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Full Article

Fusion of *Clostridium perfringens type D* and *B* epsilon and beta toxin genes and it's cloning in *E. coli*

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ABSTRACT

Designing and producing a proper fusion construction is the most important problem of producing large quantities of a properly folded functional protein. This construction should have all necessary components of a real gene. A good designed fusion gene construction could be cloned into a good and suitable host. *Clostridium perfringens* is an important pathogen of humans and livestock and produces numerous toxins including epsilon and beta which are responsible for severe diseases. In the present study a new construction containing *Clostridium perfringens type D* epsilon toxin gene and *type B* beta toxin gene was designed. At the first step two pairs of primers were used for these genes amplification. At the next step epsilon forward and beta reveres primers were used to produce a chimeric gene containing amplified partial cds of etxD and partial cds of cpbB which are linked together by the AEAAAKEAAAKA fragment as a small linker. The method was based on fusion PCR and using of Pfu DNA polymerase, which has a proofreading activity. The fusion gene inserted into pJET1.2blunt and cloned into *E.coli* strain TOP10. Based on the latest information, this is the first design and cloning of epsilon-beta fusion gene and also this is the first time that PCR fusion strategy is used for Clostriadial gene fusion, which could be used for development of a recombinant epsilon-beta fusion protein vaccine. This construction also could serve as a model for development and production of novel fusion protein for other potential proteins and toxins.

Keywords: *Clostridium perfringens*, epsilon toxin, beta toxin, cloning, fusion PCR

INTRODUCTION

Clostridium perfringens (formerly known as *Clostridium welchii*) an important pathogen of humans and livestock, is a Gram-positive, rod-shaped, anaerobic, spore-forming, heat-resistant bacterium of genus *Clostridium* (Rood & Cole 1991). *Clostridium perfringens* produces numerous toxins and is responsible for severe diseases in humans and animals

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including intestinal or food borne diseases, hemorrhagic enteritis, enterotoxemia and gangrenes. It is also a secondary pathogen in various diseases, such as necrotic enteritis (Ochi *et al* 2004, Sheedy *et al* 2004). Individual strains produce subsets of toxins (Petit L. *et al* 1999) and four of them iota (iA), alpha (cpa), beta (cpb), epsilon toxins (etxD) have been proposed to be used for classification of *Clostridium perfringens* into five isotypes A, B, C, D, and E (McDonel 1986)(McClane 2001). Among the toxigenic Clostridial species, including *Clostridium tetani*, *Clostridium botulinum* and *Clostridium difficile*, *Clostridium perfringens* is the paradigm species for genetic studies, because of it's oxygen tolerance, fast growth rate (8 to 10 min generation time in optimal conditions), and ability to be genetically manipulated (Rood 1998).

A physical map of the genome of *Clostridium perfringens* strain CPN50, associated with human disease, has been shown to possess a single circular chromosome of about 3.58 Mb by PFGE. More than 100 restriction sites and 24 genetic loci have been located on the genome. The complete 3031430 bp sequence of *clostridium perfringens* strain 13, that comprises 2660 protein coding regions and 10 rRNA genes, showing pronounced low overall G-C content (28.6%) was reported .*Clostridium perfringens* has the highest tendency to arrange the genes on the leading strands, which seems to be highly related to having the highest G+A content of the leading strands. This relationship seems to be common to other Gram-positive bacteria (Shimizu *et al* 2002).

Clostridium perfringens epsilon toxin is a potent toxin and a member of a group of highly toxic Clostridial toxins, it is the third most potent Clostridial toxin after botulinium and tetanus neurotoxins (Payne *et al* 1997). Epsilon toxin is active in a wide range of animal species and plays a central role in enterotoxemia of sheep and lambs. Epsilon toxin gene from *Clostridium perfringens type B* was cloned, then sequenced and expressed in *E.coli* (Sophie *et al* 1992). The sequence of 20 amino acids from the N terminus of *Clostridium perfringens* epsilon toxin was determined. Cloning and expression of epsilon toxin gene from *Clostridium perfringens type D* in *E.coli* was reported (Goswami *et al* 1996) (Souza *et al* 2010). Based on these studies important structures are conserved in recombinant epsilon related to the native form. Recombinant toxin was strongly recognized by anti native epsilon antibodies. Souza also hshowed that antibodies against this recombinant toxin recognized the native epsilon toxin and neutralized its action in mice. Cloned *type B* and native *type D* toxins have similar molecular weights and isoelectric points and that they both react with specific monoclonal antibodies. Epsilon toxin gene (etx) was cloned into the pET-11 plasmid of *E.coli* strain BL21 to produce the recombinant toxin. The potency of *Clostridium perfringens type D* epsilon toxoid expressed by *E.coli* evaluated by it's administration to goats, sheep, and cattle. The produced and tested recombinant epsilon toxoid was adequate for immunization of ruminants against enterotoxemia (lobatoa *et al* 2010). The potency of formaldehyde inactivated recombinant epsilon toxin as a vaccine candidate in sheep was determined. Aluminum hydroxide was used as adjuvant in this vaccine. Sheep were vaccinated by this construct in combination with sheep pox vaccine. Results showed that sheep were being protected against both sheep pox and enterotoxemia (Chandran *et al* 2010).

