Sequencing and comparative analysis of flagellin genes fliA and fliB in bovine Clostridium chauvoei isolates

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Key words: Clostridium chauvoei, flagellin, polymorphism

Abstract: BACKGROUND: Clostridium chauvoei is the etiological agent of blackleg as an endogenous infection in cattle. Flagella have been known to play a critical role in the protective immunity of animals to clostridial infections. C. chauvoei has two copies of fliC gene, namely fliA and fliB. OBJECTIVES: The aim of this study was the determination and nucleotide sequence analysis of both copies of fliC genes in vaccinal strain and Iranian C. chauvoei isolates. METHODS: Six specific primers for amplification of fliA, fliB, and flagellin (fliC) genes were designed by Oligo software. Polymerase chain reaction was performed to amplify a fragment of 700 bp for both copies of flagellin (fliA and fliB) genes. The nucleotide percentage identity and divergence among isolates were deduced using BLAST and MegAlign softwares. RESULTS: It was found that divergence in fliB was more than fliA by sequence alignment analysis. Six highly conserve regions, thirty-one SNPs and 13 amino acid polymorphisms were found in fliC gene (between fliA and fliB sequences) of Iranian C. chauvoei isolates. In comparative analysis, genomic similarity of the fliA and fliB genes between the vaccinal strain and examined field isolates was proved to be as high as 97.3 % and 98.2%, respectively. CONCLUSIONS: The fliC copies were identified as excellent biomarkers to study the molecular epidemiology and strain diversity among C. chauvoei isolates. The existence of genetic variation between two alleles of fliC gene in C. chauvoei is reported for the first time in Iran. In spite of some genetic variations, the immunologic cross protection test showed a high protection power of the local vaccine (produced by Razi Institute) against homologous and heterologous challenge.

Introduction

Blackleg in cattle has been recognized in Iran since 1938 (Ardehali et al., 1984). The disease is distributed in most cultivated areas, especially in plain rice fields, low hills, and sandy spots. The disease is generally known to affect cattle, but sheep, goats, swine, camels, deer, and mink are also susceptible (Ardehali and Darakhshan, 1975). Blackleg is a fatal disease for young animals between 10 months and two years of age (Blood et al., 1983; Mi-
yashiro et al., 2007; Uzal et al., 2003).

*Clostridium chauvoei* is a gram positive and spore forming anaerobic bacterium. *C. chauvoei* is the causative agent of blackleg with high mortality rate (Bagge et al., 2009). Death can occur due to septicemia (Kojima et al., 2001). Many Symptoms observed in blackleg are also created by *C. septicum*, *C. novyi*, and *C. perfirgens* (Kojima et al., 2001; Miyashiro et al., 2007). Distinguishing *C. chauvoei* from *C. septicum* based on physiologic and toxigenic characteristic is very difficult. Similarity, in 16s rRNA sequence between *C. chauvoei* and *C. septicum* is 99.3% that indicates similarity at phenotypic levels (Miyashiro et al., 2007).

Flagella are well controlled organelle and essential for microbial motility in many bacterial genera. While the genetics, regulation, assembly, and physical structure of the Gram negative bacterial flagellin has been extensively investigated, less is known about the flagella of Gram positive bacteria, and, in particular, clostridial flagella (Dauga et al., 1998; Ji et al., 2001). Different studies suggest that flagella are important factors in pathogenesis of bacteria (Attridge and Rowley, 1983; Morooka et al., 1985). Flagellin in *C. chauvoei* is important to induce protective immunity in host, but in contrast, in other clostridia, toxoid is very important for immunization (Tamura and Tanaka, 1984).

