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CLONING AND EXPRESSION MOST EXPECTED ANTIGENIC FRAGMENT OF BETA-TOXIN GENE FROM *CLOSTRIDIUM PERFRINGENS* TYPE B

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ABSTRACT

Clostridium perfringens type B and C is an important pathogen and produces Beta-toxin which are responsible necrotic enteritis in humans or livestock. The death in individuals with this disease are over 50%. Vaccines against *C. perfringens* type B and C are currently manufactured using Beta-toxin produced by the virulent *C. perfringens* strain itself. To achieve the effective components for the creation of immunity at the first step used different primers in various location of Beta-toxin gene (cbp) by bioinformatics tools according to the secondary protein structure. After amplication of PCR products, one regions of Beta-toxin gene with high antigenicity was cloned into pTZ57RT and sub-cloned into the expression vector pET21a(+). The cloned vector was transformed into E. coli BL21 (DE3) and successfully expressed. Protein expression was confirmed by SDS-PAGE electrophoresis and western blotting. This recombinant peptide from most antigenic region of Beta-toxin gene can be suggested for antibody production and new peptide vaccine.

Keywords: *Clostridium perfringens*, Beta-toxin gene, Cloning

INTRODUCTION

Clostridia are ubiquitous and are commonly found in the environment, soil, decaying organic matter and a member of the gut flora in humans and animals **(Cato et al., 1986)**. *Clostridium perfringens* is an anaerobic, Gram-positive, rod-shaped, spore-forming bacterium that is one of most important pathogen of humans and livestock **(Miclard et al., 2009; Rood and Cole, 1991)**.
C. perfringens produces numerous toxin which are responsible for severve diseases inclusing intestinal or foodborne in human and animals. This micro-organism is classified into five toxintype (A, B, C, D and E) based on their ability to synthesize four major toxin, namely Alpha, Beta, Epsilon and Iota **(Petit et al., 1999)**. *C. perfringens* type B and C isolates, which produce Beta-toxin (BT) that causes necrotic enteritis in human and domestic animal **(Shatursky et al., 2000; Springer and Selbitz, 1999)**.
CBP is a lethal pathogenic factor of *C. perfringens* type B which aid in the lysis of HL-60 cells by forming cation-selective pores in the cell membrane **(Nagahama et al., 2003)**. This function is necessary for both necrotizing enteritis and lethal enterotoxemia caused by *C. perfringens* **(Nagahama et al., 2008; Sayeed et al., 2008)**. The gene for the BT has 1209 base pair with 336 amino acids in BT protein. The secreted toxin has similarities (based on 17% to 28% identity) to other toxins that are known to form pores in the plasma membranes of eukaryotic cells **(Hunter et al., 1993)**. Therefore, the production of toxins in heterologous expression systems is viable altenative.
The efficiency of vaccines based *C. perfringens* recombinant Beta-toxin has been reported. The importance of the Beta-toxin in human and animal diseases has been demonstrated by immunization studies with Beta toxoid. In one study α-β fusion gene from *C. perfringens* type C was cloned and expressed in E. Coli **(Bai et al., 2006)**. In this study the expressed α-β fusion protein can be used as the immunogens peptide for immunization. They constracted a recombinant epsilon-beta fusion protein for applying in vaccine production **(Langroudi et al., 2011)**. In another study, α/β2/β1 trivalent fusion-toxin (CPAB2B1) displayed increased immunogenicity relative to CPA and CPB2B1 alone. In other work, a vaccine based on Beta toxoid of *C. perfringens* type C produced and evaluated in E. Coli. The non-toxic recombinant Beta toxoid (rBT) was innocuous for mice and induced beta antitoxin in rabbits **(Milach et al., 2012).**
The aim of this work is production a recombinant fragment of Beta-Toxin (r-f-BT) from C. perfringenes type B in E. coli. Expected to use this recombinant protein for production of antibody against Beta-toxin of C. Perfringenes type B and futher applications.

MATERIAL AND METHODS

DNA extraction

Clostridium perfringens type B strain ATCC3626 prepared from Razi vaccine and serum research Institute. The cell was grown for 18 to 20 h at 37 °C in TGY (2% Trypticase, 2% glucose, 0.5% yeast extract). Genomic DNA was extracted by standard method with phenol/chloroform/isoamyl alcohol method **(Sambrook et al., 1989)**.

