*Microbiology Research Journal International*

***23(2): 1-9, 2018; Article no.MRJI.39261***
***ISSN: 2456-7043***
*(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)*

# Development of a New Bifunctional Fusion Protein of Vaccine Strains *Clostridium perfringens* Type D and *Clostridium septicum* Epsilon-alpha Toxin Genes

**Marzieh Kamalirousta[1] and Reza Pilehchian[2*]**

*[1]Department of Biology, Kharazmi University, Alborz, Karaj, Iran.*
*[2]Specialized Clostridia Research Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Alborz, Karaj, Iran.*

***Authors' contributions***

*This work was carried out in collaboration between both authors. Author RP designed the study, performed the bioinformatics analysis, wrote the protocol and final manuscript. Author MK managed the analyses of the study, wrote the first draft of the manuscript and the literature searches. Both authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/MRJI/2018/39261
Editor(s):
(1) Lachhman Das Singla, Professor, Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, India.
Reviewers:
(1) Ivan Felismino Charas Dos Santos, UNESP, Brazil.
(2) Ketan Vagholkar, D. Y. Patil University School of Medicine, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23605

***Original Research Article***

***Received 9th December 2017***
***Accepted 16th February 2018***
***Published 13th March 2018***

## ABSTRACT

There are various difficulties regarding of produce and design of a suitable fusion protein, and functionality is the most important of this problem. For producing a functional fusion protein, it is necessary to have a proper suitable host to clone a properly designed fusion gene. *Clostridium perfringens* and *C. septicum* are important pathogens of humans and livestock and produce numerous toxins including epsilon and alpha, which are responsible for severe diseases. In this study, a new construct of *C. perfringens* type D epsilon and *C. septicum* vaccine strains alpha toxin genes designed in addition, cloned into a prokaryotic host. Online software used for *in silico*, study and the prediction of the fusion protein construct primary, secondary and tertiary structures. At the next step, specific primers used for amplification of these genes, based on nucleotide sequences

**Corresponding author: E-mail: r.pilehchian@rvsri.ac.ir, langgroudi@gmail.com;*

that retrieved from GenBank. The amplified *etx* and CSA fragments linked together by a linker based on a helix forming peptide (*i*+4) E, K. The linker introduced between two domains by fusion PCR and using *Pfu* DNA polymerase, epsilon forward and alpha reverse primers. The fusion gene ligated into pGEM-B1cloning vector and cloned into *E. coli* strain TOP10. This epsilon-alpha fusion gene could be used for development of a recombinant epsilon alpha fusion protein vaccine.

*Keywords: Anaerobes; bioinformatics; cloning; fusion PCR.*

## 1. INTRODUCTION

*Clostridium perfringens* is anaerobic spore-forming, Gram-positive, rod-shaped bacterium, of the genus *Clostridium*. The organism, which is non-motile and heat-resistant, is widely found in the world, which is the result of variation of its genomic size. *C. perfringens* often found in soil and is the normal flora of the digestive tract of humans and mammals [1].

The virulence of *C. perfringens* is depending on its ability to produce more than seventeen major and minor toxins and extracellular enzymes. The four major toxins including, alpha, beta, epsilon, and iota are used for its classification into five types A-E [2-4].

Each type of these organisms carries a different combination of toxin genes. It has now become clear that many important *C. perfringens* toxins encoded by large plasmids. *C. perfringens* strain CPN50 associated with human disease genome physical map shown to possess a single circular chromosome of about 3.58 Mb [5-7].

Epsilon toxin (ETX), a potent toxin of *C. perfringens* types D and B, is a pore-forming toxin and the third most potent Clostridial toxin after botulinum and tetanus neurotoxins [8].The toxin gene is located on a plasmid and is 1008bp in length, and translated into a mature protein containing 334 amino acids. Its molecular weight is 37 kDa which produces as a prototoxin and can be activated after treatment with a proteolytic enzyme such as trypsin [9].

ETX causes enterotoxaemia in sheep and necrotic enteritis in very young lambs and sometimes can cause similar situations in the newborn horses. ETX belongs to the aerolysin family of pore-forming toxins [10].

