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

Jabbari, A.R.^{1*}, Azizian, Kh.¹, Esmaelizad, M.²

¹Department of Anaerobic Bacterial Vaccines Research and Production, Razi vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran ²Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

Key words:

Abstract:

Clostridium chauvoei, flagellin, polymorphism

Correspondence

Jabbari, A.R. Department of Anaerobic Bacterial Vaccines Research and Production, Razi vaccine and Serum Research Institute, Karaj, Iran Tel: +98(26) 34502899 Fax: +98(26) 34552194 Email: a.jabbari@rvsri.ac.ir

Received: 27 July 2015 Accepted: 16 November 2015



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

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 *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 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 *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.

> 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 °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 *fli*C gene: Two specific primers (CFC 5[']-cat tgc tac agc agg taa ta<c>3['] and CRC 5[']-gaa cag cac cta act ttg at<c>3['] 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 *fliC* gene sequences of reference strains in the GenBank.

Results

FliC-PCR: A fragment of 1000 bp length was amplified from *C. chauvoei* by *fliC* specific (CFC and CRC) primers. Two copies of *fliC* (*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 fliC 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 *fliBs* is more than *fliAs* (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 *fliC* 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-

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

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 <i>fliB</i>
	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 <i>fliB</i>
	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 <i>fliA</i>
	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 <i>fliA</i>
	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 *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 *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.

Acknowledgments

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

References

- 1. Alm, R.A., Guerry, P. (1993) Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni*. J Bacteriol. 175: 3051-3057.
- 2. Ardehali, M., Darakhshan, H. (1975) Isolation and characterization of *Clostridium chauvoei* strains isolated from cases of blackleg in cattle in Iran. Arch Razi Inst. 27: 37-41.
- Ardehali, M., Darakhshan, H., Moosawi M. (1984) The existence and present situation of clostridial diseases of domestic animals in Iran. Arch Razi Inst. 34: 27-32.
- Attridge, S., Rowley, D. (1983) The role of the flagellum in the adherence of *Vibrio cholerae*. J Infect Dis. 147: 864.
- Bagge, E., Lewerin, S.S., Johansson, K. (2009) Detection and identification by PCR of *Clostridium chauvoei* in clinical isolates, bovine faeces and substrates from biogas plant. Acta Vet Scand. 51: 8.
- Blood, D.C., Radostitis, O.M., Henderson, J.A. (1983) Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Goats and Horses. (6th ed.) Bailliere Tindall, oxford, London, Uk.
- Dauga, C., Zabrovskaia, A., Grimont, P.A.D. (1998) Restriction fragment length polymorphism analysis of some flageelin genes of *Salmonella enterica*. J Clin Microbiol. 36: 2835-2843.
- Ji, W.S., Hu., J.L., Qiu, J.W., Peng, D.R., Shi, B.L., Zhou, S.J., Wu, K.C., Fan, D.M. (2001) Polymorphism of flagellin A gene in *Helicobacter pylori*. World J Gastroentrol. 7: 783-787.

- 9. Joys, T.M. (1988) The flagellar filament protein. Can J Microbiol 34: 452-458.
- Kojima, A., Uchida, I., Sekizaki, T., Sasaki, Y., Ogikubo, Y., Kijima, M. (2000) Cloning and expression of a gene encoding the flagellin of *Clostridium chauvoei*. Vet Microbiol. 76: 359-372.
- Kojima, A., Uchida, I., Sekizaki, T., Sasaki, Y., Ogikubo, Y., Tamura, Y. (2001) Rapid detection and identification of *Clostridium chauvoei* by PCR based on flagellin gene sequence. Vet Microbiol. 78: 363-371.
- Kostrzynska, M., Betts, J.D., Austin, J., Trust, T.J. (1991) Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. J Bacteriol. 173: 937-946.
- 13. Macnab, R.M. (2004) Type III flagellar protein export and flagellar assembly. Biochim Biophys Acta. 1694: 207-217.
- Miyashiro, S., Nassar, A., Souza, M., Carvalho, J., Adegas, J. (2007) Identification of *Clostridium chauvoei* in clinical samples cultures from blackleg cases by means of PCR. Braz J Microbiol 38: 491-493.
- Morooka, T., Umeda, A., Amako, K. (1985) Motility as an intestinal colonization factor for *Campylobacter jejuni*. J Gen Microbiol. 131: 1973-1980.
- Reid, S.D., Selander, R.K., Whittam, T.S. (1999) Sequence diversity of flagellin (*fliC*) alleles in pathogenic *Escherichia coli*. J Bacteriol. 181: 153-160.
- Sasaki, Y., Kojima, A., Aoki, H., Ogikubo, Y., Takikawa, N., Tamura, Y. (2002) Phylogenetic analysis and PCR detection of *Clostridium chauvoei*, *Clostridium haemolyticum*, *Clostridium novyi* types A and B, and Clostridium septicum based on the flagellin gene. Vet Microbiol. 86: 257-267.
- Tamura, Y., Tanaka, S. (1984) Effect of antiflagellar serum in the protection of mice against *Clostridium chauvoei*. Infect Immun. 43: 612-616.
- 19. Tanaka, M., Hirayama, N., Tamura, Y. (1987)

Production, characterization, and protective effect of monoclonal antibodies to *Clostridium chauvoei* flagella. Infect Immun. 55: 1779-1783.