Primer designing

Since the aim of producing universal antibody for all antigenic components of Beta toxin *C. perfringens* therefore fragments of cpb were aligned and conserved gene sequences were selected. Multiple sequence alignments of 11 gene sequence of *C. perfringens* type B was performed using the CLUSTAL W2 program - (include: B-CPB240, B-CPB213, B-CPB228, B-CPB236, B-CPB220, B-CPB214, B-B, B-cpb, B-C-b, B-CWB-CN-228, B-CN301), we designed universal primers from conserved regions. Different primers were designed in various location of C.perfringens cpb gene (GenBank Accession No. X83275.1) according to the secondary structure of protein (Table 1). Secondary structure of beta protein (α-helix and β-sheet regions) was obtained based on the amino acid sequence of Beta toxin using PSIPRED Bioinformatics, then primers were designed according to different situations and out of range of the α-helix and β-sheet. Peptides outside the region of helix and loop regions are antigenic peptides. For confirmation of primer designing Immune Epitope Database (IEDB) was used.

Table 1 Six oligonucleotide primers for PCR-synthesizing cpb gene.

Primer name	Primer sequense	Sequence size	Position F-R
F1cbp	aaa gag caa tgt tca ttt aac tta aca	618 bp	1-618
R1cbp	tgt aga tga ttc agc ata ttc gct		
F2cbp	act aat tct act gca att aat ttt ccg	582 bp	399-981
R2cbp	gga ata gac ttg tcc tac cca gtt		
F3cbp	agc gaa tat gct gaa tca tct aca	474 bp	591-1065
R3cbp	aat agc tgt tac ttt gtg agt aag cca		

Polymerase chain reaction (PCR)

The PCR was carried out in a final volume of 50 µl containing 1 µg template DNA samples which were extracted from the bacterial strains. The target fragment was amplified using PCR Master Mix (Bioneer). A total of 35 cycles was performed under the following conditions: 94ºC for 5 min, and 1 cycles at 94ºC for 60 sec, 56ºC for 60 sec, 72ºC for 60 sec, then 1 cycles at 94ºC for 60 sec, 54ºC for 60 sec, 72ºC for 60 sec and then 35 cycles at 94ºC for 45 sec, 52ºC for 45 sec, 72ºC for 45 sec with a final extension at 72ºC for 10 min. PCR products were detected by 1.0% agarose gel electrophoresis and photographed.

"Cloning and expression r-f-BT protein"

The PCR product of Beta-toxin gene (cbp) with one region of cbp with high antigenicity (based on antigen prediction bioinformatics tools) was selected and extracted from the gel using the DNA recovery kit (bioneer). The extracted fragment was ligated into vector PTZ57RT (InsTAclone™ PCR Cloning Kit) according to manufacturers protocol. The recombinant plasmid was transformed into competent E. coli DH5α and selected on LB agar plates containing X-gal/IPTG and ampicillin. The white clones with positive plasmid were selected and controled by PCR using its specific primers and M13 primers. Plasmid digestion was performed by EcoR1 and Sal1 restriction endonuclease according to Fermentas protocol. After agarose electerophoresis, the EcoR1-cbp.f-Sal1 was purified and subcloned into pET21a(+) (Invitrogen) to generate the vector pET-21a-cbp.f. This transformant was picked and used to inoculate LB medium. The recombinant vector pET-21a-cbp.f was transformed into E. coli BL21(DE3) and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping. BL21 cells transformed with the plasmids described above were grown in LB medium with 100 µg/mL ampicillin at 37℃ to OD600 = 0.4~0.6. At this time, the expression of the protein was induced by adding 0.1 mM Isopropylthio-beta-galactoside (IPTG). The r-f-BT protein was purified (by instruction in www.thermo.com/pierce) and examined with SDS-PAGE and western blotting.

RESULTS

The aim of this project is to provide certain fragments of the Beta-toxin with high antigenicity and under epitope-focusing. First primers were designed based on the secondary structure with PSIPRED tool (Figure 1), then the results predicted antigenic regions of Beta-toxin by semi-empirical method showed that Beta-toxin has 9 antigenic regions which is shown in table 2. Thus region between 399−981 bp which has more than 5 antigenic regions of overlap with other fragments (Figure 2). Therefore this region was selected and tranformed.