Flagellum is composed of four parts: (1) basal body consists of MS rings (*FliF*) and rod (Gram negative bacteria have L ring (*FlgH*) and P ring (*FlgI*) in addition to MS rings); (2) hook and associated hook-filament junction (*FlgK* and *FlgL*); (3) filament cap (*FliD*); and (4) Flagellar filament (*FliC*) which is composed from repeating the flagellin protein subunit, encoded by *fliC* gene (Macnab, 2004; Wilson and Miles, 1975). Comparison of the amino acid sequence of the flagella of many bacterial species has revealed a distinctive domain structure of the protein. The N- and C- terminal parts of the molecule, which are responsible for secretion and polymerization, are conserved among species, whereas the central regions, which produce the surface-exposed antigenic part of the flagellar filament, are highly variable (Reid et al., 1999).

The number of *fliC* gene copies in clostridia genera are different. The *C. chauvoei* has two copies of *fliC* gene in genomic DNA, which are named *fliA* and *fliB*. The *C. septicum* has three copies and *C. difficile* has only one copy of *fliC* gene (Sasaki et al., 2002). The particular structure of the flagellin gene with terminal conserved regions allow gene amplification and sequence analysis to study the variations in the central region.

The aim of this study was to identify molecular identification of two copies of *fliC* gene, *fliA* and *fliB*, in bovine *C. chauvoei* isolates.

**Materials and Methods**

**Bacterial strains and culture:** The *C. chauvoei* (Vaccine strain and three field isolates), which is used in this study, was collected from Anaerobic Bacterial Department of Razi Vaccine and Serum Research Institute of Iran (Table 1). All of the strains tested for biochemical identification fermented glucose, maltose, lactose, and sucrose. They did not ferment salicin, inulin, glycerol, and manitol.

**PCR amplifications (DNA extraction):** The bacterial isolates were cultured on Thio-glycolate consisted of liver broth, incubated at 37 °C for 48 hours in anaerobic condition. Bacterial cells were pelleted at 4000 rpm for 30 minutes and washed two times by sterile phosphate buffer saline. The pellets were re-suspended in 200 ul of HPLC-grade water. After boiling for 20 minutes and centrifugation, approximately 5 ul of supernatant was used as template for PCR assay.

**Amplification of *fliC* gene:** Two specific primers (CFC 5′-cat tgc tac agc agg taa ta<c>3′ and CRC 5′-gaa cag cac cta act tgg att<c>3′) were designed to amplify a fragment of 1000
bp of fltC gene. This PCR was tested for differentiation of C. chauvoei from other pathogenic clostridia, including C. septicum, C. tetani, C. novyi, and C. perfringens.

Amplification of fltC Gene Copies (fliA and fliB):

Two specific reverse primers for fliA (CRA, 5′-cca ctc tta act gtt aat act gca <t>-3′) and fliB (CRB 5′-cca ctt tta aca gtt aaa aca gca <c>-3′) were designed by Oligo software. The forward primer CFC was used commonly with both CRA and CRB reverse primers. Polymerase chain reactions were performed to amplify a fragment of 700 bp for both fliA and fliB genes.

PCR reactions were consisted of 1.5mM MgCl2; 0.5 unit Taq DNA Polymerase; 0.25 mM dNTPs; DNA template 100 ng/reaction; and 10 pmol of each primers in 50 μl total volume. The PCR program was run with initial denaturation at 95 °C for 5 min and 35 cycles of denaturation at 94 °C for 1 min; annealing temperature, 52 °C for 1 min; extension, 1 min at 72 °C; and a final extension at 72 °C for 10 min. Five microliters of the PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide (0.25 µg/ml) and documented with a gel documentation system.

Nucleotide sequencing and analysis: PCR products were purified (purification kit, Roche, Cat No. 11732668001) and were sequenced in two directions (Macrogen Co. South Korea). The nucleotide sequences of fliA and fliB genes were analyzed by Megalign software. The alignments of Iranian isolates were compared to each other and the fltC gene sequences of reference strains in the GenBank.

Results

FltC-PCR: A fragment of 1000 bp length was amplified from C. chauvoei by fltC specific (CFC and CRC) primers. Two copies of fltC (fliA and fliB) gene were amplified from PCR product of the previous step with a 700 bp size.

