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Original Article

Molecular Cloning of Clostridium Perfringens Type B Vaccine Strain Beta Toxin Gene in E. coli

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Clostridium perfringens is a gram-positive, obligate anaerobic bacterium, which is widely distributed in the environment. *C. perfringens* is subdivided into five groups (types A to E), based on its four major toxin (alpha, beta, epsilon and iota). *C. perfringens* type B beta toxin causes inflammation and bloody necrotic enteritis. Type B related enterotoxaemia is a major problem of veterinary sciences. The aim of the present study was to clone and sequence *C. perfringens* type B vaccine strain beta toxin gene. Genomic DNA was extracted using phenol-chloroform method. Beta toxin gene was amplified and ligated in pJET1.2/blunt cloning vector. The ligation product was transformed using *E. coli*/TOP10 competent cells and the recombinant pJETβ clones were chosen on LB-amp. pJETβ recombinant plasmid was extracted from recombinant bacterium host and was sequenced using universal primers. Sequencing and BLAST and phylogenetic analysis of *cpb* showed over 99% identity to other previously deposited *cpb* in the GenBank.

Keywords: *Clostridium perfringens*, beta toxin gene, cloning, vaccine strain

Clostridium perfringens, which was previously known as *Clostridium welchii*, is a gram-positive, anaerobic, spore-forming, non-motile rod-shaped bacterium (1). Its name, which means 'break through', comes from its tendency to destroy the muscle tissue in the process of Clostridial myonecrosis. The organism can be found widely in the world which is the result of variation of its genomic size. Based on the toxins it produces, it can be classified into five different types (A-E) (2). Among these five types, type B and C strains generate beta toxin (CPB) (3). Beta toxin is responsible for some diseases like bloody necrotic enteritis and enterotoxaemia in livestock. It has dermonecrotic activity and is lethal (4). The monomeric form of this toxin is poisonous, but the differentiation of oligomeric from non-toxic monomeric form is very difficult. This toxin can easily change from enabled to disabled, which indicates its high sensitivity. It is unstable in high temperatures and easily inactivated by denaturating elements and trypsin (5). Therefore, despite the low level of trypsin, trypsin limiting CPB resistance in the gastrointestinal tract contributes to the disease process (4).

The *cpb* gene is located in plasmids of variable sizes. This gene is 927 bp in length and is translated into a mature protein with 336 amino

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acids. Twenty seven of these amino acids comprise a signal sequence which is removed from the mature protein before secretion, resulting in a mature 35 kDa toxin (6, 7).

C. perfringens is a model for genetic perusals, due to its striking physiological features (8). Recently, several studies on the cloning and expression of its toxin genes have been reported. A genetic construct containing *C. perfringens* epsilon and beta toxin genes was produced in 2013. Epsilon and beta toxin genes were fused using a small linker sequence, and the fusion gene was expressed as a soluble protein in E. coli and its immunogenicity was studied in mouse. Potency of the toxin (as an antigen) induced 6 and 10 IU/ml of epsilon and beta anti-toxin in rabbit, respectively. Therefore, *E. coli* is a suitable expression host for immunogenic epsilon-beta fusion toxin of *C. perfringens* (9, 10).

Numerous things can modify the current making procedure of Clostridial vaccines and alter the vaccine effectiveness but the biological process is often economically permanent. The purpose of this study was cloning of *C. perfringens* type B beta-toxin gene in *Escherichia coli* system and sequencing of the recombinant gene to develop an effective recombinant vaccine.

Materials & methods

Bacterial strains

C. perfringens type B vaccine strain (CN228) was obtained from Razi institute (Tehran, Iran). *E. coli* strain TOP10 was applied as cloning host and *E. coli* strain Rosetta as expression host. Both bacterial strains were obtained from Razi institute (RVSRI, Tehran, Iran).

Cultivation

C. perfringens strain were cultured anaerobically utilizing anoxomat chambers (Mart ® microbelogy, Netherlands) in liver extraction media, pH 7.5 at 37 ºC for 18 h. *E. coli*/TOP10 cells were cultivated as described previously (11).

