Full Article

Molecular cloning of *Clostridium septicum* vaccine strain alpha toxin gene *in E. coli*

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ABSTRACT

Clostridium septicum a Gram positive anaerobic bacterium produces several toxins including alpha, beta, gamma and delta. *C. septicum* alpha toxin is lethal and is responsible for a serious disease known as gas gangrene. The aim of the present study was to molecular cloning and sequencing of *C. septicum* vaccine strain alpha toxin gene. Genomic DNA was extracted using standard phenol and chloroform extraction method, and the target gene was amplified through PCR by specific primers. Quality and quantity of PCR product was evaluated using agarose gel electrophoresis and confirmed with spectrophotometry. The PCR product was purified and was ligated in pJET1.2blunt cloning vector and was used for *E. coli*/TOP10 competent cells transformation. pJETαsep recombinant plasmid was purified and sequenced using universal primers. Sequencing and BLAST analysis of *csa* showed over 99% identity to other previously deposited *csa* in the GenBank. The *csa* sequence was deposited in the GenBank under accession number JN793989. *E. coli*/TOP10/pJETαsep as a recombinant bacterium could be used for purifying of recombinant *csa* gene and its expression in the suitable prokaryotic hosts.

Keywords: *Clostridium septicum*, vaccine strain, alpha toxin gene (*csa*), cloning, sequencing

INTRODUCTION

C. septicum is a large anaerobic, Gram positive, rod shaped and fermentative bacterium of genus Clostridium. Terminal spore gives the bacterium a drumstick-like shape while holding and peritrichous flagella enable the bacterium to be motile. *C. septicum* is a member of the normal gut flora in humans as well as other animals so can causes different diseases in both human and animals. *C. septicum* can produce and secretes a number of toxic proteins such as alpha, beta, gamma and delta. Alpha toxin that is the lethal cytolytic and the major virulence factor, appears to be its immunodominant extracellular antigen. (Liechti *et al* 2003, Smith-Slatas *et al* 2006). *C. septicum* alpha-toxin gene is cloned and expressed in *E. coli*. The toxin length is 443 amino acids with a fragment of 31 residue signal peptide that removes from the toxin during secretion (Ballard *et al* 1995). Alpha toxin is a cytolytic

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protein and its molecular mass is about 46.45 kDa that is an inactive protoxin and could be activated after carboxy-terminal cleavage (Gordon *et al* 1999). Alternate eukaryotic proteases such as trypsin can also activate the toxin. Regardless of the activating protease, the cleavage site is located approximately 4kDa upstream to the carboxyl terminus. Activated alpha toxin is haemolytic, and could release potassium ions from erythrocytes which would be followed by haemoglobin release. Proteolytically activated ATpro had a specific activity of approximately $1.5 \times 10(6)$ haemolytic units mg-1. But protoxin does not exhibit these properties (Ballard *et al* 1993, Kennedy *et al* 2005). The Gram negative bacterium *Aeromonas hydrophila* aerolysin protein, consists of small (SL) and large (LL) lobes. *C. septicum* alpha toxin has a single lobe homologous to LL, and binds to glycosylphosphatidylinositol (GPI) membrane anchored proteins (Gordon *et al* 1999, Hong *et al* 2002, Knapp *et al* 2012). Alpha toxin induces the oligomerization and formation of at least 1.3-1.6 nm diameter ion-permeable channels (Ballard *et al* 1993; Hang'omb *et al* 2004). Its single-channel conductance is about 175 pS in 0.1M KCl in lipid bilayer membranes. This feature is typical for a large diffusion pore. These channels display no lipid specificity so exhibit slight anion selectivity and mostly remain in the open configuration, (Knapp *et al* 2010).

In Iran anaerobic bacteria had been studied since 1960 and during last five decades conventional anaerobic vaccine had been produced against clostridial diseases (Rafyi *et al* 1961). *C. septicum* had been isolated from the cases of malignant edema of cattle, and its properties had been studied (Ardehali *et al* 1974). In the present article, cloning of *C. septicum* vaccine strain alpha toxin gene in *E. coli* as a candidate for recombinant vaccine production is reported. Alpha toxin gene is cloned in *E. coli* and its cloning and sequence specificities are studied.

MATERIALS AND METHODS

Materials. Plasmid pJET1.2/blunt, *Pfu* DNA polymerase, dNTPs, T4 DNA ligase, *Nde*I and *Xho*I restriction endonuclease and GeneRuler™ 100 bp Plus DNA Ladder were purchased from Fermentas. SiZer™ 1000 plus DNA marker solution (catalogue number 24075) was purchased from Intron, High Pure PCR Product Purification Kit for DNA fragments recovery was purchased from Vivantis. SDS, Proteinase K, lysozyme and plasmid extraction kit were prepared from CinnaGen.