Clostridium perfringens beta toxin causes enterotoxaemia and necrotic enteritis in lambs, piglets and calves (Hogh 1976). In human beta toxin is known to produce necrotic enteritis (Granum 1990). Beta toxin is known as one of the combined Leucocidin/ASH4 hemolysin, domain and also Staphylococcal bi-component toxin family members, which are beta-channel forming cytolysins. Beta toxin gene sequencing has revealed similarity (17-29%) with alpha toxin, gamma toxin and leukocidin from *Staphylococcus aureus* (Hunter *et al* 1993).

The beta toxin gene from *Clostridium perfringens type C* was cloned and expressed as a glutathione S-transferase fusion protein in E. coli (Steinpórsdótti *et al*

1995). The DNA sequence was determined and compared to the *type B* sequence. Two nucleotide differences were found in the protein coding sequence, resulting in one amino acid difference between the two proteins. The purified beta-toxin fusion protein is not toxic in mice, but rabbit antiserum raised against it neutralizes the toxic effect of *clostridium perfringens type C* culture filtrate in mice. *Clostridium perfringens* recombinant beta toxin has been expressed and secreted from *Bacillus subtilis* by Steinthorsdottir in 1998. Biological activity was tested in vivo and in vitro. Gene encoding beta toxin was amplified by polymerase chain reaction from *Clostridium perfringens type C* isolated and cloned in pUC19 vectors. The nucleotide sequence was identical with *clostridium perfringens type B* beta toxin gene sequence. The Southern hybridization using labeled beta toxin gene probe revealed the presence of positive signals only in beta producing *Clostridium perfringens* (Goswami *et al* 2002).

In the present study we designed a new structural model containing *Clostridium perfringens type D* epsilon toxin gene partial cds and *Clostridium perfringens type B* beta toxin gene partial cds which are fused together using a small linker. A bioinformatics approach was used for in silico analysis of the structure of designed chimeric fusion gene.

MATERIALS AND METHODS

Materials. Taq and Pfu DNA polymerase, dNTPs, T4 DNA ligase, *NdeI* and *XhoI* restriction endonuclease, GeneRuler™ 100 bp Plus DNA Ladder, GeneJET™ Gel Extraction Kit for DNA fragment recovery, plasmid pJET1.2/blunt and LB ampicillin FastMedia™ were purchased from Fermentas Company. Pwo DNA polymerase was purchased from Roche Company. Proteinase K, SDS, RNase A, lysozyme and plasmid extraction kit (Mini Prep) were purchased from CinnaGen Company. *E. coli* strain TOP10 was prepared from National institute of genetic engineering and biotechnology. Other reagents if not described, were of analytic purity.

Bacterial strains. *Clostridium perfringens type B* strain CN228, *type C* strains CN301 and *type D* strain CN409 were prepared from Razi vaccine and serum research Institute.

Cultivation. *Clostridium perfringens* strains were cultured anaerobically using Anoxomat chambers (Mart® Microbiology, Netherlands) at 37°C in a liquid medium containing casein hydrolysate (3%), yeast extract (0.5%), glucose (0.5%), cysteine hydrochloride (0.05%), that was adjusted to pH 7.5 by the addition of Na_2HPO_4. For the isolation of DNA, cells of *Clostridium perfringens* were cultured in 20 ml of this broth for 18 hours and then separated from the culture fluid by centrifugation for 30 minutes at 5000 RPM at room temperature. *E.coli* strain *TOP10* was stored and cultured as described previously (Sambrook 1989), (Brown 2006)

Isolation of genomic DNA. High molecular weight genomic DNA from *Clostridium perfringens type B, type C and, type D* was isolated according to the modified method described earlier (Hochulli *et al* 1987). Cells from a 20 ml overnight culture were harvested by centrifugation and suspended in 300 µl THE (which contained 1 mg lysozyme per ml). Cells were added 300 µl of 10% SDS, and 5 µl RNase-A and then incubated for 30 minutes at 37°C. Proteinase K (50 mg/ml) was added and the mixture was incubated for 90 min at 37°C and extracted twice with phenol and chloroform. 60 µl sodium acetate (1:10 V/V) and 600 µl isopropanol (1 V/V) were added and incubated at 20°C and DNA precipitated by centrifugation.