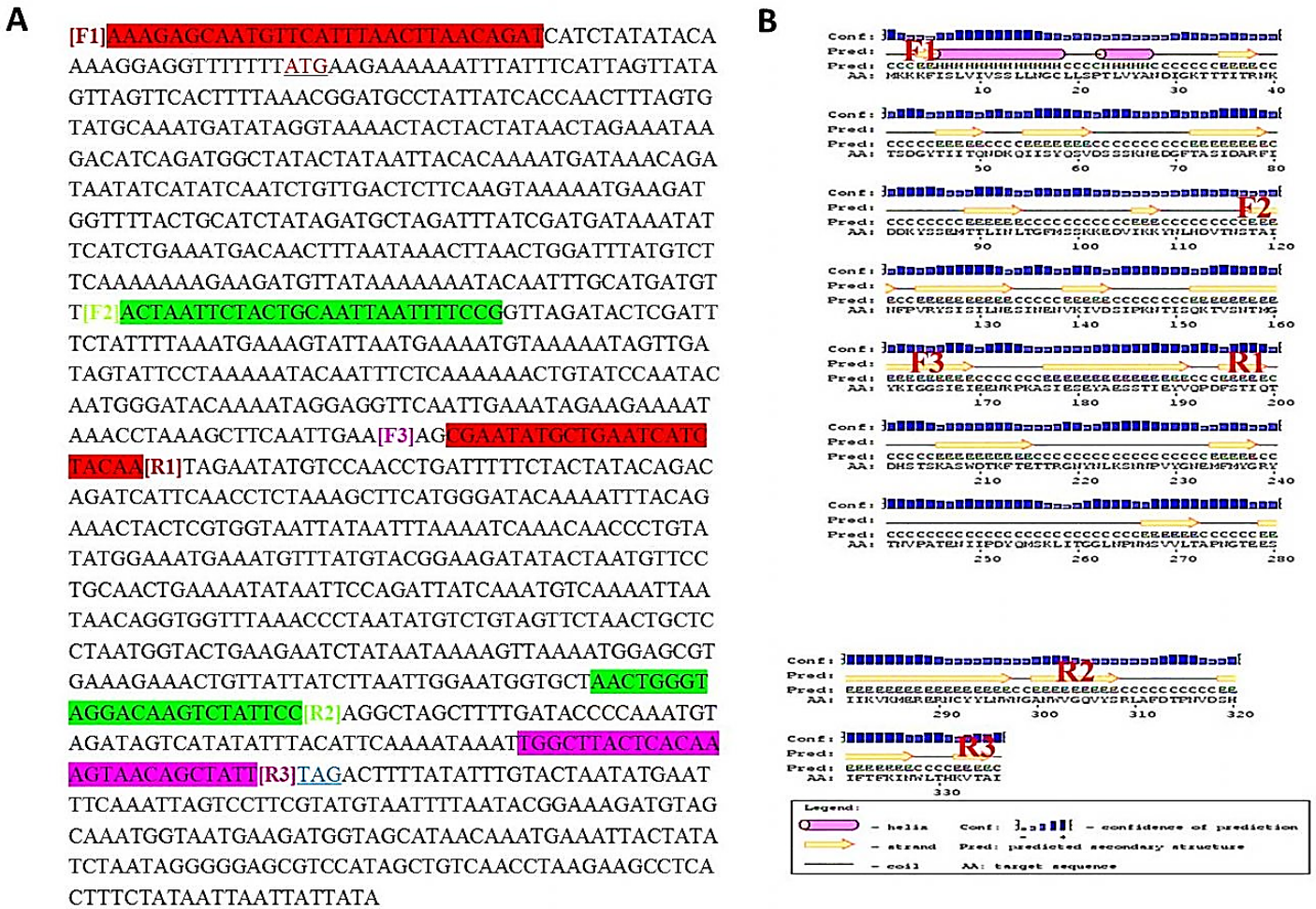

Figure 1 Bioinformatic tools for PCR Primer design **A.** Nucleotide sequence of Beta-toxin gene cpb from *C. perfringens* type B strain ATCC3626 and Suggested position for primers **B.** Primers was designed based on the secondary structure with PSIPRED tool.

Table 2 Predicted peptides of Beta-toxin by semi-empirical method

No.	Start Position	End Position	Peptide	Peptide Length
1	5	28	FISLVIVSSLLNGCLLSPTLVYAN	24
2	55	63	IISYQSVDS	9
3	103	114	EDVIKKYNLHDV	12
4	120	132	INFPVRYSISILN	13
5	138	145	NVKIVDSI	8
6	188	196	IEYVQPDFS	9
7	266	273	NMSVVLTA	8
8	303	314	VGQVYSRLAFDT	12
9	317	324	VDSHIFTF	8