- 20. Tino, T. (1977) Genetics of structure and function of bacterial flagella. Annu Rev Genet. 11: 161-182.
- Uzal, F.A., Paramidani, M., Assis, R., Morrris, W., Mivakawa, M.F. (2003) Outbreak of *Clostridial myocarditis* in calves. Vet Rec. 152: 134-136.
- Wilson, G.S., Miles, A.A. (1975) Topley and Wilson's Principles of Bacteriology, Virology and Immunology. (6th ed.). The Williams and Wilkins Co, Baltimore, Maryland, USA.

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

احمدرضا جباری (* خلیل عزیزیان (مجید اسمعیلی زاد ۲

۱) بخش تحقیق و تولید واکسن های باکتریایی بی هوازی، موسسه تحقیقات واکسن و سرم سازی رازی، سازمان تحقیقات، ترویج و أموزش کشاورزی، کرج، ایران ۲) بخش بیوتکنولوژی، موسسه تحقیقات واکسن و سرم سازی رازی، سازمان تحقیقات، ترویج و أموزش کشاورزی، کرج، ایران

(دریافت مقاله: ۴ مرداد ماه ۱۳۹۴، پذیرش نهایی: ۲۸ آبان ماه ۱۳۹۴)

*چکید*ہ

زمینه مطالعه: کلستریدیوم شونی عامل شاربن علامتی است که بعنوان یک عفونت درونزا در گاو شناخته شده است. فلاژلین (IiC) بعنوان عامل اصلی در ایجاد ایمنیت حفاظتی در دامها علیه عفونتهای کلستریدیایی نقش دارد. کلستریدیوم شونی دو کپی از ژن فلاژلین (*Tilc*) که *Hid و Hib تامیده می*شوند را دارا می باشد. **هدف: تعیین توالی و آنالیز نو کلئوتیدی هر دو کپی ژن** *Iil در سویه واکسینال و جدایههای فیلدی کلستریدیوم شونی هدف این مطالعه بوده است. روش کار: شش پرایمراختصاصی برای تکثیر رون فلاژلین و دو کپی آن با استفاده از نرم افزار الیگو طراحی گردید. واکنش زنجیرهای پلیمراز بمنظور تکثیر یک قطعه ۲۰۰ جفت بازی برای هر دو ژن <i>Hid و Kil و Hib ب*کار گرفته شد. درصد تشابه و اختلاف نو کلئوتیدی بین جدایهها با استفاده از نرم افزارهای TSA و ملاژلین و دو کپی آن با استفاده از نرم افزار الیگو طراحی گردید. واکنش زنجیرهای پلیمراز بمنظور تکثیر یک قطعه ۲۰۰ جفت بازی برای هر دو ژن *Hid و Hib ب*کار گرفته شد. درصد تشابه و اختلاف نو کلئوتیدی بین جدایهها با استفاده از نرم افزارهای TBA می باشد. شش ناحیه کاملاً حفاظت شده، سی و یک تغییر تک نو کلئوتیدی نشان داد که میزان تنوع در ژن *Til ج*دایههای می باشد. شش ناحیه کاملاً حفاظت شده، سی و یک تغییر تک نو کلئوتیدی نشان داد که میزان تنوع در ژن *Til ج*دایههای می باشد. شش ناحیه کاملاً حفاظت شده، سی و یک تغییر تک نو کلئوتیدی می آنا و *Hif و Hil بینو* و این اینو کار و ای بان از ایران کار ای باز از ایران ای در ژن *Til ج*دایههای می باشد. شش ناحیه کاملاً حفاظت شده، سی و یک تغییر تک نو کلئوتیدی می آنه و التان داد که میزان تنوع در ژن *Hil ج*دایهای ایرانی کلستریدیوم شولی شناسایی گردید مقایسه توالی نو کلئوتیدینهای *Hil و Hil با بینوان م*ار کرهای خیلی مناسب برای مطالعات اپیدمیولوزیک و شناسایی تنوع در جدایههای کلستریدیوم شولی شناخته شد. وجود تنوع ژنتیکی در بین دو آل از ران اولین بار از ایران گزارش می شود علی رغم وجود تفاوتهای کلستریدیوم شولی شناخته شد. وجود تنوع ژنتیکی در بین دو آل ار برای اولین بار از ایران گزارش می شود علی رغم وجود تفاوتهای گلستریدیوم مولی شدو و می ولوگ نشان داد ده قرات می می می ساسب برای اولین بار ایران گزارش می شود علی رغم وجود تفاوتهای کلستریدیوم مولی می تور آزمایش ایمییت می مور و ت

واژه های کلیدی: کاستریدیوم شوای، فلاژلین، پلی مورفیسم

*) نویسنده مسؤول: تلفن: ۹۹۵۰۲۸۹۹ (۲۶) ۳۴۵۵۲۱۹۴ نمابر: ۳۴۵۵۲۱۹۴ (۲۶) ۳۴۵۵۲۱۹۲ Email: a.jabbari@rvsri.ac.ir