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Original article

# Polymorphism of Flagellin gene (fliC) of *Clostridium chauvoei* isolates from Iran

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ABSTRACT

Flagellums have been reported to play a critical role in the protective immunity in host. In clostridia genera it is coded by flagellin (fliC) gene. Previous southern blot analysis has suggested that Flic gene may has two copies in genome of *C. chauvoei*. We studied sequences of two copies (fliA and fliB) of flagellin gene in Iranian isolates. In this study, four specific primers for fliA, fliB copies and two universal primers for flagellin (fliC) were designed. Polymerase chain reactions were performed to amplification of fliA and fliB. Two copies of flagellin gene were sequenced and compared with other sequences in GenBank. In phylogenic tree analysis, fliA and fliB sequences were located in separated (clusters) branches. Nucleotide sequence alignment showed that more divergence in fliBs than fliAs sequences. Six highly conserve regions and thirty-one single nucleotide polymorphisms (include thirty specific nucleotide pattern) were found between fliA and fliB sequences. These findings are the first report of Thirty-one novel Single Nucleotide Polymorphisms in fliC gene of Clostridium chauvoei isolates from Iran.

## 1. Introduction

*Clostridium chauvoei* is a gram positive and spore forming anaerobic bacterium. *C. chauvoei* is causative agent of blackleg in cattle, sheep and other ruminants with high mortality rate (Bagge et al., 2009). Death can occur due to septicemia (Kojima et al., 2001). Flagellum of *C. chauvoei* is important to induce protective immunity in host, but in contrast in other clostridia, toxoid is very important to immunization (Tamura et al., 1984). Many Symptoms observed in blackleg also are created by *C. septicum*, *C. noveyi* and *C. perfirgens* (Miyashiro et al., 2007; Kojima et al., 2001). Distinguish of 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 indicate similarity at phenotypic levels (Miyashiro et al., 2007). Different studies suggested that may be flagella are important factor to pathogenesis in bacteria (Attridge et al., 1983; Morooka and Umeda et al., 1985). Flagellum composed of four parts: 1) basal body consist 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). 4) Flagellar filament (FliC) which composed from repeating of flagellin protein subunit, encoded by fliC gene (Macnab et al., 2004; Wilson et al., 1975). The number copies of fliC gene in clostridia genera are different. The *C. chauvoei* has two copies of fliC gene in genomic DNA, which named fliA and fliB. The *C. septicum* has three copies and C. difficile has only one copy of fliC gene (Sasaki et al., 2002). In this study sequence of two copies of fliC gene in Iranian isolates of *C. chauvoei* were identified.

## 2. Materials and methods

### 2.1. Bacterial culture and DNA extraction

All Clostridium species which is used in this study were collected from Anaerobic Bacterial Vaccines Department of Razi Vaccine and Serum Research Institute of Iran (Table 1). Eight different clostridia on Thio-glycolate consist of liver in anaerobic condition provided by Anoxomat were cultured. Bacterial cultures performed in pH 6-7 for 72 hours. Cells were pelleted at 4000 rpm for 30 minute and washed two times by phosphate buffer. Genomic DNA was extracted by DNA extraction kit (Fermentase).

### 2.2. PCR amplification

Two specific primers were designed for fliC (CFC, 5′-cat tgc tac agc agg taa ga<c> 3′ and CRC, 5′-gaa cag cac cta act ttg at<c>3), used to amplify 1000bp of fliC to differentiation of C. chauvoei from Clostridium septicum, Clostridium tetani, Clostridium novyi and Clostridium perfrigens (Figure 1B). PCR reactions consist of 1.5mM of MgCl2; 0.5 unit Taq DNA Polymerase enzyme; 0.25 mM dNTP, DNA template 100 ng/reaction and10 pmol of each primer in 50 µl total volume. Denaturation was 94◦C for 5 minutes, second denaturation 94◦C for 1 minute annealing temperature 52◦C for 1minute; extension 72◦C for 1 minutes for 35 cycles and final extension in 72◦C for 10 minutes.

