Sequencing and comparative analysis of flagellin genes *fli*A and fli*B* in bovine *Clostridium chauvoei* isolates

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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 *fli*C gene, namely *fliA* and *fliB*. **OBJECTIVES:** The aim of this study was the determination and nucleotide sequence analysis of both copies of *fli*C genes in vaccinal strain and Iranian *C. chauvoei* isolates. **METHODS:** Six specific primers for amplification of *fliA*, *fliB*, and flagellin (*fli*C) 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 *fli*C 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 *fli*C 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 *fli*C 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 (*Fli*F) 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 (*Fli*D); and (4) Flagellar filament (*Fli*C) which is composed from repeating the flagellin protein subunit, encoded by *fli*C 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 *fli*C gene copies in clostridia genera are different. The *C. chauvoei* has two copies of *fli*C gene in genomic DNA, which are named *fliA* and *fliB*. The *C. septicum* has three copies and *C. difficile* has only one copy of *fli*C 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 *fli*C 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 ºC 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 *fli*C gene: Two specific primers (CFC 5´-cat tgc tac agc agg taa ta<c> 3´ and CRC 5́-gaa cag cac cta act ttg at<c>3́) were designed to amplify a fragment of 1000

bp of *fli*C gene. This PCR was tested for differentiation of *C. chauvoei* from other pathogenic clostridia, including *C. septicum*, *C. tetani*, *C. novyi*, and *C. perfrigens*.

Amplification of *fli*C 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 cct 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 *fli*C gene sequences of reference strains in the GenBank.

Results

***Fli*C-PCR:** A fragment of 1000 bp length was amplified from *C. chauvoei* by *fli*C specific (CFC and CRC) primers. Two copies of *fli*C (*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 *fli*C gene copies (*fliA* and *fliB*) of *C. chuvoei* 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*s is more than *fliA*s (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 *fli*C 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-

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

Isolate code	Clostridum sp	Source	Organ/tissue	District
CH 721	*C. chauvoei*	cattle	Muscle Muscle	Saveh
CH 740	*C. chauvoei*	cattle	Muscle	Semnan
CH 743	*C. chauvoei*	cattle	Muscle	Ilam
CH 701	*C. chauvoei*	vaccine	Muscle	Haydarabad
SEP 907	*C. septicum*	sheep	Abomasum	Haydarabad
CPA105	*C. perfringens*	Cattle	Intestine	Tehran
TT502	*C. tetani*	soil	-	NA
NA814	*C. novyi*	sheep	Liver	Isfahan

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

	Percent of Identity											
		1	2	3	4	5	6	7	8	9	10		
Divergence	1		97.9	93.5	93.3	93.9	93.4	90.2	89.5	91.8	88.7	1	Ab058932
	2	0.2		92.6	93.3	91.7	91.1	88.6	87.9	91.0	86.7	2	Ch 743 *fliB*
	3	0.0	0.0		90.7	98.2	88.4	85.6	85.0	86.6	92.5	3	Ch 721 *fliB*
	4	4.1	4.3	4.3		92.7	87.1	85.6	84.9	87.1	86.9	4	Ch 740 *fliB*
	5	0.2	0.3	0.0	4.2		87.7	84.1	83.5	85.2	91.5	5	Ch vac *fliB*
	6	7.4	7.4	7.3	11.1	8.1		96.8	95.9	97.3	93.2	6	Ab 058931
	7	8.0	8.2	8.3	11.9	9.1	2.8		97.0	99.0	89.0	7	Ch 721 *fliA*
	8	8.7	9.7	8.6	13.0	9.3	3.9	1.4		93.8	88.1	8	Ch 740 *fliA*
	9	6.8	7.1	6.5	11.1	7.3	1.9	1.9	4.0		90.1	9	Ch vac *fliA*
	10	6.8	7.0	5.6	11.0	6.7	2.0	3.0	3.6	1.3		10	Ch 743 *fliA*
		1	2	3	4	5	6	7	8	9	10		