Two specific primers (CFC and CRC) could differentiate C. chauvoei from C. septicum, C. novyi type A, C. tetani, and C. perfringens.

Nucleotide sequence analysis: The dendrogram based on nucleotide sequences of fltC gene copies (fliA and fliB) of C. chauvoei is shown in Figure 1. All fliA and fliB sequences were located in two separated branches. Multiple alignment of fliA and fliB sequences recognized thirty-one single nucleotide polymorphisms: one SNP in nucleotide position 150, three SNPs in nucleotide positions (187-189), five SNPs in nucleotide position (484-495), two SNPs in position (515-516), three SNPs in positions (530-535), four SNPs in position (552-561), eight SNPs in positions (571-585), and five SNPs in nucleotide positions (592-604) were identified between fliA and fliB in all C. chauvoei. Six highly conserved regions between fliA and fliB at nucleotide positions (134-149), (153-186), (190-238), (271-483), (496-514), and (517-529) were observed. Nucleotide sequence alignment showed that the divergence in fliB is more than fliA (Table 2). Insilico translation of nucleotide sequences observed 13 single amino acid polymorphisms (SAPs) between fliA and fliB protein sequences.

Discussion

Flagellin, as a main virulence factor of C. chauvoei, is known as the cause of blackleg in animals (Alm and Guerry, 1993). Tamura et al (1984) demonstrated that the fltC protein has immunogenicity and protective roles (Kojima et al., 2000; Tamura and Tanaka, 1984; Tanaka et al., 1987). Sequence of N-terminal of flagellin protein has been used to obtain relations between several bacteria (Sasaki et al., 2002). Flagellin in flagellar structure has hairpin model in which the N-C terminal folds to inner flagellum and the central domain is exposed to environment. Diversity in internal domains causes antigenic diversity in Entero-
bacteriaceae (Joys, 1988; Kostrzynska et al., 1991; Tino, 1977). In some species of bacteria, such as Escherichia coli and Salmonella, only one of the flagella subunits is involved in the organization of flagellum, but in many of the bacteria, flagellar filament is organized from multi-flagella subunits, such as campylobacter and some of the clostridia.

Terponema pallidum has periplasmic flagella, this flagella is composed of several flagellin subunits (Alm and Guerry, 1993). Sasaki et al. (2002) demonstrated that one or more tandem

Table 1. Explanation of Clostridium species which were used in this study.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Clostridium sp</th>
<th>Source</th>
<th>Organ/tissue</th>
<th>District</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH 721</td>
<td><em>C. chauvoei</em></td>
<td>cattle</td>
<td>Muscle</td>
<td>Saveh</td>
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<tr>
<td>CH 740</td>
<td><em>C. chauvoei</em></td>
<td>cattle</td>
<td>Muscle</td>
<td>Semnan</td>
</tr>
<tr>
<td>CH 743</td>
<td><em>C. chauvoei</em></td>
<td>cattle</td>
<td>Muscle</td>
<td>Ilam</td>
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<tr>
<td>CH 701</td>
<td><em>C. chauvoei</em></td>
<td>vaccine</td>
<td>Muscle</td>
<td>Haydarabad</td>
</tr>
<tr>
<td>SEP 907</td>
<td><em>C. septicum</em></td>
<td>sheep</td>
<td>Abomasum</td>
<td>Haydarabad</td>
</tr>
<tr>
<td>CPA105</td>
<td><em>C. perfringens</em></td>
<td>Cattle</td>
<td>Intestine</td>
<td>Tehran</td>
</tr>
<tr>
<td>TT502</td>
<td><em>C. tetani</em></td>
<td>soil</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>NA814</td>
<td><em>C. novyi</em></td>
<td>sheep</td>
<td>Liver</td>
<td>Isfahan</td>
</tr>
</tbody>
</table>

Table 2. The percent of identity and the sequence distances among the C. chauvoei isolates which was designed by MegAlign software.