Bacterial strains. *C. septicum* vaccine strain (CN913) and *E. coli* strain TOP10 were obtained from Razi Vaccine and Serum Research Institute (RVSRI).

Cultivation. *C. septicum* bacteria were cultured anaerobically at 37 °C in a liquid medium containing casein hydrolysate (3%), yeast extract (0.5%), glucose (0.5%) and cysteine hydrochloride (0.05%). pH was adjusted at 7.5 by addition of Na_2HPO_4. *E. coli*/TOP10 cells were cultured as described previously (Sambrook *et al* 1989).

Isolation of genomic DNA. For isolation of genomic DNA, the *C. septicum* vaccine strain was inoculated in 50 ml of above mentioned culture media and was incubated overnight and subsequently was centrifuged for 30 minutes at 2,370×g at room temperature. High molecular weight genomic DNA from *C. septicum* was isolated according to the method described previously (Pilehchian Langroudi *et al* 2013). Cells from overnight suspension were harvested by centrifugation and suspended in 300 µl TAE buffer (containing 1 mg/ml lysozyme). Three hundred microliters of 10% SDS solution, and 5 µl of RNase-A were added and the mixture was incubated for 30 minutes at 37 °C. Five microliters Proteinase K (50 mg/ml) was added and the mixture was incubated for 90 minutes at 37 °C and DNA was extracted twice with phenol and chloroform. Sixty microliters sodium acetate (1:10 V/V) and 600 µl isopropanol (1V/V) were added and incubated at 20°C and DNA precipitated by centrifugation.

Primers. *Nde*I and *Xho*I restriction endonuclease sequences and their flanking regions respectively were included in the 5' end of forward primer and 3' end of reverse primer. The designed sequences of *csa* primers are as follows Forward primer:

5' GAGCATATGTCAAAAAAATCTT 3'
Reverse primer:
5' CCCTCGAGTATATTATTAATTA 3'

PCR amplification of *csa*. Each PCR reaction was consisted of 0.25μl dNTPs, 10μ 10X PCR buffer and 1.5μl MgCl$_2$, 0.4μl *Pfu* DNA polymerase, 1μl of 100ng/μl appropriate template DNA, 0.5μ of 10 pmol/μl forward primer and 0.5μl of 10 pmol/μl reverse primer. Sterile DW was added up to 20μl reaction systems. After initial denaturation at 95 °C for 1 minute, thirty cycles of PCR were performed (denaturation at 95 °C for 1 minute, annealing at 52 °C for 1 min, and extension at 72 °C for 3 minutes) and continued with 10 minutes final extension at 72 °C. After analyzing on 1% agarose gel electrophoresis, the PCR product was excised from the gel, and was purified using the GeneJET™ gel extraction Kit for DNA fragment recovery according to the manufacturer's recommendations.

Cloning vector construction and ligation reaction. Linearized pJET1.2blunt plasmid containing ampicillin resistant gene was used as cloning vector and purified gene was ligated in it. Two microliters of 10X reaction buffer, 1μl cloning vector and 1μl T4 ligase and 16μl blunt end purified *csa* gene were mixed in a 0.2 ml thin walled micro tube and incubated for overnight at 14 °C.

Transformation. *E. coli*/TOP10 was converted to competent cells and its efficiency was tested. Ten microliters of ligation mixture directly was used for transformation of 100μl of competent cells. Cell was cultured on a plate containing LB ampicillin and was incubated at 37 °C for overnight. The complete technique was done as described previously (Pilehchian Langroudi *et al* 2011).

Verification of recombinant vector construction. After one night, colony PCR was carried out for eight of the colonies containing pJETαsep recombinant plasmid. pJET1.2blunt universal forward and reverse sequencing primers were used according to the manufacturer's recommendations for PCR reaction. For negative control, non-recombinant cloning vector also was subjected to the same PCR procedure. The same

colony was cultured on a new plate containing LB ampicillin. *E. coli*/TOP10 competent cells were also cultured on the same plate. Plasmid extraction was carried out by CinnaGen plasmid extraction kit (Mini Prep). Plasmid digestion was done using *Nde*I and *Xho*I restriction endonucleases according to Fermentas protocol. PCR was carried out for *csa* gene using purified plasmid.

Sequence analyzing of amplified and purified PCR products. Recombinant vectors and PCR products were sequenced after separation by agarose gel electrophoresis. Nucleotide sequencing was carried out by SEQLAB (Sequence Laboratories Goettingen GmbH).