Primers. *Clostridium perfringens* epsilon-toxin gene complete cds M80837.1 and *Clostridium perfringens type B* beta-toxin gene, complete cds L13198.1 respectively were used for epsilon toxin and beta toxin gene. The designed sequences of epsilon toxin primers are as follows:

Forward primer:

5' AAT CAT ATG AAA AAA AAT CTT GTA AAA AGT 3'

Reveres primer:
5' TTT CGC CGC CGC TTC CGC TTT TAT TCC TGG TGC CTT AAT 3'
The sequences of beta toxin primers are as follows:
Forward primer:
5' GCG GAA GCG GCG GCG AAA GAA GCG GCG GCG AAA GCG AAT GAT ATA GGT AAA ACT 3'
Reveres primer:
5' AAT CTC GAG AAT AGC TGT TAC TTT GTG 3'

PCR amplification of epsilon toxin and beta toxin genes. The following components were added into a 500 µl microcentrifuge tube. Each PCR reaction contained 0.1 µmol forward primer, 0.1 µmol reveres primer, 0.25 mmol dNTPs, 1.5 mmol MgCl2,10 µl 10×PCR buffer, 2 unit pf DNA polymerase and appropriate template DNA. Sterile DEPC treated distilled water was added into a 50 µl reaction system. Thirty cycles of PCR were performed (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min).

Sequence analysing of amplified and purified PCR products. The PCR products in the size of 1 kb were excised from agarose gel following electrophoresis, and purified by using the GeneJET™ Gel Extraction Kit for DNA fragment recovery according to the manufacturer's recommendations. Nucleotide sequencing was carried out by SEQLAB (Sequence Laboratories Goettingen GmbH).

Epsilon-beta chimeric fusion gene construction. Fusion PCR was carried out for epsilon-beta fusion gene construction. Purified *clostridium perfringens* epsilon and beta gene were used in a two steps PCR reaction. The 1st step reaction was designed as a 3 cycle reaction and started in a 0.2 ml thin walled micro tube using all needed components except of primers. After 3 cycles epsilon forward and beta reveres primers were added to the same micro tube and 2nd step of PCR reaction was continued for 20 more cycles. The blunt end PCR product in the size of 2 kb (fusion gene) was excised from agarose gel following electrophoresis, and purified by using the GeneJET™ Gel Extraction Kit for DNA fragment recovery according to the manufacturer's recommendations.

Verification of epsilon-beta fusion gene construction by internal primers. Nested PCR was carried out for epsilon-beta fusion gene construction using epsilon forward and beta reveres primers which had been published previously. The epsilon toxin gene forward primer and the beta toxin gene reveres primer respectively has been designed based on GenBank sequences M95206 and X83275, (Christoph ,2004). These internal primers are as followed
Epsilon toxin gene forward primer:
5' TGGGAACTTCGATACAAGCA3'
Beta toxin gene reveres primer:
5' TGAACCTCCTATTTTGTATCCCA3'

Cloning vector construction and ligation reaction set up. Fermentas linearized pJET1.2/blunt plasmid containing ampicillin resistant gene was used as cloning vector and purified fusion gene was ligated in it. 2 µl 10X reaction buffer, 1 µl pJET1.2/blunt, 1µl T4 DNA ligase and 16 µl blunt end purified fusion gene were mixed in a 0.2 ml thin walled micro tube and incubated over night at 12-14 °C.

Transformation. *E. coli* strain TOP10 was used to produce competent cells and its efficiency tested. Ligation mixture (10 µl) directly used for transformation of 100 µl of bacterial strains. Cell were cultured on a plate containing LB ampicillin FastMedia™ and incubated at 37 °C for one night. The complete technique was done as described previously (Hanahan 1983, 1985).

Verification of fusion construction. After one night, colony PCR was carried out for one of the colonies containing pJET1.2/blunt recombinant plasmid. pJET1.2/blunt forward and reveres sequencing primers were used according to the manufacturer's recommendations for PCR reaction mixture and program. For negative control pJET1.2/blunt non recombinant plasmid vector also was subjected to the same PCR procedure. The same colony was cultured on a new plate containing LB ampicillin FastMedia™. One *E. coli* strain TOP10 competent but not

transformed colony, also cultured on the same plate. Plasmid extraction carried out by CinnaGen plasmid extraction kit (Mini Prep). Plasmid digestion was done using *NdeI* and *XhoI* restriction endonuclease according to Fermentas protocol. Two set of PCR was carried out for epsilon-beta fusion gene construction using purified plasmid. Internal epsilon forward and beta reveres primers and original epsilon forward and beta reveres primers were used for this purpose. All of the above recombinant plasmids and PCR products analyzed after separation by agarose gel electrophoresis.

RESULTS

Epsilon toxin gene of *Clostridium perfringens type D* and beta toxin gene of *types B* and *C* were amplified using two pairs of primers designed according to nucleotide sequences of M80837.1 and L13198, Figure 1 shows agarose gel electrophoresis of these genes.

Sequencing analysis of amplified and purified PCR products. Sequencing data of *Clostridium perfringens type D* etx gene showed 984 bp, and *Types B* and *C* cpb gene showed 927 bp length. Sequencing results of *types B* and *C* cpb genes showed complete identities between these two genes.