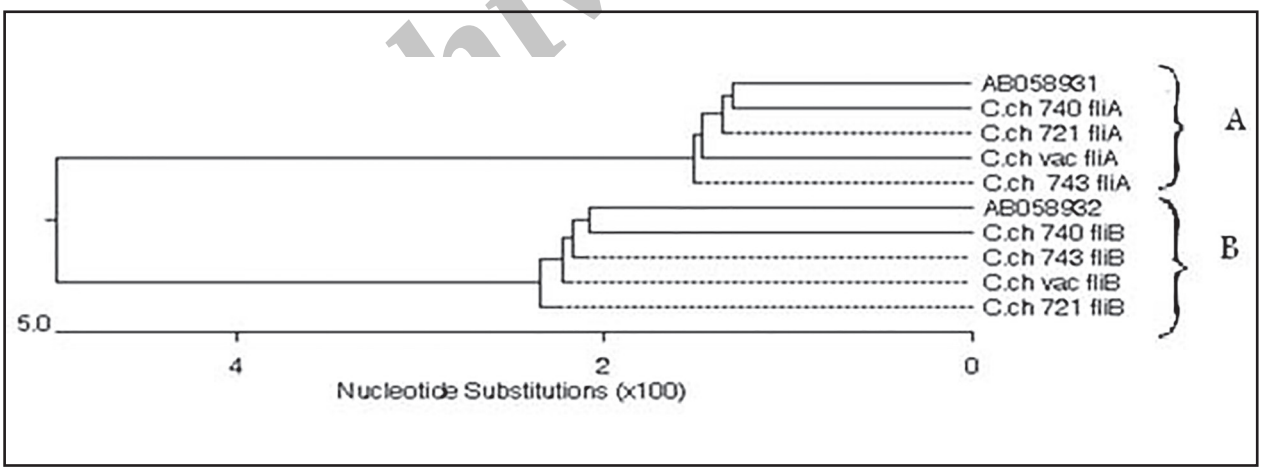

Figure 1. Dendrogram was designed by MegAlign software, based on nucleotide sequences of *fliA* and *fliB* of *C. chuvoei*.

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

copies of *fli*C in different clostridia belonged to cluster I are available, as *C. chauvoei* and *C. novyi* type A, have two copies of *fli*C gene and *C. septicum* has three copies of *fli*C gene. Kojima (2000) sequenced only one copy of *fli*C gene in *C. chauvoei*. In this study two copies of *fli*C gene in four Iranian vaccine and field isolates of *C. chauvoei* were sequenced. Phylogenic 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 *fli*C gene (Table 2). Six highly conserved regions were detected in different copies of *fli*C 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 *fli*C gene, which were identified in this study, might help us to better understand the *fli*C 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 *fli*C 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.

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مجله طب دامی ایران، ۱۳۹۵، دوره ۱۰، شماره ۱، ۳۲-۲۷

تعیین توالی و آنالیز مقایسه‌ای ژن‌های فلاژلین *FliA* و *FliB* در جدایه‌های گاوی کلستریدیوم شوای

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چکیده

زمینه مطالعه: کلستریدیوم شوای عامل شاربن علامتی است که بعنوان یک عفونت درون‌زا در گاو شناخته شده است. فلاژلین (liC) بعنوان عامل اصلی در ایجاد ایمنیت حفاظتی در دام‌ها علیه عفونت‌های کلستریدیایی نقش دارد. کلستریدیوم شوای دو کپی از ژن فلاژلین (*fliC*) که *fliA* و *fliB* نامیده می‌شوند را دارا می‌باشد. **هدف:** تعیین توالی و آنالیز نوکلئوتیدی هر دو کپی ژن *fliC* در سویه واکسینال و جدایه‌های فیلدی کلستریدیوم شوای هدف این مطالعه بوده است. **روش کار:** شش پرایمراختصاصی برای تکثیر ژن فلاژلین و دو کپی آن با استفاده از نرم افزار الیگو طراحی گردید. واکنش زنجیره‌ای پلیمراز بمنظور تکثیر یک قطعه ۷۰۰ جفت بازی برای هر دو ژن *fliA* و *fliB* بکار گرفته شد. درصد تشابه و اختلاف نوکلئوتیدی بین جدایه‌ها با استفاده از نرم افزارهای BLAST و MegAlign محاسبه گردید. **نتایج:** یافته‌های آنالیز توالی‌های نوکلئوتیدی نشان داد که میزان تنوع در ژن *fliB* بیشتر از *fliA* می‌باشد. شش ناحیه کاملاً حفاظت شده، سی و یک تغییر تک نوکلئوتیدی، سیزده اختلاف اسید آمینه‌ای در ژن *fliC* جدایه‌های ایرانی کلستریدیوم شوای شناسایی گردید. مقایسه توالی نوکلئوتیدین‌های *fliA* و *fliB*، نشان داد که قرابت سویه واکسینال با جدایه‌های فیلدی به ترتیب ۹۷/۳ و ۹۸/۲٪ می‌باشد. **نتیجه‌گیری‌نهایی:** کپی‌های ژن *fliC* بعنوان مارکرهای خیلی مناسب برای مطالعات اپیدمیولوزیک و شناسایی تنوع در جدایه‌های کلستریدیوم شوای شناخته شد. وجود تنوع ژنتیکی در بین دو آلل ژن *fliC* برای اولین بار از ایران گزارش می‌شود. علی رغم وجود تفاوت‌های ژنتیکی مذکور، آزمایش ایمنیت متقاطع قدرت حفاظت واکسن تهیه شده از سویه بومی (توسط موسسه رازی) را در چالنج با سویه‌های همولوگ و هترولوگ نشان داد.

واژه های کلیدی: کلستریدیوم شوای، فلاژلین، پلی مورفیسم

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