DNA extraction and gene amplification

Whole genomic DNA was extracted by phenol-chloroform method (12). Primers were designed for separation of beta toxin coding gene from *C. perfringens* genomic DNA. The complete nucleotide sequence of *cpb* gene were retrieved from the GenBank. The primary data for primer design was GenBank HQ179547 accession number. *cpb* gene was amplified by PCR using specific forward 5′ AAT CAT ATG AAT GAT ATA GGT AAA ACT 3′ (including *Nde*I restriction site) and reverse 5′ AAT CTC GAG AAT AGC TGT TAC TTT GTG 3′ (including *Xho*I restriction site) primers using *Pfu* DNA polymerase enzyme for achievement of blunt-end PCR product.

Cloning

Linearized pJET1.2/blunt vector (Fermentas) was used as cloning vector. Amplified *cpb* gene was ligated into pJET1.2/blunt to produce pJETβ recombinant vector. pJETβ was transformed into *E. coli* strain TOP10 by electroporation method. After transformation, screening of recombinant clones was performed by antibiotic resistance (culture of suspension on LB-amp agar plate and incubation at 37°C) and colony PCR. After overnight growth on selective media, colony PCR was carried out for six of the colonies containing pJETβ recombinant plasmids. pJET1.2/blunt universal forward and reverse sequencing primers were used for colony PCR reaction according to the manufacturer's recommendations. For negative control, non-recombinant cloning vector was also subjected to the same PCR procedure. The same

colony was cultured on a new plate containing LB ampicillin, and later plasmid extraction was carried out.

Recombinant pJETβ extraction and analysis

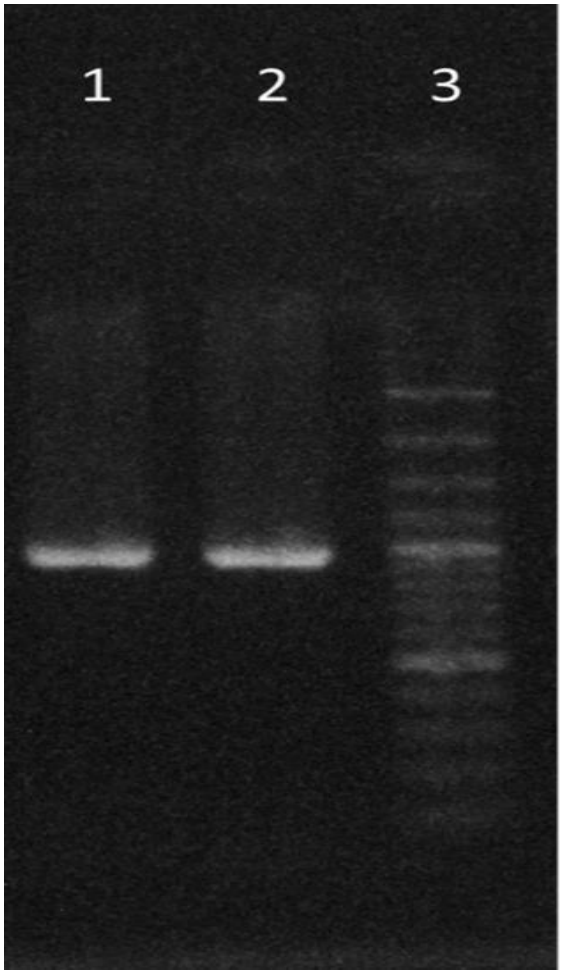

Figure 1. 1% agarose gel electrophoresis of *C. perfringens cpb* gene. Lanes 1 & 2: PCR products (beta toxin type B); lane 3: 100 bp plus DNA molecular weight marker.

Recombinant pJETβ was extracted and purified using plasmid extraction kit (Fermentas) according to the manufacturer instructions and digested with *Nde*I and *Xho*I restriction enzymes (Fermentas). Nucleotide sequencing of the purified recombinant vector was carried out (Source Bioscience co, UK). Then BLAST and phylogenetic analysis was accomplished.

C. perfringens cpb gene was amplified using one pair of primers. (Figure 1) shows 1% agarose gel electrophoresis of this amplified gene.

During ligation procedure, beta toxin gene (927 bp) was ligated into pJET1.2/blunt cloning vector (2974 bp) yielding pJETβ recombinant vector (3901 bp). (Figure 2) shows the structure of recombinant vector.