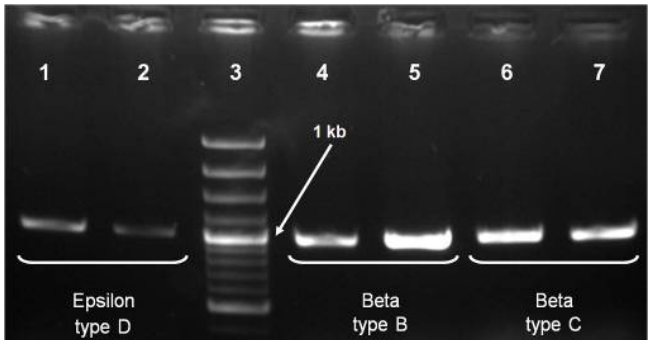

Figure 1. Agarose gel electrophoresis of *Clostridium perfringens type D* epsilon toxin gene (etx) and *Clostridium perfringens type B* and C beta toxin genes (cpb).

Epsilon-beta chimeric fusion gene construction. At the next step epsilon forward and beta reveres primers were used to produce a chimeric gene containing amplified partial cds of etxD and partial cds of cpbB, which are linked together by the AEAAAKEAAAKA fragment as a small linker. The fusion construction length is 1947 bp. The method was based on fusion PCR and using of Pfu DNA polymerase, which has a proofreading activity. Figures 2 shows fusion PCR product loading on gel electrophoresis.

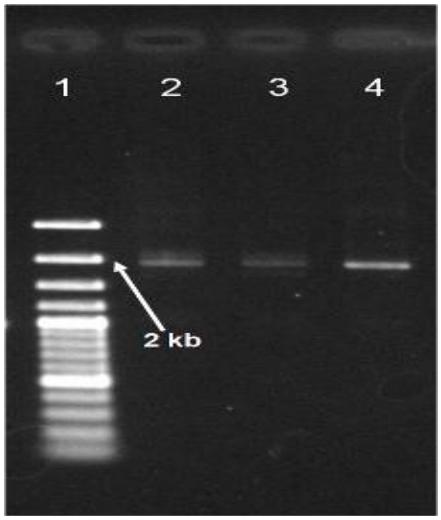

Figure 2. Epsilon-beta fusion construction PCR product on gel
Lane 1: 100 bp plus DNA Ladder
Lanes 2 and 3: Epsilon-beta fusion construction (non purified)
Lane 4: Epsilon-beta fusion construction (purified)

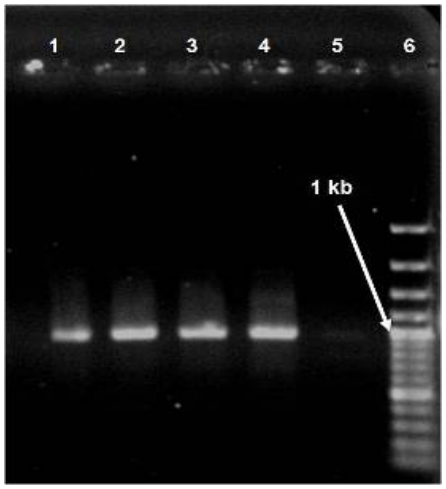

Figure 3. Result of nested PCR of epsilon-beta fusion gene construction using internal epsilon forward and beta reveres primers.
Lanes 1and 2: Products of nested PCR from double purified fusion gene*
Lane 3: Products of nested PCR from double purified fusion gene**
Lane 4: Products of nested PCR from purified fusion gene***
Lane 6: 100 bp plus DNA Ladder
*:Double purified fusion gene has been amplified by Pwo DNA polymerase

**:Double purified fusion gene has been amplified by Pfu DNA polymerase
***:Purified fusion gene has been amplified by Taq DNA polymerase