Two specific primers were designed for fliA (CFC 5′-cat tgc tac agc agg taa ga<c> 3′ and CRA, 5′-cca ctc tta act gtt aat act gca <t>-3') and fliB (CFC and CRB, 5′-cca cct tta aca gtt aaa aca gca <c>-3′) by oligo software. Polymerase chain reactions were performed to amplifying 700bp of fliA and fliB.

### 2.3. Sequencing and sequence analysis:

PCR products were electerophoresed in 1٪ agarose gel for an hour and purified by PCR product purification kit (Roche). Purified PCR products were sequenced by Macrogen Company. All fliA and fliB sequences were analyzed by Megalign softwares.

## 3. Results

A fragment with 1000bp length was amplified from *C. chauvoei* by specific outer primers (CFC and CRC) (Figure 1). These amplicons contain different copies of fliC (mixed of fliA and fliB) in which a common forward primer (CFC) and specific reverse primers (CRA and CRB) were used (Figure 1B). In second step, two specific fragments fliA and fliB with 700bp length were amplified from PCR products in previous step.

Dendrogram was designed based on fliA and fliB nucleotide sequences from Iranian isolates and two reference sequences of fliC in GenBank by MegAlign software. All fliA and fliB sequences were located at two separated branches, A and B respectively (Figure 3).

**Table 1**
Different *Clostridium spp* strains which were used in the study.

| Code of the strains | Bacterial species | Source |
|---|---|---|
| CH721 | *C. chauvoei*,721 | cattle |
| CH740 | *C. chauvoei*,740 | cattle |
| CH743 | *C. chauvoei*,743 | calf |
| CH709 | *C. chauvoei*,*vac* | cattle |
| Sp913 | *C. septicum* | cattle |
| CP409 | *C. perfringens* | sheep |
| CN211 | *C. tetani* | soil |
| CN811 | *C. novyi* | sheep |

Multiple Alignments of fliA and fliB sequences recognized thirty one single nucleotide polymorphisms, one SNP in nucleotide positions 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 position (571-585) and five SNPs in nucleotide position (592-604) were characterized between fliA and fliB in all *C. chauvoei* (Figure 2). Six highly conserved regions were observed between fliA and fliB at nucleotide positions (134-149), (153-186), (190-238), (271-483), (496-514) and (517-529) (Figure 2). Nucleotide sequence alignment showed that fliBs have more divergence than fliAs (Table 2).