*C. septicum* is a motile bacterium with peritrichous flagella. This organism is fermentative anaerobic and can be found in any anoxic position including organic compounds. *C. septicum* produces several toxins including alpha, beta, gamma, and delta. Alpha toxin (AT) is a lethal virulence factor of *C. septicum* and responsible for a serious disease known as gas gangrene. The toxin utilizes its pathogenesis by pore formation on the host cell surface and also by a range of effects on the target cell [11]. Secreted AT is an inactive protoxin and proteolytic hydrolysis is required for its activation. Protoxin connects to the GPI-anchored proteins on the target cell membrane. Separation of 45 amino acid sequences of the C-terminal by the host cell Furin proteases, actives the toxin [12, 13].

The purpose of this study was to design a new fusion structural model consisting of *C. perfringens* type *D* epsilon toxin and *C. septicum* alpha toxin genes. Bioinformatics approach used for *in silico* analysis of the chimeric fusion gene structure.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Strains

*C. perfringens* type *D* strain CN409 and *C. septicum* strain CN913, *E. coli* strain Top10, were prepared from Razi vaccine and serum research Institute.

### 2.2 Designing Fusion Gene

Nucleotide sequences retrieved from GenBank. In the new construction, *etc* and *CSA is* linked together using a small hydrophobic linker, which optimized for expression in *E. coli cloning* host cell. *Nde*I and *Xho*I restriction sites and their flanking regions performed respectively at the 5'end of *etx* and the 3'end of *csa*. After *in silico* analysis of the chimeric fusion protein biochemical structure, it was revealed that the designed fusion gene length is 2358 base pairs.

### 2.3 Fusion Protein Tertiary Structure Prediction

Phyre[2] online program used for fusion protein tertiary structure prediction[14]. Full length translated fusion protein sequence of 786 amino

acids uploaded. Three templates (c3c0mB.pdb, d3c0na2.pdb and d1uyja.pdb) were selected to model fusion protein based on heuristics to maximize confidence, percentage identity and alignment coverage. 144 residues were modeled by *ab initio*.

## 2.4 Primers

Primers designing performed using GenBank retrieved complete CDs of *C. perfringens* type D epsilon toxin gene (HQ179546.1) and *C. septicum* alpha toxin gene (JN793989). The designed primers are as follows:

*etx* forward primer:

5' TGG GAA CTT CGA TAC AAG CA 3'

*etx* reverse primer:

5' TGA ACC TCC TATTTT GTATCC CA 3'

*ca* forward primer:

5´GAG CAT ATG TCA AAA AAA TCT T3´

*ca* reverse primer:

5´CCC TCG AGT ATA TTA TTA ATT A3´

## 2.5 Primers for Epsilon-alpha Fusion Gene

*Nde*I restriction site designed for forward epsilon primer and *Xho*I restriction site was located in reverse alpha primer. Linker sequence designed in reverse epsilon and forward alpha primers.

Forward primer:

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

Reverse primer:

5´TTT CGC CGC CGC TTC CGC TTT TAT TCC TGG TGC CTT AAT 3´

The sequences of alpha toxin primers are as follows:

Forward primer:

5´GCG GAA GCG GCG GCG AAA TCA AAA AAA TCT T3´

Reverse primer:

5´CCC TCG AGT ATA TTA TTA ATT A3´

## 2.6 Cell Cultivation and Genomic DNA Isolation

Vaccine strains of *C. perfringens* type D and *C. septicum* cultured in anaerobic condition using Anoxomat anaerobic jars at 37°C in a liquid Brain-heart medium, and pH 7.5 for 18 hours then separated from the culture fluid by centrifugation for 15 minutes at 5000 RPM at room temperature. High molecular weight genomic DNA from *C. perfringens* type *D* and *C. septicum* were isolated according to the modified method described earlier [15]. Cells from a 20 ml overnight culture harvested by centrifugation and suspended in 300 µl TE. 300 µl of 10% SDS, and 5 µl RNase-Added, then cells incubated for 30 minutes at 37°C. 50 mg/ml proteinase-K added and the mixture incubated for 90 min at 37°C and DNA 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. Purified DNA diluted in 30 µl DNase free water.

## 2.7 PCR Amplification

Amplification of epsilon and alpha gene was performed using specific primers and special PCR reagents including PCR buffer, appropriate template DNA, *Pfu* DNA polymerase, dNTPs, Mg SO4 and sterile DNase free water in 0.2 µl thin layer micro tube. Thirty cycles of PCR performed. For epsilon toxin gene (denaturation at 95°C for 1 minute, annealing at 50°C for 1 min, and extension at 72°C for 2 min)and (denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1:30 min for alpha-toxin gene)as described previously[16].