RESULTS

C. septicum csa gene *was* amplified using one pair of primers which had been designed according to nucleotide sequences of S75954(3), figure 1 shows 1% agarose gel electrophoresis of this amplified gene. During ligation alpha toxin gene (1332 bp) was ligated into pJET cloning vector (2974 bp) and pJETαsep recombinant vector (4306 bp) was produced, figure 2 shows recombinant vector structure. Overnight culture of transformed bacteria on LBA ampicillin showed recombinant *E. coli*/TOP10/pJETαsep colonies. Colony PCR of eight recombinant colonies using *csa* forward and reverse primers showed *csa* DNA fragments on 1% agarose gel electrophoresis (figure 3).
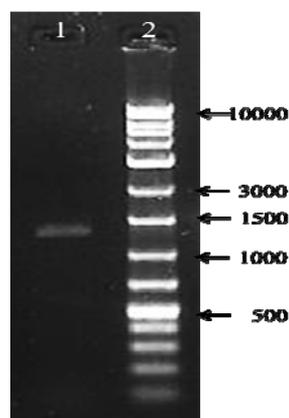
Figure 1. 1% agarose gel electrophoresis of *C. septicum csa* gene. Lane 1: PCR products. Lane 2: 1000 plus DNA molecular weight markers.

Sequencing analysis of amplified and purified PCR products. Recombinant pJETαsep plasmid of *E.*

*coli/*TOP10/pJETαsep was extracted and purified. Sequencing analysis of purified pJETαsep showed *csa* gene. The nucleotide sequence was deposited in the GenBank. Purified recombinant plasmid was digested using *Nde*I and *Xho*I, figure 4 shows that *csa* gene coding part is released after digestion.
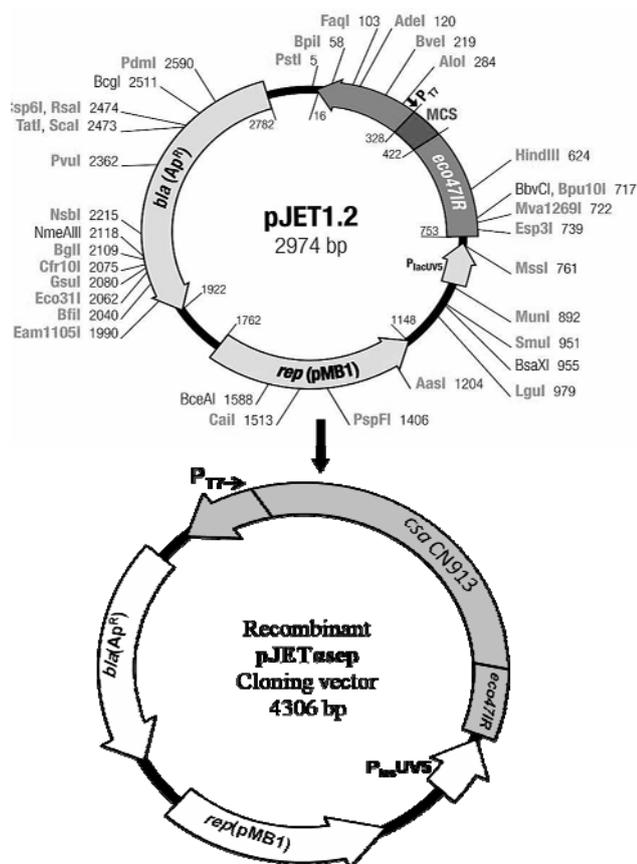

Figure 2. Recombinant pJETαsep cloning vector structure shows alpha toxin gene derived from *C. septicum* CN913 (csa CN913).
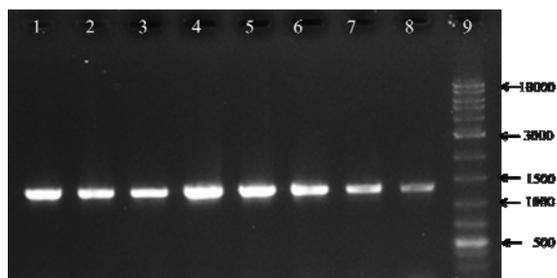

Figure 3. Colony PCR analysis of eight recombinant *E. coli/* TOP10/pJETαsep colonies. Lane 1-8: PCR products of three recombinant colonies. Lane 9: 1000 plus DNA molecular weight markers.
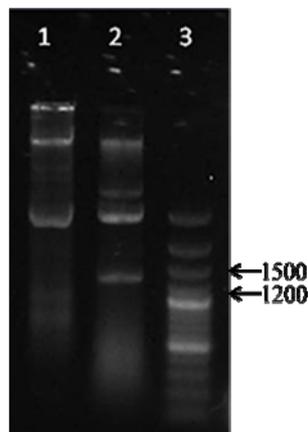

Figure 4. Purified recombinant pJETαsep analysis after digestion by *Nde*I *and Xho*I. Lane 1: uncut supercoiled recombinant plasmid. Lane 2: digested recombinant plasmid shows *csa* gene between 1200 and 1500 bp. Lane 3: 100 bp plus DNA molecular weight markers.