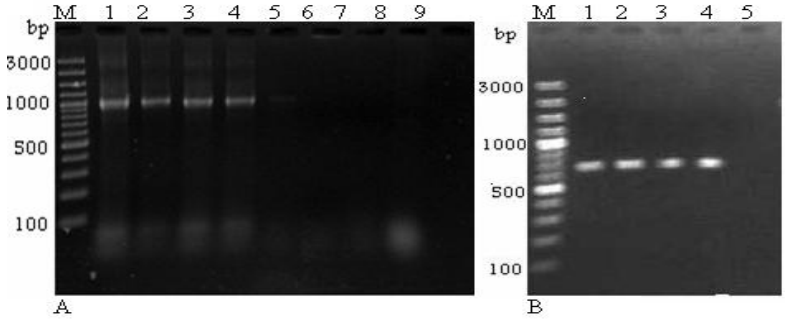
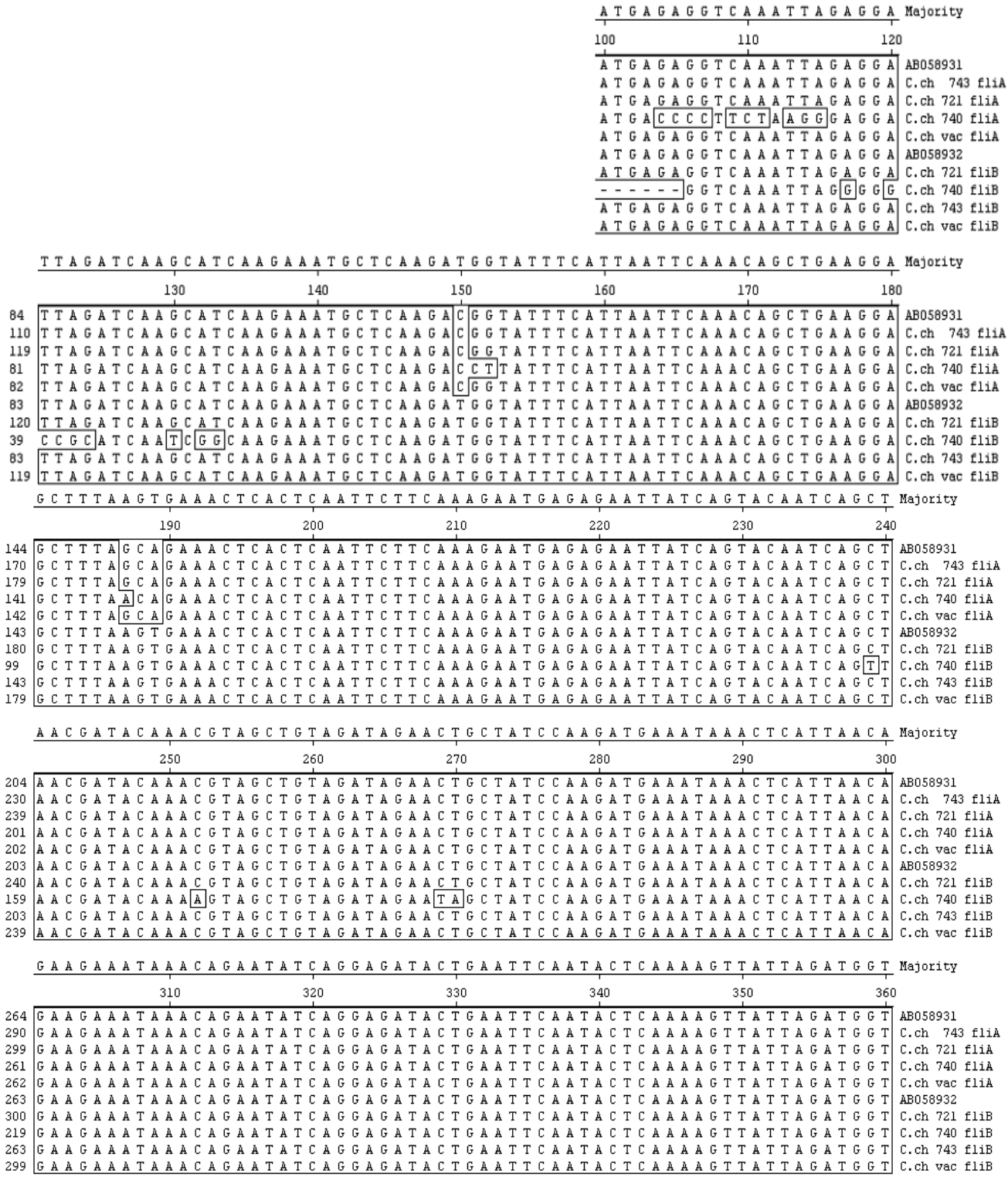



**Fig. 1.** A. PCR products of flagellin gene which were amplified by outer primers (CFC and CRC) that differentiated *C. chauvoei* from *C. septicum*, *C. novyi* type A, *C. tetani* and *C. perfringens*. Lane M, size marker. Lane 1 to 4 *C. chauvoei*. Lane 5, *C .septicum*. Lane 6, *C. novyi* type A. lane 7, *C. tetani*. Lane 8, *C. perfringens* B. PCR products of fliA and fliB genes by CFC, CRA and CFC, CRB primers respectively. Lane M: DNA marker .lane 1 and 2 fliA, lane 3and 4 fliB.