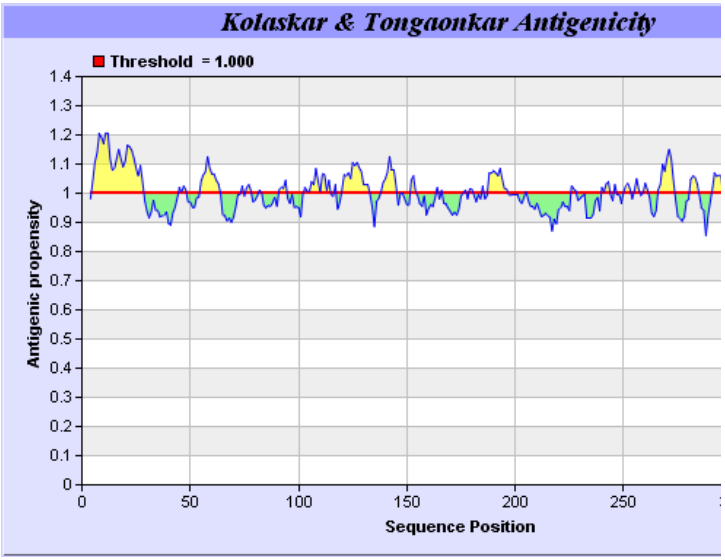

Figure 2 Semi-empirical method for prediction of antigenic regions of Beta-toxin *C. perfringens*.

"Gene cloning and expression of r-f-BT gene" in E. coli BL21 (DE3)

The r-f-BT gene from *C. perfringens* type B strain ATCC3626 was isolated from genomic DNA-extracted template by PCR amplification. The reaction yielded more products. Electrophoresis of PCR product confirmed the length of PCR fragment, which is shown in figure 3A. One of the fragments of Beta-toxin approximately 582 bp with high antigenicity was ligated to the cloning vector pTZ5R/T with T4 DNA ligase. The cloning vector containing the r-f-BT gene was introduced into competent E. coli DH5α cells by CaCl2 transformation. Transformed E. coli were grown at 37℃ in medium containing X-gal/IPTG and ampicillin. The positive plasmids were identified via sequential digestion with EcoR I and Sal I and r-f-BT protective antigen gene about 582 bp was obtained (Figure 3B).

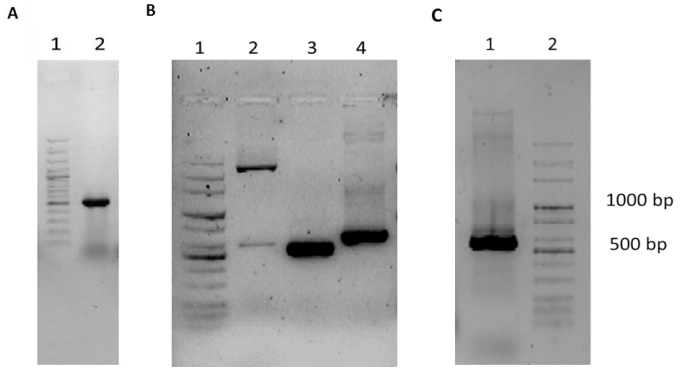

Figure 3 Agarose gel electrophoresis of r-f-β gene was amplified by PCR **A.** Lane 1: DNA molecular marker (GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp), Lane 2: 582bp. **In B.** Lane 1: DNA molecular marker. lane 2: pTZ57CPB/EcoR1 + Sal I. Lane 3, 4: Colony PCR results with Primer F2R2 (582bp) and M13 universal primers (741bp). **In C.** Cloned fragment into PET21 (a+) was confirmed by specific Primer F2R2.

The recombinant plasmid pET-21a-cbp.f was transformed into E. coli BL21(DE3) and the recombinant strain BL21(DE3) was obtained. Then SDS-PAGE and Brown band at the position of the reaction in western blotting confirmed the successful cloning and expression. The r-f-BT protein was produced in E. coli with an apparent molecular weight of 23 kD was observed (Figure 4).