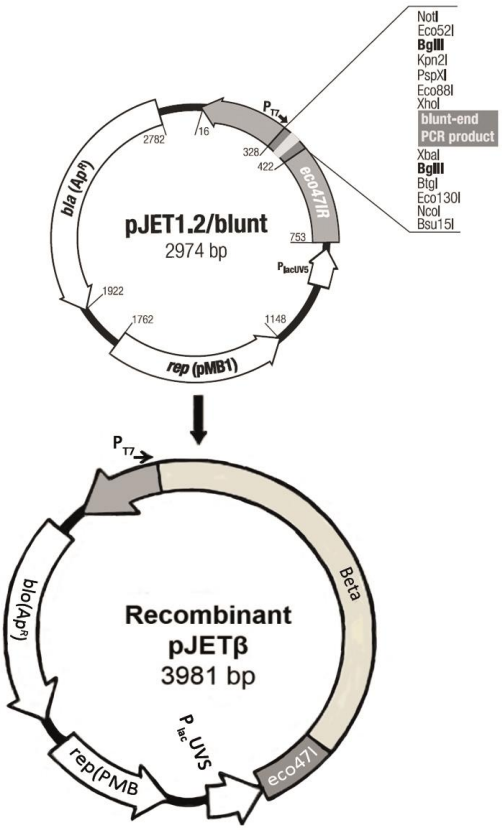

Figure 2. Recombinant pJETβ cloning vector structure shows beta toxin gene derived from *Clostridium perfringens* CN228.

Overnight culture of transformed bacteria on LB ampicillin showed recombinant *E. coli*/TOP10 /pJETβ colonies. Colony PCR of six recombinant colonies using *cpb* forward and reverse primers confirmed the presence of a 927 bp DNA fragment on 1% agarose gel electrophoresis (figure 3). (Figure 4) shows the fragments generated by the ecombinant pJETβ plasmid after enzymatic digestion.

Sequencing analysis of purified pJETβ confirmed *cpb* gene. (Figure 5) and (Table 1) shows that BLAST and phylogenetic analysis of *cpb* confirm over 99% identity to other previously deposited *cpb* in the GenBank.

Discussion

C. perfringens is a potent pathogen because of its ability to generate several toxins. Some Clostridial toxins are active at the moment of secretion like beta toxin which is the major toxin of

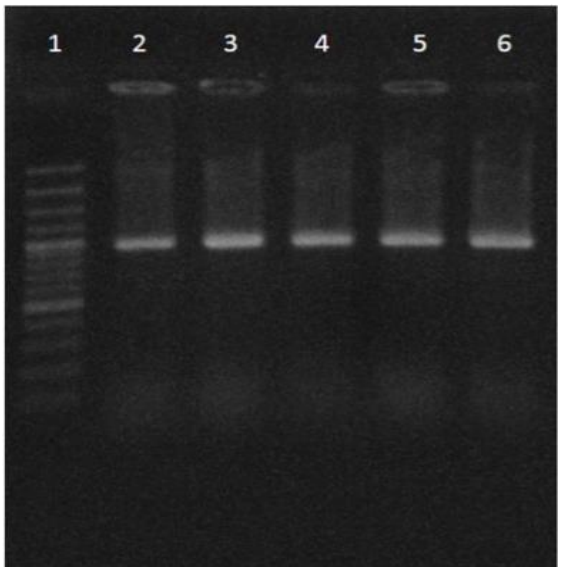

Figure 3. Colony PCR analysis of six recombinant *E. coli*/TOP10/pJETβ colonies. Lane 1: 100 bp plus DNA ladder; lanes 2-6: PCR products of six recombinant colonies.

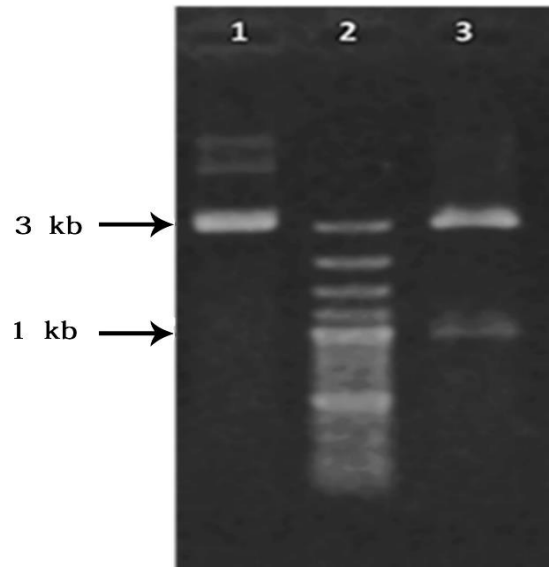

Figure 4. pJETβ plasmid extraction result. Lane 1: uncut recombinant pJETβ with 3 kb size; lane 2: 100 bp plus DNA ladder; lane 3: digested pJETβ with 1 kb insert size.