ATGAGAGGTCAAATTAGAGGA Majority
100 110 120
ATGAGAGGTCAAATTAGAGGA AB058931
ATGAGAGGTCAAATTAGAGGA C.ch 743 fliA
ATGAGAGGTCAAATTAGAGGA C.ch 721 fliA
ATGACCCCTTCTAAGGGAGGA C.ch 740 fliA
ATGAGAGGTCAAATTAGAGGA C.ch vac fliA
ATGAGAGGTCAAATTAGAGGA AB058932
ATGAGAGGTCAAATTAGAGGA C.ch 721 fliB
------GGTCAAATTAGGGG C.ch 740 fliB
ATGAGAGGTCAAATTAGAGGA C.ch 743 fliB
ATGAGAGGTCAAATTAGAGGA C.ch vac fliB
TTAGATCAAGCATCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA Majority
130 140 150 160 170 180
84 TTAGATCAAGCATCAAGAAATGCTCAAGACGGTATTTCATTAATTCAAACAGCTGAAGGA AB058931
110 TTAGATCAAGCATCAAGAAATGCTCAAGACGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch 743 fliA
119 TTAGATCAAGCATCAAGAAATGCTCAAGACGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch 721 fliA
81 TTAGATCAAGCATCAAGAAATGCTCAAGACCTTATTTCATTAATTCAAACAGCTGAAGGA C.ch 740 fliA
82 TTAGATCAAGCATCAAGAAATGCTCAAGACGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch vac fliA
83 TTAGATCAAGCATCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA AB058932
120 TTAGATCAAGCATCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch 721 fliB
39 CCGCATCAATCGGCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch 740 fliB
83 TTAGATCAAGCATCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch 743 fliB
119 TTAGATCAAGCATCAAGAAATGCTCAAGATGGTATTTCATTAATTCAAACAGCTGAAGGA C.ch vac fliB
GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT Majority
190 200 210 220 230 240
144 GCTTTAGCAGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT AB058931
170 GCTTTAGCAGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch 743 fliA
179 GCTTTAGCAGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch 721 fliA
141 GCTTTAACAGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch 740 fliA
142 GCTTTAGCAGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch vac fliA
143 GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT AB058932
180 GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch 721 fliB
99 GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGTT C.ch 740 fliB
143 GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch 743 fliB
179 GCTTTAAGTGAAACTCACTCAATTCTTCAAAGAATGAGAGAATTATCAGTACAATCAGCT C.ch vac fliB
AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA Majority
250 260 270 280 290 300
204 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA AB058931
230 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch 743 fliA
239 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch 721 fliA
201 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch 740 fliA
202 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch vac fliA
203 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA AB058932
240 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch 721 fliB
159 AACGATACAAAAGTAGCTGTAGATAGAATAGCTATCCAAGATGAAATAAACTCATTAACA C.ch 740 fliB
203 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch 743 fliB
239 AACGATACAAACGTAGCTGTAGATAGAACTGCTATCCAAGATGAAATAAACTCATTAACA C.ch vac fliB
GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT Majority
310 320 330 340 350 360
264 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT AB058931
290 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 743 fliA
299 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 721 fliA
261 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 740 fliA
262 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch vac fliA
263 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT AB058932
300 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 721 fliB
219 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 740 fliB
263 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch 743 fliB
299 GAAGAAATAAACAGAATATCAGGAGATACTGAATTCAATACTCAAAAGTTATTAGATGGT C.ch vac fliB