Having the *etx* and *csa* amplified fragments, at the next step, special fusion PCR procedure carried out as described previously[16].

## 2.8 Sequence Analyzing of Amplified and Purified PCR Products

After PCR, amplification 1% agarose gel electrophoresis was used for its qualification. The blunt-ended PCR products in size of about 1 kb were purified using gel extraction Kit for DNA fragment recovery according to the

manufacturer's recommendations. To confirm a nucleotide sequence, extracted nucleotide sequences sent to Bioneer Company for sequencing.

### 2.9 Chimeric Fusion Gene Construction and confirmation

Purified *C. perfringens* epsilon gene and *C. septicum* alpha gene were linked together using fusion PCR in a two steps PCR reaction as described previously [16] In brief, 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 cycle's epsilon forward and alpha, reverse primers added to the same micro tube and 2nd step of PCR reaction continued for 20 more cycles. The blunt end PCR product in the size of about 2.5 kb (fusion gene) extracted from agarose gel electrophoresis, and purified. Nested PCR carried out for epsilon-alpha fusion gene construction using epsilon forward and reverse primers. Sequencing of purified fusion gene performed.

### 2.10 Cloning Vector Construction and Transformation

A linearized pGEM-B1 plasmid containing ampicillin resistant gene used as cloning vector and purified fusion gene ligated in it. 2 µl 10X reaction buffer, 1 µl pGEM-B1, 1µl T4 DNA ligase and 13 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. In order to produce competent cells *E. coli* strain TOP10 used. The ligation mixture was used directly for transformation (10/100 µl) of bacterial strains. Cells cultured on an LB-ampicillin plate and incubated over-night at 37°C.

### 2.11 Confirmation of Fusion Construction

After one night, colony PCR carried out for the colonies containing pGEM-B1 recombinant plasmid using pGEM-B1 forward and Reverse sequencing primers according to the manufacturer's recommendations. PGEM-B1 non-recombinant plasmid vector also used as negative control. One *E. coli* strain TOP10 competent but not transformed colony also cultured on the same plate. Plasmid extracted at the next step. Plasmid digestion was done using *Nde*I and *Xho*I restriction endonucleases. Then two sets of PCR was carried out using internal epsilon forward and alpha reverse primers and original epsilon forward and alpha reverse primers for epsilon-alpha fusion gene construction with the purified plasmid. Agarose gel electrophoresis analysis for all of the above recombinant plasmids and PCR products was performed. Sequencing extracted recombinant plasmids performed to confirm ligation and transformation in Bioneer Company.

## 3. RESULTS

Analyzing of the epsilon-alpha fusion protein, using the Phyre[2] online program and three templates, showed 82% of residues modeled at >90% confidence, Fig. 1 Indicating where the sequence was covered by each template, color-coded by the confidence of the match to that template overall. Fig. 2 shows tertiary structure prediction of the fusion protein.

The sequence of the epsilon-alpha fusion gene was deposited in GenBank under accession number KU726861.

After isolation of genomic DNA, PCR procedures optimized for amplification of epsilon toxin gene and alpha toxin genes, using two pairs of special primers. Fig. 3 shows 1% agarose gel electrophoresis of gradient PCR.

At the next step epsilon forward and alpha reveres, primers used to produce ε-α chimeric gene.

The result of nested PCR of epsilon-alpha fusion gene construction, using internal epsilon forward and reverse primers showed successful amplification of the desired fragment (Fig. 4).

An overnight culture of *E. coli* strain TOP10, which transformed directly with ligation product, showed colonies on LB-AMP agar plate. Screening gel electrophoresis (colony PCR) using epsilon primers showed ~1 kb DNA fragments (Fig. 5).

Recombinant pGEMεα extracted from *E. coli*/TOP10/pGEMεα and digested with *Nde*I and *Xho*I restriction endonucleases (Fig. 6).

