and had been formerly identified by polymerase chain reaction (PCR) reactions, were used in the present study. *Campylobacter* species were subjected to detect *wlaN*, *cgtB*, and *waaC* genes. The PCR products of three LOS-genes were subjected for restriction fragment length polymorphism (RFLP) using HindIII and AluI restriction enzymes in separate reactions. The most prevalent RFLP patterns in the combination of 2 enzymes were subjected for sequencing and sequence analysis software.

Results and Conclusions: Patterns of RFLP for PCR products of all 69 *wlaN* and 29 *cgtB* genes were similar yet among 86 *waaC* gene PCR products, 6 different RFLP patterns were obtained. In conclusion, PCR-RFLP analysis demonstrated considerable variation in gene content and overall sequence heterogeneity in the animal-associated *C. jejuni* and *C. coli* LOS biosynthesis genes.

Keywords: *Campylobacter*, Virulence Factors, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism

1. Background

The infections caused by thermophilic campylobacters, mainly *Campylobacter jejuni*, are recognized by symptoms ranging from mild diarrhea to serious neuropathies (1). Also, the pathogenesis of *Campylobacter* infections is not clearly understood, yet several virulence-associated genes have been described, most of which are associated with pathogenicity (2). One of the most important locuses of the *Campylobacter* genome is a highly changeable region, known as the lipooligosaccharide (LOS) biosynthesis locus, which has an effective role in some aspects of pathogenicity (3, 4). Various genetic features and variability of the LOS locus allow *Campylobacter* to establish a molecular mimicry between LOS of the bacterial cell wall and gangliosides in human peripheral nerves, which plays an important role in the pathogenesis of an acute para-

lytic neuropathy, known as Guillain-Barre' Syndrome (5). Several genetic mechanisms are used by *Campylobacter* to turn on or turn off a gene to modulate the substrate specificities of the glycosyltransferases for LOS synthesis. Mechanisms include variation in homopolymeric tracts, single-base deletions, insertions and mutations, which cause the expression of different LOS structures (3, 6). Since different foods with animal origin have been incriminated as the main source for *Campylobacter* infections in humans (7), investigation of the LOS-associated virulence markers of *Campylobacter* strains isolated from food animals, such as broilers, cattle, and sheep is important.

2. Objectives

The present research was conducted to evaluate the genetic diversity of 3 LOS-associated genes (*wlaN*, *cgtB*, and

waaC) in *C. jejuni* and *C. coli* isolated from poultry, cattle, and sheep fecal samples, using the comparative polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

3. Methods

3.1. Bacterial Strains and Positive Controls

In the present study, the researchers used a total of 100 *Campylobacter jejuni* and *C. coli* strains, which had been isolated and recognized in previously published researches (8, 9). The isolates had been identified using cultivation and polymerase chain reaction (PCR) procedures with specific primers. Strains with positive results for presence of *cgtB*, *wlaN*, and *waaC*, LOS-associated genes were subjected to PCR-restriction fragment length polymorphism, using previous or new specific primers. Detailed information about positive strains for presence of the genes used in this study and their origins are listed in Table 1. In addition, the type strains *C. jejuni* (ATCC 33291) and *C. coli* (RTCC 2541) were included as positive controls for PCR reactions.

3.2. DNA Concentration and PCR-RFLP Assay

Bacterial DNA, which had been previously extracted and purified by using the phenol-chloroform procedure, was used as DNA template in PCR reactions. The concentration of the DNA was estimated by spectrophotometry at 260 and 280 nm before PCR examination. The previously positive strains for presence of *cgtB*, *wlaN* and *waaC* genes (8, 9) were subjected to amplification of the complete sequence of the genes, using specific primers listed in Table 2. The PCR amplifications were performed in a final volume of 25 μ L. The reaction mixtures consisted of 2 μ L of the template DNA, 2.5 μ L 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), 1 μ L dNTPs (50 μ M), 1 μ L of 1U Ampli Taq DNA polymerase, and 1 μ L of 25 pmol from the forward and reverse primers (CinnaGen, Iran), shown in Table 2, and the volume of the reaction mixtures were increased to 25 μ L 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 2 for 1 minute, and extension at 72°C for 1 minute. Final extension was carried out at 72°C for 7 minutes and the PCR products were remained in the thermal cycler at 4°C until they were collected.