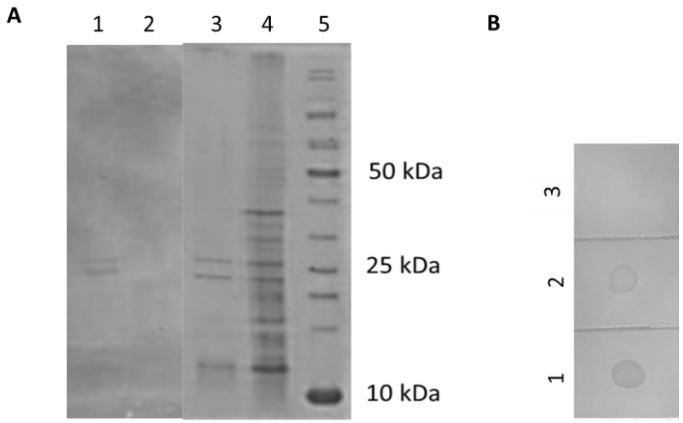

Figure 4 Characterization of r-f-BT producing in E. coli. **A.** Western blot and SDS-PAGE 12% analysis respectively, Lane 1: r-f-BT protein in reaction with native Beta-toxin antibodies. Lane 2: untransformed E. coli Bl21 (DE3) star extract (negative control). Lane 3: r-f-BT protein 23 kDa purified from E. coli inclusion bodies, Lane 4: Supernatant from soluble fraction of recombinant E. coli. Lane 5: Fermetas unstained protein marker. **B.** The results of dot blot analysis using native Beta-toxin antibodies. Lane 1: r-f-BT protein, Lane 2: BT *C. perfringens* (positive control), Lane 3: negative control.

DISCUSSION

Beta-toxin is one of the lethal toxins produced by *C. perfringens* type B and C strains **(Hunter et al., 1993)** Beta-toxin of *C. perfringens* (CBP) type B is caused the principal disease such as lamb dysentery in Great Britain and South Africa **(Niilo, 1980)**. Moreover, the toxoid vaccines majority of the commercial vaccines containing *C. perfringens* types B and C antigens, used in domesticated did not induce the minimum titers of b antitoxin, they are required to be tested for safety, residual toxicity and potency **(Milach et al., 2012;Titball, 2009)**.

Vaccine based on recombinant Beta toxoid (rBT) produced and evaluated in Escherichia coli, the non-toxic rBT was innocuous for mice and induced b antitoxin in rabbits. In other study the Beta-toxin gene fused to the glutathione-S-transferase (GST) was cloned and expressed in E. coli. The purified fusion protein is not toxic in mice and raised rabbit antisera against it specifically neutralises the toxic effect BT of *C. perfringens* type C culture filtrate in mice. Accordingly, the recombinant toxin proteins instead of their native toxins, are promising alternatives to the control of diseases caused by *Clostridium perfringens* **(Milach et al., 2012; Steinpórsdótti et al.,2006).** Other result suggested that recombinant toxoids are potential vaccine candidates against Clostridial toxins **(Zeng et al., 2011)**.

Due to the strong toxicity of Beta-toxin, we decided to evaluate a recombinant form of the toxin (rec-β) as a potential antigenic toxoid for production of a recombinant vaccine against C. perfringenes in future studies, after immunogenicity assay. As regards, the technology of recombinant protein antigens for immunization goes to identification main epitopes of protein antigens. Therefore antibody production is more successful with segments which contain epitope-focused antigens. The aim of this project is cloning a small fragment of the Beta-toxin with high antigenicity and epitope-focusing. First we designed primers according to the sequence and secondary structure of proteins that after protein structures are preserved**;** Beta-toxin has been shown more β-sheet than α–helix by secondary structure prediction. According to previous research, most pore-forming protein toxins have extensive β-sheet in their structure which can create suitable antigenic effect **(Parker and Feil, 2005)**. The variable regions were located in the external loop structures, while the predicted β-strands were formed by conserved sequences. The primers designing was done in external loop position. Epitope analysis plays an important role in the development of effective vaccine and diagnostic tools for different infection. In one study using different bioinformatics tools, one of the B cell epitopes of epsilon toxin comprising the region (Etx40-62) was identified. The rLTB.Etx40-62 fusion protein thus can be evaluated as a potential vaccine candidate against *C. perfringens* **(Kaushik et al., 2013)**.

In the present study we describes the successful isolation and cloning f-BT gene from strain of *C. perfringens*. We constructed a r-f-BT protein from *C. perfringens* type B. Ultimately western blot of r-f-BT protein showed that the antibodies specifically recognize antigen which it is attached. In summary, our findings demonstrate that r-f-BT of *C. perfringens* was capable of reacting with native Beta-toxin antibodies. The recombinant toxins with epitope-focused also be used to produce monoclonal antibody for immunoassay or possible therapy.

CONCLUSION

Herein, we reported that a r-f-BT of *C. perfringens* type B has been cloned and expressed in E. coli BL21, the achievement of this study was the production of r-f-BT with high antigenicity. These recombinant toxin (r-f-BT) proteins can replace the natural protein and can be used immunological detection of specific antibodies against the Beta toxin and vaccine research. These approaches were successful in maintaining the antigenicity of the epitope using bioinformatics tools, significantly minimize the time and efforts in generating recombinant protein with high antigenicity.

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