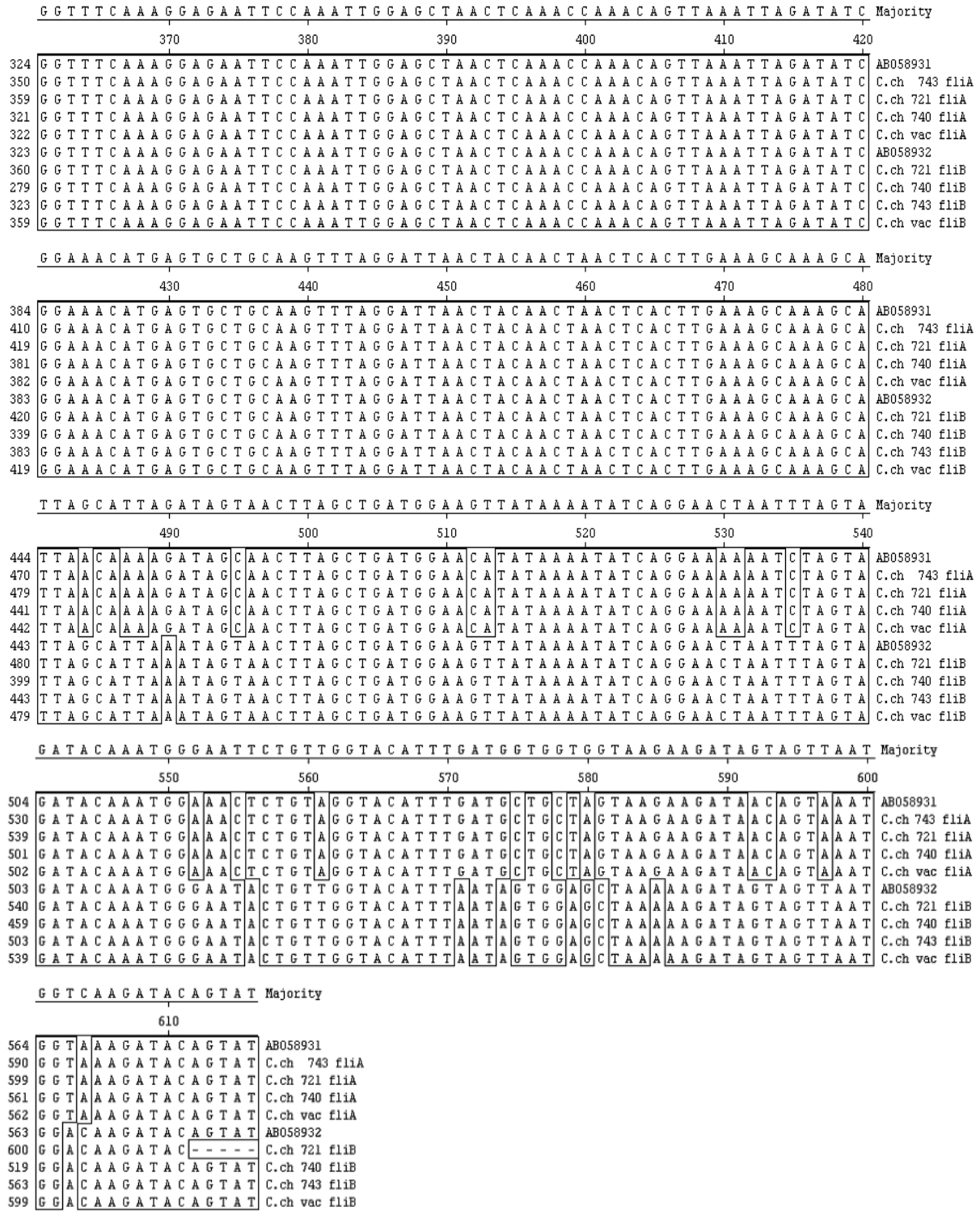



**Fig. 2.** Nucleotide sequence alignment of fliA and fliB of Iranian isolates of *C.chuvoei* by Meg A Align software. Sequences of Iranian isolates were compared with only sequences of fliA and fliB (accession numbers AB058931 and AB058932) from GenBank.