<table>
<thead>
<tr>
<th>Percent of Identity</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH 721 <em>fliA</em></td>
<td>97.9</td>
<td>93.5</td>
<td>93.3</td>
<td>93.9</td>
<td>93.4</td>
<td>89.2</td>
<td>89.5</td>
<td>91.8</td>
<td>88.7</td>
<td>1</td>
</tr>
<tr>
<td>CH 740 <em>fliB</em></td>
<td>92.6</td>
<td>93.3</td>
<td>91.7</td>
<td>91.1</td>
<td>88.6</td>
<td>87.9</td>
<td>91.0</td>
<td>86.7</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CH 743 <em>fliB</em></td>
<td>90.7</td>
<td>98.2</td>
<td>88.4</td>
<td>85.6</td>
<td>85.0</td>
<td>86.6</td>
<td>92.5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH vac <em>fliB</em></td>
<td>92.7</td>
<td>87.1</td>
<td>85.6</td>
<td>84.9</td>
<td>87.1</td>
<td>86.9</td>
<td>4</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CH 740 <em>fliA</em></td>
<td>97.3</td>
<td>93.2</td>
<td>2.8</td>
<td>97.0</td>
<td>99.0</td>
<td>89.0</td>
<td>7</td>
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<tr>
<td>CH 743 <em>fliA</em></td>
<td>87.3</td>
<td>93.8</td>
<td>88.1</td>
<td>89.1</td>
<td>90.1</td>
<td>9</td>
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</tbody>
</table>

Figure 1. Dendrogram was designed by MegAlign software, based on nucleotide sequences of *fliA* and *fliB* of *C. chauvoei*.
copies of fliC in different clostridia belonged to cluster I are available, as C. chauvoei and C. novyi type A, have two copies of fliC gene and C. septicum has three copies of fliC gene. Kojima (2000) sequenced only one copy of fliC gene in C. chauvoei. In this study two copies of fliC gene in four Iranian vaccine and field isolates of C. chauvoei were sequenced. Phylogenetic analysis showed more than five percent divergence between fliA and fliB sequences placed in two branches A and B (Fig. 1).

Comparing Iranian isolates to Japanese strain (AB058931-2) showed high similarity (93-97%) in both fliA and fliB copies of fliC gene (Table 2). Six highly conserved regions were detected in different copies of fliC gene. On the other hand, thirty-one single nucleotide polymorphisms including thirty specific nucleotide patterns were observed between fliA and fliB of C. chauvoei. Nucleotide sequence alignment showed more divergence in fliB (> 2%) than fliA sequences (Table 2).

In this study, a set of primers (CFC and CRC) are promising primers to differentiate C. chauvoei from C. septicum, C. novyi type A, C. tetani and C. perfringens. On the other hand, conserved regions, single nucleotide polymorphisms, and specific nucleotide patterns of fliC gene, which were identified in this study, might help us to better understand the fliC gene structure and design better specific primers and probes for diagnostic techniques such as real time PCR.

Although physiological traits, biochemical tests, and toxins are still used to characterize C. chauvoei, this information does not possess the discrimination required for source attribution and epidemiological investigations (Ji et al., 2001). The existence of genetic variation between two copies of fliC in C. chauvoei is reported for the first time in Iranian isolates. This finding can be considered as a scientific base for molecular identification and discrimination among the field isolates. It will be helpful to find out the genetic diversity of the C. chauvoei isolates according to their different origin, host, and geographical areas.

Acknowledgments

The authors wish to thank the Razi Vaccine and Serum Research Institute for Grant supporting this project.

References

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تعیین توالی و آنالیز مقایسه‌ای زن‌های فلاژلین FliB و FliA در جدایه‌های گاوی کلستریدیوم شوای
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مجله طب دامی ایران، ۱۳۹۳، ۱۲ (۸)، ۷-۲۲