## 4. DISCUSSION

A fusion protein construction is consisting of two proteins or protein domains, which linked together by a linker fragment. Fusion protein technology is the strategy to achieve rapid,

cheap and efficient expression of proteins. In this study DNA sequence retrieved from GenBank reveled that C*. perfringens* type D epsilon toxin and *C. septicum* alpha toxin genes are 1008 and 1332 bp, respectively [15,16]. C*. perfringens* type D inactivated epsilon prototoxin has 334 amino acids. A fragment of 32 amino acids from the start codon is a signal peptide when removed, mature inactivated toxin produced [17]. *C. septicum* alpha toxin is a proteolytically activated pore-forming toxin, belongs to the aerolysin-like toxins family. AT has 443 amino acids. The first 31 amino acids of the sequence are a signal peptide. Residues 203 to 232 alternated between the aqueous milieu and the membrane core insert into membranes, consistent with the formation of an amphipathic transmembrane beta-hairpin [12]. There is a putative functional region at the proximal region of AT to the receptor-binding domain but distal from the pore-forming domain that is proposed to regulate the insertion of the transmembrane beta-hairpin of the pre-pore oligomer[18]. Previously it was reported that the sequence of a helix forming peptide "An $(EAAAK)_nA$" (n=2-5) is a proper linker for joining to different protein to produce a functional fusion protein[19].