The PCR products of *wlaN*, *cgtB*, and *waaC* genes were subjected for RFLP using HindIII and AluI restriction enzymes (Thermo scientific, Fermentas) in separate double repeat reactions. The amplicons were digested with 4 U of

each restriction enzyme in 1.5 μ L of 10x restriction buffer (Buffer R for HindIII and B1 buffer for AluI) and then incubated at 37°C for 15 hours, according to the manufacturer's guidance. The digested PCR products (15 μ L) were immediately separated on 2.5% agarose gel stained with ethidium bromide and photographed under UV transilluminator. The 100-bp DNA ladders were used as molecular markers to evaluate the size of the bands (Table 3).

3.3. Sequencing and Alignment of Virulence Genes' PCR Products

From PCR products of LOS-associated genes (*wlaN*, *cgtB*, and *waaC*), amplicons with the most prevalent RFLP pattern using combination of HindIII and AluI restriction enzymes were subjected for sequencing (Takapouzist Inc., Iran). To assess the diversity of the genes, they were compared with the GenBank databases, using the BLAST program maintained by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and multiple sequence alignments were made by the ClustalW method, using the MEGA5 software (10).

3.4. Statistical Analysis

Statistical analysis was performed using the SPSS version 12.0.1 software. Discrete variables and proportions were compared using the Chi-square test with the significance level defined at $P \leq 0.05$.

4. Results

4.1. The RFLP Results of LOS Genes

Among 69 *wlaN* gene PCR products, all RFLP patterns using 2 restriction enzymes (HindIII and AluI) were similar (Figure 1). Furthermore, all 29 *cgtB* gene PCR products showed similar RFLP patterns using 2 restriction enzymes (Figures 2 and 3). Among 86 *waaC* gene PCR products, 6 different RFLP patterns were obtained using 2 restriction enzymes in combination. Three patterns were obtained using each of the restriction enzymes separately (Figures 1 and 2) and the most prevalent pattern was H1A1 (detected in 40% of the *waaC* amplicons). These patterns and their frequency were H1A1 (34), H2A3 (17), H3A2 (15), H1A2 (14), H1A3 (3), and H2A2 (2).

4.2. Nucleotide Sequence Accession Numbers and Sequence Analysis

Nucleotide sequences were deposited with GenBank section of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and assigned accession numbers were as follows: *wlaN* gene, KY965833; *cgtB* gene, KY965832; *waaC* H1A1 pattern, KP715105; *waaC* H2A3

Table 1. Number Virulence-Associated Genes Amplicons and Their Sources Among Campylobacter Isolates

Species	Biological Origin	No. of Strains Generating Specific Gene Amplicon		
		<i>wlaN</i>	<i>CgtB</i>	<i>WaaC</i>
<i>C. jejuni</i>	Cattle	10	5	10
	Sheep	5	7	8
	Chicken	22	8	25
<i>C. coli</i>	Cattle	4	0	7
	Sheep	1	5	7
	Chicken	27	4	28
total		69	29	85

Table 2. Nucleotide Sequences Used as Primers in the PCR Reaction for RFLP of the LOS Genes

Name of Primer	Sequence (5' to 3')	Target Gene	Annealing Temperature, °C	Product Size, bp	Reference
<i>wlaN</i> F	AGGGTTTAAATAGTTGCAATTTCTC	<i>wlaN</i> (RFLP)	50	912	(10)
<i>wlaN</i> R	ATGAAATTTTAAATCTTTACGGAATTA				
<i>waaC1</i>	TAATGAAAATAGCAATTGTTCTG	<i>waaC</i> (RFLP)	42	1029	(10)
<i>waaC2</i>	GATACAAAATCACTTTTATCGA				
<i>cgtB</i> F	TTAAGAGCAAGATATGAAGGTG	<i>cgtB</i> (RFLP)	56	562	(11)
<i>cgtB</i> R	GCACATAGAGAACGCTACAA				