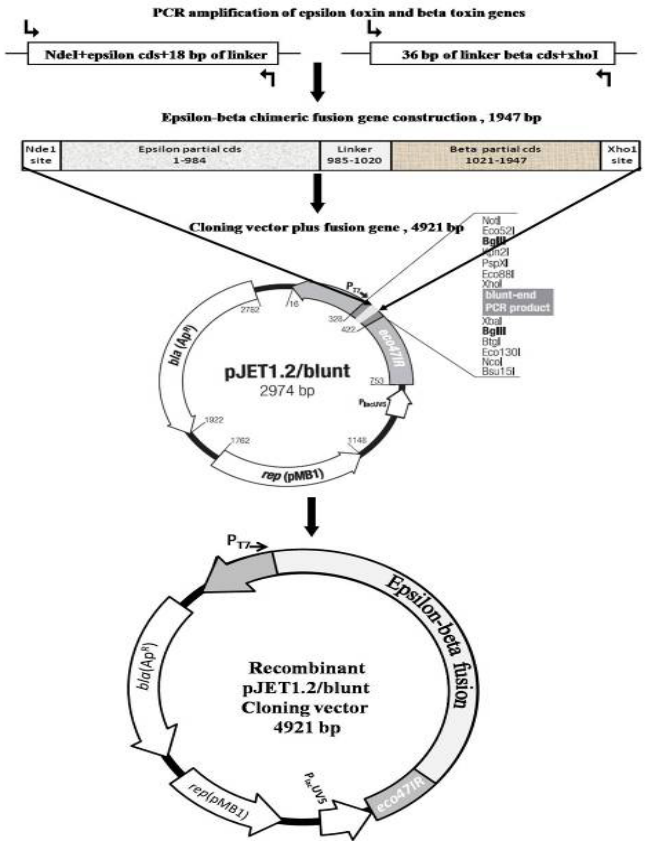

Figure 4. Recombinant pJET1.2/blunt vector .at the 1st step epsilon and beta gene amplified .At the 2nd step fusion gene is constructed. At the 3rd step, fusion gene construction is inserted to the pJET1.2/blunt in the blunt-end PCR product site. Recombinant plasmid vector size is 4921 bp.

Verification of epsilon-beta fusion gene construction by internal primers. The length of amplified fragment between epsilon forward and beta reveres primers which used for nested PCR was ~1kb. Figure 3 shows gel electrophoresis separation of epsilon-beta fusion gene construction nested PCR.

Cloning vector construction and Transformation. During ligation epsilon-beta fusion gene inserted into pJET1.2/blunt (2974 bp linear) vector at the blunt ends and produced a 4921 bp circular recombinant plasmid, Figure 4 shows recombinant pJET1.2/blunt vector diagram. Over night culture of *E. coli* strain TOP10 which had been transformed directly with this ligation product, showed colonies and good condition of growth on LB agar containing ampicillin.

while *E.coli* strain TOP10 nontransformed competent colony did not grown on the LB agar with out ampicillin. Extracted uncut recombinant cloning vector showed two sharp bands above 3 kb on electrophoresis

Verification of fusion construction. PCR product gel electrophoresis of recombinant plasmid colony PCR showed ~2 kb DNA fragment. Negative control PCR product showed one 700 bp bond. Culture result of the same colony on a LB agar plate containing ampicillin, showed good and suitable growth of bacteria, gel.When this recombinant cloning vector was digested by *NdeI* and *XhoI* restriction endonuclease showed one sharp 2 kb and one sharp 3 kb, figure 5 shows this data. PCR product of recombinant cloning vector using internal epsilon forward and beta reveres primers showed one sharp 1 kb band but our primer amplified 2kb pcr product.

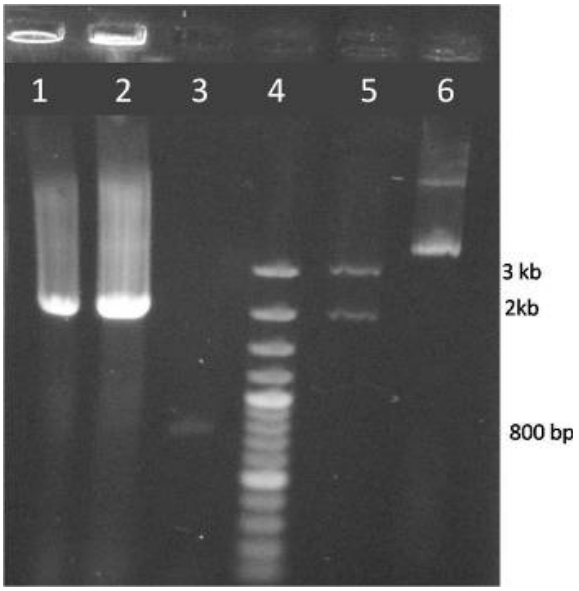

Figure 5. Gel electrophoresis data for verification of fusion construction
Lane 1and 2: PCR product gel electrophoresis of recombinant plasmid colony PCR
Lane 3: Negative control PCR product
Lane 4: 100 bp plus DNA Ladder
Lane 5: Extracted digested recombinant cloning vector
Lane 6: Extracted uncut recombinant cloning vector

DISCUSSION

Expressing and purifying large quantities of functional properly folded protein is bottle neck in structural and functional genomic studies. Expression difficulties typically include poor yield and formation of insoluble aggregates. But the most important problem is designing and producing a proper fusion construction which should have all needed components of a real gene.