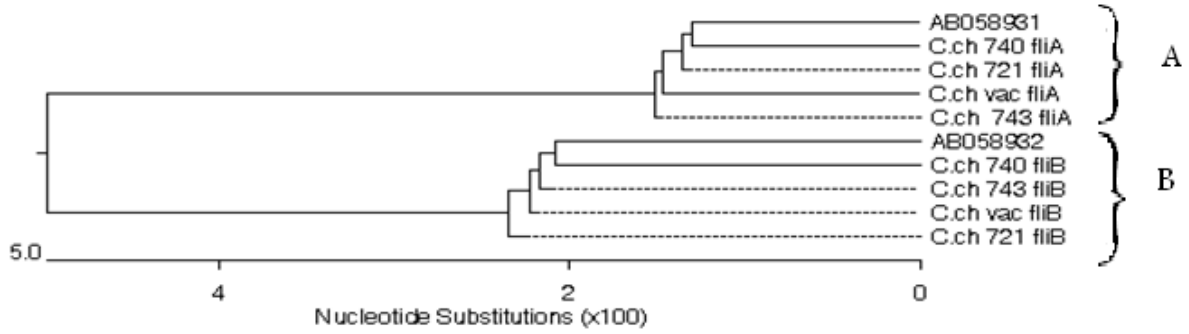



**Fig. 3.** Dendrogram was designed by MegAlign software, based on nucleotide sequences of fliA and fliB of C.chuvoei.

**Table 2**
Sequence distances table was designed by MegAlign software.

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **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** | ab058931 |
| **7** | 8.0 | 8.2 | 8.3 | 11.9 | 9.1 | 2.8 | | 97.5 | 95.0 | 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 fli A |
| **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** | | |

**4. Discussion**

FliC protein is virulence factor in some bacteria (Alm et al., 1993; Carsiotis et al., 1984). Tamura et al demonstrated that mice treated by anti-flagella serum are resistant to *C. chauvoei* (Tamura et al., 1984) and Immunogenicity in flagellated strain *of C. chauvoei* is 100-fold more than unflagellated strain (Tanaka et al., 1987). These two evidences indicate that the fliC protein has immunogenicity and protectivity roles (Kojima et al., 2000; Tamura et al., 1984; Tamura et al., 1992). The terminal domains of flagellin proteins have role for polymerization of flagellum (Alm et al., 1993). N–terminal part of flagellin is conserved and has regulatory function. Sequence of N–terminal of flagellin protein has been used to obtain relation between several bacteria (Sasaki et al., 2002). Flagellin in flagellar structure has hairpin model that N-C terminal fold to inner flagellum and central domain exposed to environment. Diversity in internal domains causes antigenic diversity in Enterobacteriaceae (Tino et al., 1977; Joys et al., 1988; Kostrzynska et al., 1991). In Some species of bacteria such as Escherichia coli and Salmonella, only one of the flagellin subunit involved for organization of flagellum but in many of bacteria, flagellar filament organized from multi flagellin subunit such as campylobacter and some of clostridia (Alm et al., 1993). Terponema palidum has periplasmic flagella, this flagella composed of several flagellin subunit (Alm et al., 1993) Sasaki et al demonstrated that one or more tandem copy of fliC in different clostridia belong of cluster I are available, as *C. chauvoei* and *C. noveyi* type A, have two copies of fliC gene and *C. septicum* has three copies of fliC gene (Sasaki et al., 2002). Kojima (2000) sequenced only one copy of fliC gene in *C. chauvoei*.

In our study, two copy of fliC gene contain fliA and fliB were amplified in Iranian isolates of C. chauvoei. In phylogenic analysis, showed more than five percent divergence between fliA and fliB sequences and were placed

in divided branches A and B (Figure 2, 3). Comparisons of Iranian isolates to Japanese strain (AB058931-2) were showed high similarity (93-97%) in both, fliA and fliB copies of FliC gene (Table 2). Six highly conserve regions were detected in different copies of fliC gene (Figure 2). On the other hand, thirty-one single nucleotide polymorphisms (SNPs) (include thirty specific nucleotide pattern) were observed between fliA and fliB of *C. chauvoei*. Nucleotide sequence alignment showed that more divergence in fliBs (> 2%) than fliAs sequences (Figure 3, Table 2).

Molecular analysis of fliA and fliB help us to better understanding of structure of different copies of FliC gene in *C. chauvoei* in Iranian isolates, on the other hand, thirty specific nucleotide pattern finding might help to design molecular techniques to diagnosis of *C. chauvoei* based on FliC gene in future.

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