Table 3. Distribution of the *waaC* Different RFLP Patterns Among Isolates

Origin (Number of Positives)	Species	<i>waaC</i> RFLP Pattern					
		H1A1	H1A2	H1A3	H2A2	H2A3	H3A2
Chicken (53)	<i>C. jejuni</i>	9	6	2	0	8	0
	<i>C. coli</i>	11	3	0	3	2	9
Cattle (17)	<i>C. jejuni</i>	4	2	0	0	4	0
	<i>C. coli</i>	3	1	0	0	0	2
Sheep (15)	<i>C. jejuni</i>	4	2	1	0	2	0
	<i>C. coli</i>	3	0	0	0	1	4
Total (86)		34	14	3	3	17	15

pattern, KR150515, and *waaC* H3A2 pattern, KR150516. The sequences of *waaC* amplicons showed more than 97% homology with the sequences previously reported for Campylobacter strains. The sequences of *wlaN* and *cgtB* amplicons showed more than 93% homology with the sequences of previously reported sequences (Figures 3 - 5).

4.3. Statistical Analysis

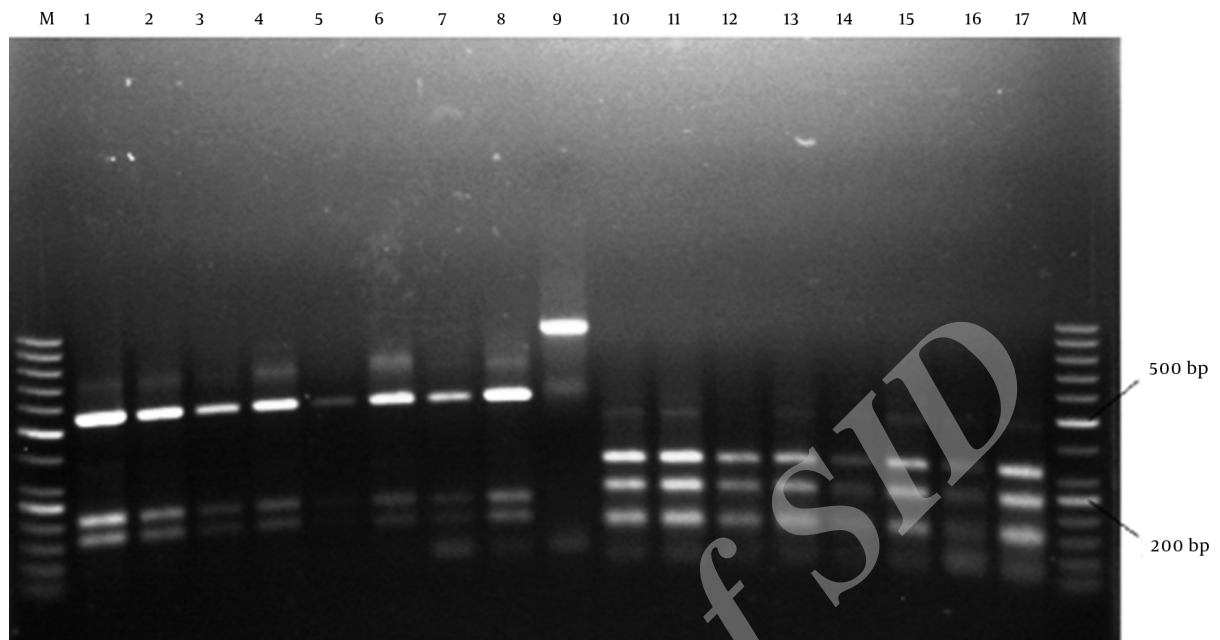
The predominant pattern of *waaC* in PCR-RFLP, using 2 restriction enzymes among both *C. jejuni* and *C. coli*, was H1A1. The H1A3 pattern was not present in *C. coli* isolates and

patterns H3A2 and H2A2 were not present in *C. jejuni* isolates. The H2A3 and H1A2 patterns were significantly higher in *C. jejuni* isolates compared with *C. coli* strains ($P < 0.05$). There were no significant correlations between *waaC* RFLP-pattern and Campylobacter origin.