A good designed fusion construction could be clone into a good and suitable host, and then expression would be the next step. In the present study a new construction containing *Clostridium perfringens type D* epsilon toxin gene partial cds and *Clostridium perfringens type B* beta toxin gene partial cds was designed. After PCR amplification, these two genes fused together using a small linker based on the fusion PCR method. A bioinformatics approach was used to in silico analysis of the structure of designed chimeric fusion gene before producing epsilon beta fusion construction. Tick cell wall of gram positive bacteria is one of the problems for genomic DNA extraction in these microorganisms. We examined some procedures to solve this problem and finally we used SDS and 1 mg lysozyme per ml of TAE solution. RNase-A and DEPC treated water also used for removing all unwanted material such as RNA in the final DNA extract. Genomic DNA of *Clostridium perfringens* used for amplification of epsilon and beta toxin genes using forward and reveres primers designed from the available sequence. We used *Clostridium perfringens* epsilon-toxin gene complete cds M80837.1 for epsilon toxin gene reference and *Clostridium perfringens type B* beta-toxin gene, complete cds L13198.1 for beta toxin gene reference. Blast in GenBank revels these are the only two sequences that have the complete cds with full annotation of signal and mature peptides. Two pairs of epsilon and beta toxin protective antigen gene primers were synthesized which allowed the open reading frame (ORF) of epsilon and beta toxin to be amplified. The *NdeI* cleavage site and first part of the designed linker were inserted to the 5' and 3' ends of epsilon toxin genes. The second part of the designed linker and *XhoI* cleavage site were inserted to the 5' and 3' ends of beta toxin genes via PCR. The epsilon-toxin gene from *Clostridium perfringens type B* has been cloned and expressed in *E. coli* by using plasmid vector pUC18 (Hunter *et al* 1992). The data obtained from three M13 subclones were sequenced independently showed the nucleotide and derived amino acid sequences of the etx gene are located between the start codon at base 188 and the stop codon at base 1174 (986 bp). Possible Shine-Dalgarno (S.D.) and -10 and -35 consensus sequences controlling the transcription and translation of the etx gene and ORF1 were demonstrated and the experimentally N-terminal amino acid sequence of epsilon-toxin Determined. Beta toxin is synthesized as a 336 amino acid protein. The first 27 residues of it constitute a signal peptide. The secreted protein has a predicted molecular mass of 34861 Da and a pI of 5.5 (Hunter *et al* 1993). The amplified and purified PCR products Sequences were analyzed. According to the results, the sequences identities of the PCR products were confirmed by comparison of the sequences with previous reports obtained from the GenBank and the EMBL and the PDB databases. Amplified Sequence of epsilon toxin gene showed a 1008 bp fragment which has 6 parts. These parts are consisting of flanking region (nucleotides 1-3), Nde1 cleavage site (nucleotides 4-9), epsilon signal peptide (nucleotides 7-102), epsilon mature peptide (prototoxin 103-990), mature peptide (toxin 142-990) and linker for fusion (nucleotides 990-1008) with beta toxin gene. Amplification sequence of beta toxin gene showed a 972 bp which has 4 parts of, linker (nucleotides 1-36) for fusion with Epsilon toxin gene; mature peptide (toxin 37-963), *XhoI* cleavage site (nucleotides 964-969), and flanking region (nucleotides 970-972). The three sequences were submitted to GenBank (accession numbers HQ179546, HQ179547 and HQ424445). A procedure for precise assembly of linear DNA constructs as long as 20 kb has been proposed previously (Shevchuk *et al* 2004). The method, which called long multiple fusion, has been used to assemble up to four fragments simultaneously (for final 10.8 kb

product), offering an additional improvement on the combination of long PCR and overlap extension PCR using Pfu DNA polymerase. In the present study the method was based on fusion PCR and using of Pfu DNA polymerase, which has a proofreading activity. The fusion gene length is 1947 bp, which within nucleotides 1 to 984 is belonging to epsilon partial cds, nucleotides 985 to 1020 (36 bp) is linker sequence which is optimized for *E. coli* as:
GCGGAAGCGGCGGCGAAAGAAGCGGCGGCGA AGCG, and nucleotides 1021 up to 1947 (927 bp) is belong to beta partial cds. *NdeI* restriction site and it's flanking region at the 3' of epsilon and *XhoI* restriction site and it's flanking region for the 5' end of beta also are present. According to the aim of the next step of the research, the designed chimeric gene should be ligated into PET26b(+) expression vector and then *E. coli* strain *BL21* would be transformed by this DNA insert, so the *NdeI* and *XhoI* restriction sites and their flanking regions at the 3' of epsilon and 5' end of beta genes are designed, respectively. These sequences are necessary for inserting of fusion gene into PET26b(+) expression vector. The approach for verification of epsilon-beta fusion gene construction was as followed. Epsilon forward primer, which used for nested PCR, begins amplification at base number 464 on epsilon toxin gene downstream to 5' end of fusion construction. Beta reveres primer, which used for nested PCR, begins amplification at base number 1443 on beta toxin gene upstream to 3' end of fusion construction. So, the length of amplified fragment between these two primers is 980 bp and the designed linker is included in it (figure 3).
PJET1.2/blunt is a 2974 bp linearized plasmid vector which is a positive selection cloning vector. This vector contains a lethal gene which is disrupted by ligation of DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need of blue/white screening. The vector contains an expanded multiple cloning site, as well as a T7 promoter for in vitro transcription and ampicillin resistant gene. The blunt ends of vector contain 5-phosphoryl groups and are located at bp 371-372 in the lethal gene, during ligation epsilon-beta fusion gene inserted into this vector at the blunt ends and produced a 4921 bp circular recombinant plasmid. After transformation only the bacteria which has been transformed by recombinant plasmid vector, showed growth on the ampicillin containing LB agar. After obtaining epsilon-beta fusion, the construction was verified. pJET1.2/blunt sequencing forward and reveres primers are located at the left and right sides of multiple cloning site of this plasmid. As the recombinant plasmid colony PCR produced ~2 kb DNA fragment, but non recombinant plasmid as negative control for this PCR showed a 700 bp DNA fragment, it could be accepted that this 2 kb fragment is epsilon-beta fusion construction. For obtaining more reason on this statement we examined the growth condition of the same colony and *E.coli* strain TOP10 nontransformed colony on LB agar plate containing ampicillin. Since *E.coli* strain TOP10, has not ampicillin resistant plasmid and in this step we used a nontransformed colony as negative control, so only the colony of transformed cells showed suitable growth after an over night culture. Additionally both extracted uncut and digested recombinant cloning vectors were loaded on gel electrophoresis. Results showed two sharp bands above 3 kb in the case of uncut vector, but one sharp 2 kb and one sharp 3 kb in the case of digested vector. As the pJET1.2/blut size is 2974 bp and the epsilon beta fusion gene size is 1947 bp, so these data demonstrate that the recombinant vector, which is inserted by epsilon-beta fusion gene, has transformed the *E.coli* strain TOP10. Finally PCR procedure using internal primers and fusion gene as template resulted in amplification of a ~1kb DNA fragment. After amplification and sequence analyzing this fragment showed 980 bp length which our designed linker is included in it. In this study we developed a new approach for fusion of *Clostridium perfringens type D* and *B* epsilon and beta toxin genes based on fusion PCR strategy. According on the latest information, this is the first design and cloning of epsilon-beta fusion gene and also this is the first time that PCR fusion