C. perfringens type B and C. This toxin causes enterotoxaemia and necrotic enteritis in livestock and human (13). In 1957, *C. Perfringens* Iranian variant type B was isolated from intestinal contents of enterotoxemia of sheep and goats. Furthermore, three strains of isolated *C. welchii* type B were differed from the classical type B strains based on [kappa] production and absence of [lambda] and hyaluronidase toxins. Two of the strains were isolated from young goats and the other from an adult sheep (14). Therefore, using type B in enterotoxemia tetra vaccine design in Iran is necessary.

The cloning vector used in this study was pJET1.2/ blunt which is a novel positive selection blunt end vector. Using this vector has advantages over the vectors with sticky end. In vectors with sticky end, self-ligation is common. In blunt end vectors immediately after finding the desired gene amplify-ication, with the use of enzymes like *Pfu* or other enzymes with proof reading nature, ligation can be completed. Any DNA fragment, either blunt or sticky-end, can be successfully cloned using this vector. The pJET1.2/blunt vector has 2974 bp length and contains a lethal gene which is located between nucleotide 371 and 372 (9). After insertion of DNA at this site, the lethal gene will be destroyed so any cell that have received recombinant plasmids have the ability to reproduce. Using this vector, white and blue colonies are no longer necessary (15).

The result of colony PCR performed with colonies grown as template, showed 927 bp fragment and the PCR with extracted plasmid as template and display of approximately 927 bp frag-ment on agarose gel confirmed the previous result. These findings suggest that beta-toxin is in the recombinant vector and was transformed into the *E. coli*/TOP10 bacteria.

Souza *et al.* have previously cloned the *etx* gene into vector pET-11a and the recombinant

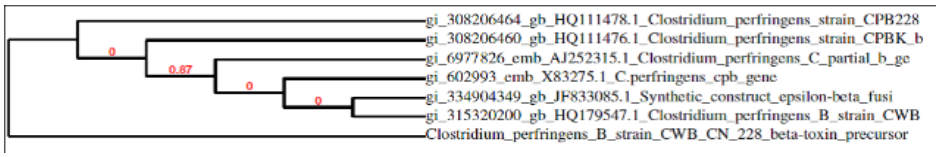

Figure 5 . Phylogenetic analysis of *cpb* based on data of Table 1 (22, 23).

Table 1. BLAST analysis of *cpb* confirms over 99% identity to other previously deposited *cpb* in the GenBank (18)

Accession number	Description	Query cover	Identity	References
HQ179547.1	*Clostridium perfringens* B strain CWB CN228 beta-toxin precursor, gene, partial cds	84%	99%	(12)
JF833085.1	Synthetic construct epsilon-beta fusion protein gene. Partial cds	83%	99%	(19)
L13198.1	*Clostridium perfringens* type B beta-toxin gene, complete cds	83%	99%	(6)
AJ252315.1	*Clostridium perfringens* C partial b gene for beta toxin	83%	99%	(20)
X83275.1	*C. perfringens* cpb gene	83%	99%	(21)
HQ424445.1	*Clostridium perfringens* strain CN301 beta-toxin precursor (cpb) gene, partial cds	66%	99%	(19)
HQ111476.1	*Clostridium perfringens* strain CPBK beta-toxin gene, partial cds	37%	98%	Unpublished data
HQ111478.1	*Clostridium perfringens* strain CPB228 beta-toxin gene, partial cds	36%	98%	Unpublished data

epsilon toxin was expressed in inclusion bodies and was used successfully for animal immunization (16).

In a study by Tang *et al*. *C. perfringens* type C enterotoxin isolated from goat was detected, cloned and sequenced (17). The result showed sequence and amino acid identity with reference strain in GenBank.

In a study by Bai *et al*. on cloning of α-β fusion gene from *C. perfringens* and its expression, the gene was cloned using plasmid pZCPAB and expressed α-β fusion protein was identified by molecular assays like ELISA (10).

One of the factors that play an important role in the effectiveness of the vaccine is the vaccine strain's toxigenesis power. This reason led us to choose *C. perfringens* vaccine strain (CN228) in the current research.

Acknowledgments

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Conflict of interests

The authors declared no conflict of interests.

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