5. Discussion

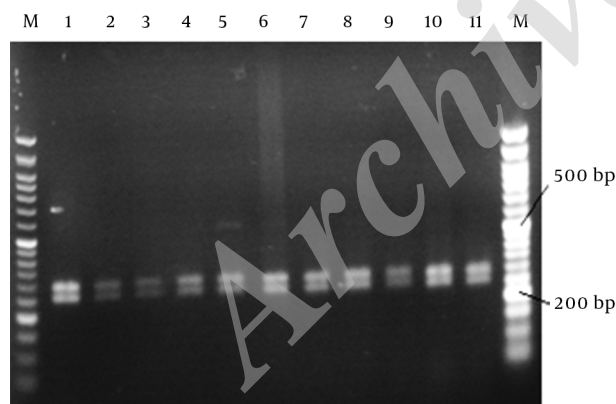
Ganglioside mimicry by Campylobacter Lipooligosaccharide (LOS) is considered an important factor in triggering of the Guillain-Barre and Miller-Fisher neuropathy

Figure 1. RFLP Result of *wlaN* Gene Using Two Restriction Enzymes



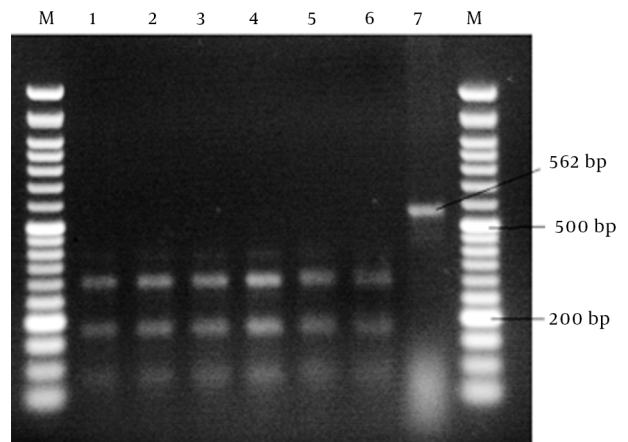
Lanes 1 - 8, amplified *wlaN* gene digested with HindIII; Lane 9, *wlaN* PCR product; lanes 10 - 17, pattern of cut-off sites of AluI; lanes M, DNA marker (Vivantis, Malaysia).

Figure 2. RFLP Result of *cgtB* Gene Using HindIII Restriction Enzyme



Lanes 1 - 11, digestion pattern of PCR products; lane M, DNA marker (SM0331; Fermentas).

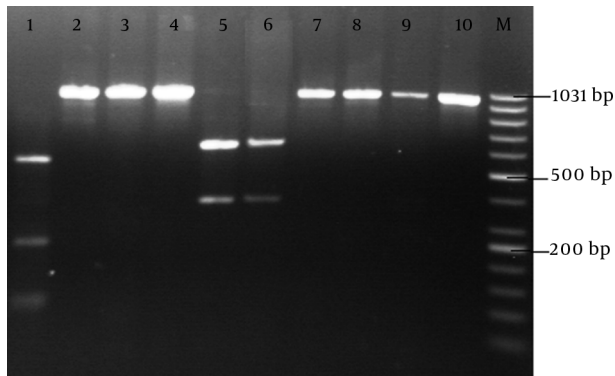
Figure 3. RFLP Result of *cgtB* Gene Using AluI Restriction Enzyme



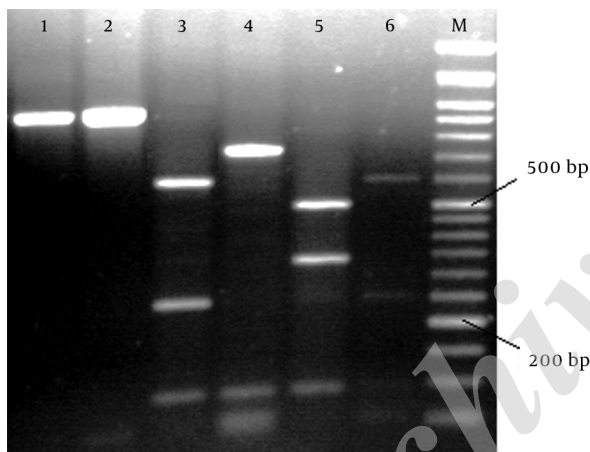
Lanes 1 - 6, pattern of digestion of PCR products; lane 7, *cgtB* PCR product lane M, DNA marker (SM0331; Fermentas).