strategy is used for Clostridial gene fusion, which could be used for development of a recombinant epsilon-beta fusion protein vaccine. Also it could serve as a model for development and construction of novel fusion protein for other potential proteins and toxins.

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References

Brown, A.S., Habeeb, A.F.S.A. (1977) Structural studies on toxin of *Clostridium perfringens type D*. Localization of the site of tryptic scission necessary for activation of ε - toxin. *Biochemical and Biophysical Research Communications* 73: 889-896.

Brown, T.A., 2006Gene cloning & DNA analysis (an introduction) *fifth edition Blackwell publishing*9600 Garsington Rode, Oxford OX4 2DQ, UK..…

Chandran, D., Naidu, S.S., Sugumar, P., Rani, G.S., Vijayan, S.P., Mathur, D., Garg, L.C., Srinivasan, V.A. (2010). Development of a recombinant epsilon toxoid vaccine against enterotoxemia and its use as a combination vaccine with live attenuated sheep pox virus against enterotoxemia and sheep pox, *Clinical Vaccine Immunology* 17(6):1013-6.

Cheung, J. K. & Rood, J. I., (2000) The VirR Response Regulator from *Clostridium perfringens* Binds Independently to Two Imperfect Direct Repeats Located Upstream of the pfoA Promoter, *Journal of Bacteriology* 182, 57–66.

Christoph, G. Baums, Ulrich Schotte,Gunter Amtsberg, Ralph Goethe , (2004) Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates, *Veterinary Microbiology* 100, 11–16

Lobato FC, Lima CG, Assis RA, Pires PS, Silva RO, Salvarani FM, Carmo AO, Contigli C, Kalapothakis E. 2010 Aug 31. Potency against enterotoxemia of a recombinant *Clostridium perfringens type D* epsilon toxoid in ruminants, *Vaccine* 28, 6125–6127.

Goswami, P.P., Girish, K.S., Chaudhuri, P., Tiwari, V., Akare, S.J., Harbola, P.C. (2002) Cloning and sequencing of beta toxin gene of *Clostridium perfringens* type C. *Indian journal of experimental biology* 40(1):109-10.

Goswami, P. P., Rupa, P.,.Prihar N. S., Garg,L.C., (1996) Molecular Cloning of *Clostridium perfringens* Epsilon Toxin Gene and Its High Level Expression in E. coli, *Biochemical and Biophysical Research Communications* 226, 735–740 ARTICLE NO. 1422.

Granum, P.E. (1990) *Clostridium perfringens* toxins involved in food poisoning. *International Journal of Food Microbiology* 10, 101-11.

Habeeb, A.F.S.A., Lee, C.L., Atassi, M.Z., (1973) Conformational studies on modified proteins and peptides VII Conformation of s-prototoxin and s-toxin from *Clostridium perfringens*. Conformational changes associated with toxicity. *Biochemica et Biophysica Acta* 322: 245-250.

Hanahan, D. (1983) Studies on transformation of *E. coli* with plasmids. *Journal of molecular biology* 166(4):557-80.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In: Glover D M, editor; Glover D M, editor. DNA cloning: a practical approach. Vol. 1. Oxford, United Kingdom: *IRL Press,* pp. 109–135.