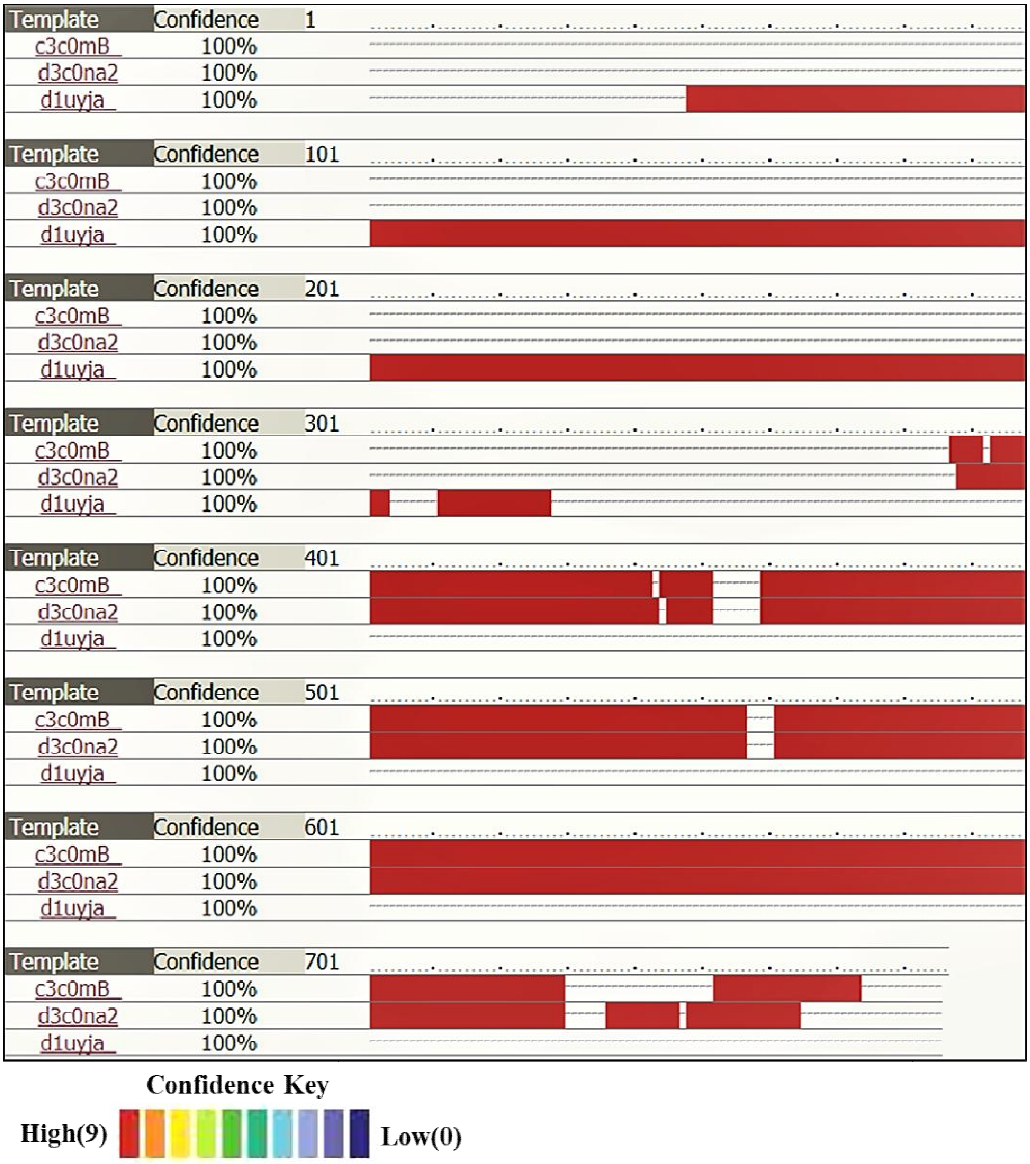


**Fig. 1. Confidence of epsilon-alpha fusion protein Tertiary structure, based on three templets**

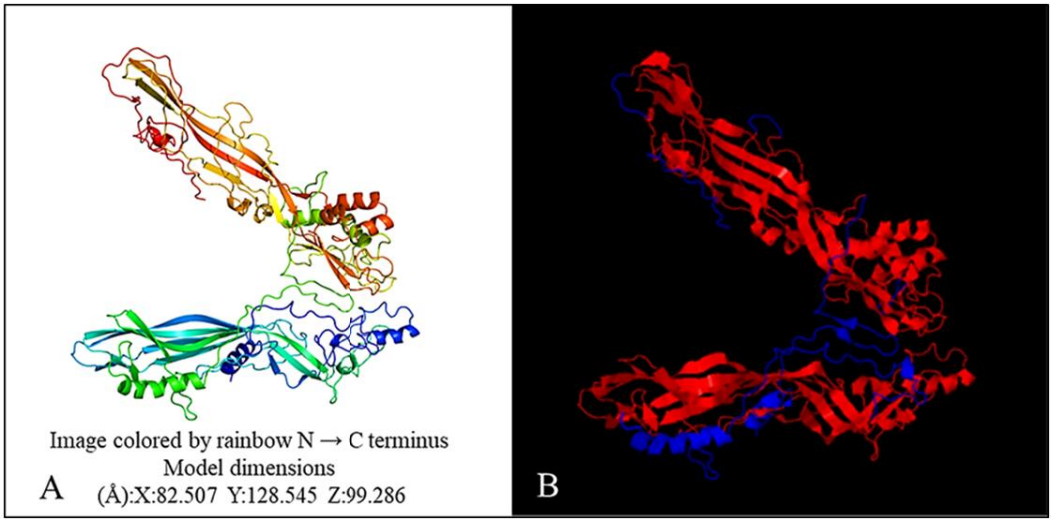


**Fig. 2. The tertiary structure of fusion protein prediction by the phyre[2] online program. Colored by (A), rainbow and (B) confidence**

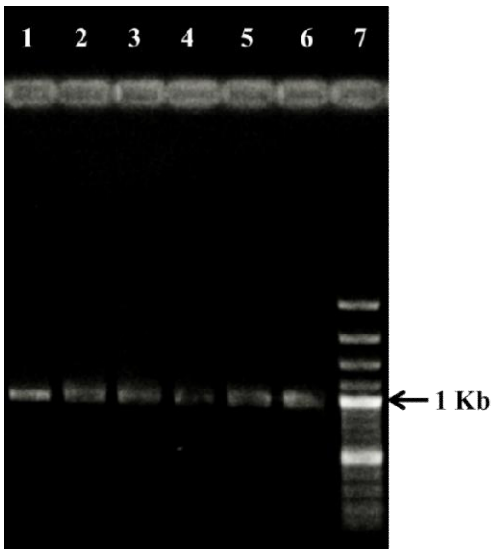


**Fig. 3. 1% agarose gel electrophoresis optimizing of *C. perfringens* type D epsilon toxin gene**

*Lanes 1-2, etxD amplification at 52.8°C; Lanes3-4, etxD amplification at 54.5°C; Lanes5-6, etxD amplification at 56.4°C; lane 3, 100 bp plus DNA size marker*

It seems that the first effort to produce clostridium fusion protein was at 1995. The beta-toxin gene of *C. perfringens* type C cloned and expressed as a glutathione S-transferase fusion protein in *E. coli*, the nits DNA sequence determined and compared to the type B sequence. Results showed two nucleotide differences in the protein coding sequences so one amino acid difference between the two proteins [20].