syndromes after *Campylobacter* infections. Differences in the gene content of the LOS loci provide a basis for diversities in LOS outer cores among *C. jejuni* strains, which resulted in grouping to 8 LOS classes A-H (11), based on the organization of about 40 distinct genes. Muller et al. reported strain-specific differences in the occurrence

of *cgtB* and *wlaN* genes, both of which encode a beta-1, 3-galactosyltransferase, connected with different abilities to colonize the chick gut and to invade Caco-2 cells (12). The results of a study performed by Muller et al. indicated that 80% of strains with no or weak colonization and inva-

Figure 4. RFLP Results of *waaC* Gene Using HindIII Restriction Enzyme

Lanes 2 - 4 and 7 - 9, pattern 1 (H1); lanes 5 and 6: pattern 2 (H2); lane 1, pattern 3 (H3); lane 10, *waaC* PCR product without effect of enzyme; lane M, DNA marker.

Figure 5. RFLP Results of *waaC* Gene Using the AluI Restriction Enzyme

Lanes 1 - 2, *waaC* PCR product without enzyme effect; lane 4, pattern 1 (A1); lane 5, pattern 2; (A2); Lanes 3 and 6, pattern 3 (A3) Lane M, DNA marker.

sion ability have no functional β -1, 3-galactosyltransferase encoded by *cgtB* or *wlaN* (6). Moreover, Feodoroff et al. showed that the presence of *cgtB* was associated with bloody stools, yet the other putative virulence factors did not correlate with any specific clinical findings (13). Moreover, the phase-variable expression of *wlaN* results in alternate ganglioside-mimicking LOS structures (14). Such differences, like gene variations and presence or absence of the gene in isolates with different origins or various geographic areas, could alter *Campylobacter* antigenic properties and change the ability of strains to infect their hosts. The researchers sequenced only one isolate of *wlaN* positive samples because all strains had similar RFLP pattern with two restriction enzymes. Indeed, all PCR products of

cgtB and *wlaN* genes in the study showed a similar RFLP pattern using HindIII and AluI restriction enzymes. In contrast, *cgtB* gene PCR product was smaller in size than the PCR product of Godschalk et al. due to a difference in oligonucleotides used as primers (15). However, for *wlaN* gene, the researchers used the same primers yet the results in contrast, showed one RFLP pattern, which was due to the number of restriction enzymes used compared with 4 restriction enzymes used by Godschalk et al. (16). At either end of the LOS biosynthesis loci are the heptosyltransferase genes, like the *waaC* gene, which encodes a heptosyltransferase I, that surround regions exhibiting significant variation in ORF content. Thus, these organisms likely synthesize novel LOS structures (17).

From 3 LOS-associated genes in the present study only inner core associated *waaC* gene, showed different RFLP patterns using HindIII and AluI restriction enzymes in combination. This phenomenon may be due to variations in the gene. Surprisingly, the *C. coli* isolates RFLP pattern of *waaC* gene ($P < 0.05$) were dissimilar compared with *C. jejuni* strains RFLP pattern of *waaC* gene and some RFLP patterns were only found in *C. jejuni* isolates or showed low presence in *C. coli* isolates (H1A3 and H2A3), and some RFLP patterns were present only in *C. coli* isolates (H2A2 and H3A2). This phenomenon could be due to differences between *C. jejuni* and *C. coli* in LOS structure formation. In summary, PCR-RFLP analysis of the three LOS-associated genes of thermophilic *Campylobacter* spp. isolated from food animal fecal samples showed significant dissimilarity in *waaC* gene content. Sequence results of the current study showed the presence of homopolymeric tract (poly C and poly G) only in *wlaN* gene of *Campylobacter jejuni* obtained from a poultry origin. A large number of potential genes contain homopolymeric tracts in *C. jejuni* (18). This apparently chimeric arrangement is suggestive of some form of recombination and variation. Parkhill et al. demonstrated that homopolymeric tracts are associated with several loci in the *C. jejuni* genome and subject to high frequency mutation (as high as one in every 2 cells in a population) (18). These variations resulted in changes of the open reading frame and in expression of intact or truncated proteins. Despite the presence of such sequence in the *wlaN* sequence, there was diversity in *wlaN* amplicons using AluI and HindIII enzymes.

5.1. Conclusions

In conclusion, the study showed that *Campylobacter* spp. isolated from different sources have various LOS properties and variations of the genes occurred mainly in inner core-related genes. In addition, *C. coli* and *C. jejuni* have significantly various RFLP pattern in association with the *waaC* gene.

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