DISCUSSION

Genomic DNA extraction of Gram positive bacteria is difficult because there is a thick cell wall in these bacteria. Some procedures were examined to remove this thick cell wall and finally 10% SDS solution and 1 mg/ml lysozyme in TAE buffer was used for this purpose (Pilehchian Langroudi *et al* 2011). *C. septicum* genomic DNA was used for amplification of alpha toxin gene; forward and reverse primers were designed from the available sequence. *C. septicum csa* complete *cds* S75954 was used (Ballard *et al* 1995) as reference gene. BLAST in GenBank reveals that this is the only sequence which has the complete cds with full annotation of protoxin and mature peptides. One pairs of primers were synthesized and amplification of the open reading frame of *csa* gene was done. The *Nde*I cleavage site and its flanking region were inserted at the 5' end of *csa* gene by the PCR procedure. The *Xho*I cleavage site and its flanking region were inserted at the 3' end of *csa* gene by the PCR procedure. Alpha toxin gene during ligation (the blunt PCR product) at the blunt terminals of linearized pJET was inserted and 4306 bp circular pJETαsep was produced. After transformation only those bacteria which had transformed by recombinant plasmid vector, was grown on the ampicillin containing LB agar. Screening of the recombinant *E. coli*/TOP10/ pJETαsep by colony PCR, produced 1332 bp DNA fragment, but in the case of non-recombinant

plasmid as negative control was not produced. At the next step, the pJETαsep was purified and was double digested whit *Nde*I and *Xho*I, and then both undigested and digested fragments were extracted and loaded on the agarose gel electrophoresis. As it is shown in figure 4 in the case of digested vector, one ~1.3 kb band could be seen. Using universal forward and reverse sequencing primers of the vector, the purified construct of pJETαsep was sequenced. According to the results, the csa sequences in comparison with the previous sequences reports obtained from the GenBank database were confirmed and a 1332 bp fragment was showed on the *csa* amplified sequence. ATG is the start codon of this sequence, and the alpha toxin protein (443 amino acids) will be produced in translation. The *csa* sequence as a complete cds was deposited in the GenBank under accession number JN793989. In 1994 a genomic library of *C. septicum* (NCTC547 strain) in *E. coli* was made, and when alpha toxin gene was inserted in pCS21 and at least one open reading frame fragment, containing 1380 bp was shown. After expression, molecular weight of alpha toxin protein was estimated about 48.9 kDa (Imagawa *et al* 1994). Gordon in 1997 reported that alpha toxin molecular mass is about 46.45 kDa. The difference between these two molecular mass, was resulted from the cleavage of the second protein. There is a variation of *C. septicum* alpha toxin genes, which were classified into 10 patterns. They found that, nucleotide sequences at the 3' end of the sequences respectively coincided with those of groups F2 and F4 (Imagawa *et al* 1994, Ballard *et al* 1995, Amimoto *et al* 2006). In 2012 Assist reported that the *C. septicum* strains can be classified in two groups. The first group including two vaccine strains, showed the majority of the sequences and conservation. The second group showed sequences with significant changes in the nucleotide and amino acid sequences numbers. In our study recombinant pJETαsep was sequenced. One fragment consisting 1332 bp of alpha toxin gene, that 100% is identical whit nucleotide sequences of

Imagawa (1994) and Ballard (1995), is shown, but molecular mass and genetic diversity of alpha toxin is not investigated, and it needs further researches. In 2012 Mukamoto had been reported that there is a tryptophan-rich region near the C-terminal of *C. septicum* alpha toxin protein sequence. This region is consisting of residues 302 up to 312, which in it W307 and W311 are essential and residue 309 must be hydrophobic with an aromatic side chain. Residue 308 has no important role. Based on this study, WDW-W sequence in the tryptophan-rich region has an important cytotoxic effect in the alpha toxin whole structure. In our study the unique tryptophan rich region (NYSE WDWKWV) between residues 332-343, is found, but its effects are not investigated.

The present study was based on using *Pfu* DNA polymerase, *Pfu* has a proofreading activity, so the csa gene that was obtained in our vaccine strain, is 1332 bp. *Nde*I restriction site at the 5' terminal, and *Xho*I restriction site at the 3' terminal are also located in this gene. For inserting of *csa* gene in the expression vector and for produce the prokaryotic recombinant expression hosts that may be used for recombinant vaccine production purpose, these sequences are necessary.

Acknowledgment

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