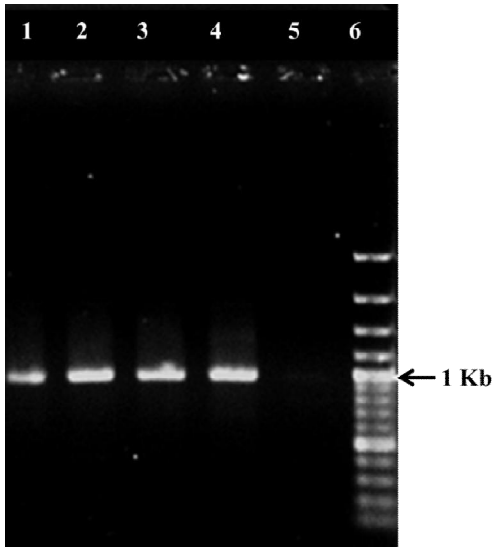


**Fig. 4. Nested PCR of epsilon-alpha fusion gene construction using internal epsilon forward and reverse primers**

*Lanes 1-5, Products of nested PCR from purified fusion gene; lane 6: 100 bp plus DNA size marker*

An inter-generic recombinant alpha domain fusion protein (r-alphaCS) reported at 2012 to detect and neutralize the *C. perfringens* and *S. aureus* alpha toxins. The fusion protein showed specific immune response against the two bacteria alpha toxins. The antisera also neutralized the toxicities of both the native

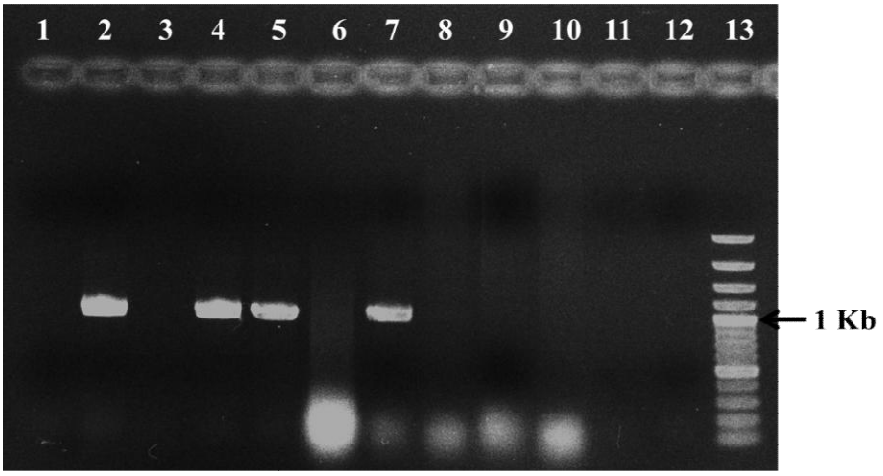


**Fig. 5. 1% gel electrophoresis for the screening of ε-α fusion construction**
*Lanes 1-12, PCR product of recombinant E. coli/TOP10/pGEMεα colony PCR, lane 13, 100 bp plus DNA size marker*

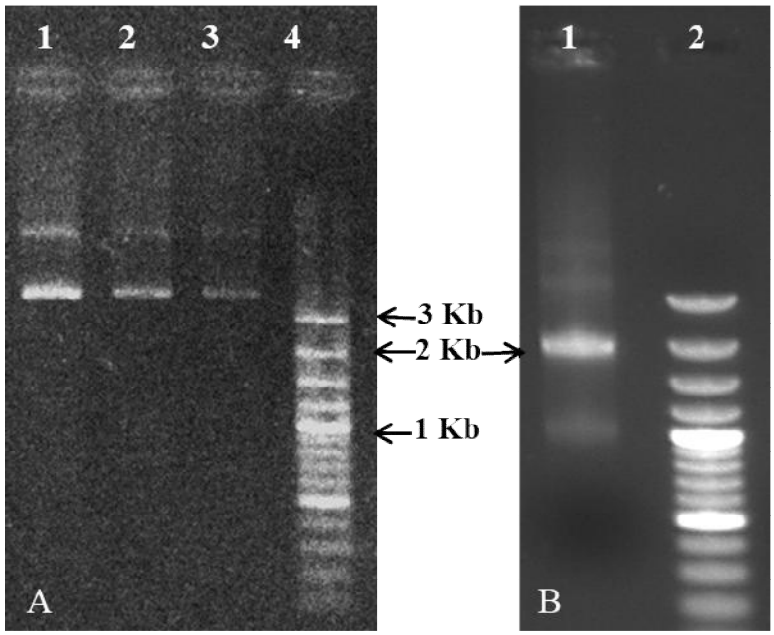


**Fig. 6. 1% gel electrophoresis of purified recombinant cloning vector, A (intact) and B (digested*)**
*Lanes 1, GeneRuler™ 1kb DNA Size marker;Lane2,intact pGEMεα; lane3, digested cloning vector showing epsilon-alpha fusion gene.*
**Enzymatic digestion using NdeI and XhoI restriction endonuclease*

wild-type toxins *in vitro*. Results revealed that the fusion protein was a nontoxic competitive inhibitor of staphylococcal alpha-hemolysin [21].