Hunter, S.E.C., I. N. Clarke, D.C. Kelly, and R. W. Titball, (1992), Cloning and Nucleotide Sequencing of the *Clostridium perfringens* Epsilon Toxin Gene and Its Expression in *E. coli*, *Infection and Immunity* p. 102-110

Hunter, S.E.C., Brown, J.E., Oyston, P.C.F., Sakurai, J. and Titball, R.W. (1993) Molecular genetic analysis of the beta toxin of *Clostridium perfringens* reveals sequence homology with the alpha-toxin, gamma-toxin and leukocidin of *Staphylococcus aureus*. *Infection and Immunity* 61, 3958-3965.

Hoch, J. A. (2000) Two-component and phosphorelay signal transduction. *Curr. Opin. Microbiol.* 3, 165–170.

Hochulli, E., Dobeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *Journal of Chromatography* 41, 177–184.

Hogh, p. (1976) experimental studies on serum treatment and vaccination gainst *Clostridium perfringens type C* infection

in piglets. Developments in biological standardization 32,6-76.

McClane, B.A. (2001) *Clostridium perfringens*. Doyle MP, editor. Food microbiology: fundamentals and frontiers. Washington, DC: ASM Press, p. 351–72.

McDonel, J.L., Dorner, F, Drew, H. (1986) Toxins of *Clostridium perfringens types A, B, C, D, and E. Pharmacology of bacterial toxins. Oxford: Pergamon Press,* p. 447–517.

Miyata, S., Matsushita, O., Minami, J., Katayama, S., Shimamoto, S., Okabe, A., (2001) Cleavage of a C-terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon toxin in the synaptosomal membrane. *The journal of Biological Chemistry* 276, 13778–13783.

Ochi S., Oda, M., Matsuda, H., Ikari, S., Sakurai, J. (2004) *Clostridium perfringens* alpha-toxin activates the sphingomyelin metabolism system in sheep erythrocytes. *The journal of Biological Chemistry 279: 12181-12189.*

Petit, L., Gibert, M., Popoff, M.R. (1999) *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiology* 7: 104–110.

Payne, D.W., Oysten, P. (1997) The *Clostridium perfringens* epsilon toxin. The Clostridia: Molecular biology and pathogenesis. *Academic Press Limited: London, pp. 439-447.*

Rood, J.I., Cole, S.T. (1991) Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiology and Molecular Biology Review* 55:621–648.

Rood, J.I. (1998) Virulence genes of *Clostridium perfringens*. *Annual Review of Microbiology* 52, 333–360.

Sambrook, J., Fristch, E.F., Maniatis, T., Molecular Cloning. (1989)2nd Ed. Cold Spring Harbor. New York: Cold Spring Harbor Laboratory Press.

Sheedy, S.A., Ingham, A.B., Rood, J.I., Moore, R.J. (2004) Highly conserved alpha-toxin sequences of avian isolates of *Clostridium perfringens*. *Journal of Clinical Microbiology* 42: 1345-1347.

Shevchuk, N.A., Bryksin, A.V., Nusinovich, Y.A., Cabello, F.C., Sutherland, M. Stephan Ladisch, M.S. (2004) Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Res*earch 32 (2), e19.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proceedings of the National Academy of Sciences* 99, 996-1001.

Souza, A.M., Reis, J.K.P., Assis, R.A., Horta, C.C., Siqueira, F.F., Facchin, S., Alvarenga, E.R., Castro, C.S., Salvarani, F.M., Silva, R.O.S., Pires, P.S. , Contigli, C. , Lobato F.C.F. ,and Kalapothakis, E. (2010),Molecular cloning and expression of epsilon toxin from *Clostridium perfringens type D* and tests of animal immunization, *Genetics and Molecular Research* 9 (1): 266-276.

Steinpórsdótti, V., Fridriksdóttir, V., Gunnarsson, E., Andrésson, O.S., (1995) Expression and purification of *Clostridium perfringens* beta-toxin glutathione S-transferase fusion protein, *FEMS Microbiology Letters, Volume 130, Issue 2-3, pages 273–278*.

Steinthorsdottir, V, Fridriksdottir, V. (1998), Eggert Gunnarsson, Oèlafur S. Andreèsson, Site-directed mutagenesis of *Clostridium perfringens* beta-toxin: expression of wild-type and mutant toxins in Bacillus subtilis, *FEMS Microbiology Letters 158, 17-23.*

Titball, R.W. (2009), *Clostridium perfringens* vaccines, *Vaccine* 27, D44–D47.

Uzal, F.A., Kelly W.R., Morris W.E., Assis R.A. (2002). Effects of intravenous injection of *Clostridium perfringens type D* epsilon toxin in calves. *Journal of comparative pathology* 126 (1):71–5.

Uzal, F.A., Kelly,W.R., Morris W.E., Bermudez J., Baison M. (2004). The pathology of peracute experimental *Clostridium perfringens type D* enterotoxemia in sheep. *Journal of Veterinary Diagnostic Investigation* 16, (5) 403–11.