In 2013 the helix forming peptide linker fragment "AEAAAKEAAAKA" used successfully to produce fusion protein as a candidate for the Clostridial vaccine, this report showed a proper fusion protein of *C. perfringens* type D and *C. septicum* epsilon and beta toxins with suitable immunological effects [22].

In 2016 a bivalent chimera of *C. perfringens* beta (CPB) and iota (CPI) constructed by splicing the

non-toxic C terminal binding regions. Flexible glycine linker (G4S) used for this purpose and two regions fused together using overlap-extension PCR. The results revealed that the fusion protein is non-toxic and could competitively inhibit binding of CPB to host cell receptors thereby reducing its cytotoxicity [23].

In our work, a new construction of C*. perfringens* type D epsilon and *C. septicum* alpha toxin genes designed and produced. After *in silico* analysis and tertiary protein structure prediction by Phyre[2] (Figs. 1 and 2), each epsilon and alpha genes separately amplified (Fig. 3). Amplified *etx* was 1008 bp with 6 domains including a flanking region (nucleotides 1-3), *Nde*I 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). Amplified *csa* sequence was 1332 bp with four domains including, linker (nucleotides 1-36), mature peptide (toxin 37-963), *Xho*I cleavage site (nucleotides 964- 969), and flanking region (nucleotides 1330-1332). The new fusion construct, produced by fusion PCR and evaluated using colony PCR procedure (Figs. 4 and 5). The epsilon-alpha fusion gene specified as a fragment of 2358 bp consisting of *at* nucleotides 1-987, linker 988-1024 (36bp) and *csa* 1024-2358.The chimeric fusion gene cloned into a pGEM-B1 cloning vector and *E. coli*/TOP10transformed by it. Extracted and purified recombinant plasmids sequencing showed that the recombinant pGEMεα is 5361 bp consisting of pGEM-B1 (3003) bp and the chimeric fusion gene (2358 bp).

Based on previous studies, epsilon alpha fusion gene can be expressed in *E. coli*. Therefore, this method would be beneficial to produce these toxins in a safe production host and in an immunologic form.

## 5. CONCLUSION

This is the 1st time that *Clostridium perfringens* and *Clostridium septicum* epsilon-alpha fusion gene are designed and cloned into a suitable vector. This construct will be further used to sub-clone in an expression host and will be expressed to produce epsilon-alpha fusion protein.

## ACKNOWLEDGEMENT

The authors wishes to express their warm thanks to all of the scientific members and staffs of anaerobic vaccine production department of Razi Vaccine and Serum Research Institute.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Rood JI, Cole ST. Molecular genetics and pathogenesis of *Clostridium perfringens.* Microbiol Rev. 1991;55(4):621-48.
2. McClane B. *Clostridium perfringens*. Food microbiology: Fundamentals and frontiers, ed. M. Doyle. Washington DC: ASM Press; 2001.
3. Hatheway CL. Toxigenic clostridia. Clin Microbiol Rev. 1990;3(1):66-98.
4. McDonel J. Toxins of *Clostridium perfringens* types A, B, C, D, and E. Pharmacology of bacterial toxins, ed. F. Dorner and H. Drew. Oxford: Pergamon Press; 1986.
5. Miyamoto K, et al. Sequencing and diversity analyses reveal extensive similarities between some epsilon-toxin-encoding plasmids and the pCPF5603 *Clostridium perfringens* enterotoxin plasmid. J Bacteriol. 2008;190(21):7178-88.
6. Miyamoto K, et al. Complete sequencing and diversity analysis of the enterotoxin-encoding plasmids *in Clostridium perfringens* type A non-food-borne human gastrointestinal disease isolates. J Bacteriol. 2006;188(4):1585-98.
7. Li J, et al. Toxin plasmids of *Clostridium perfringens.* Microbiol Mol Biol Rev. 2013; 77(2):208-33.
8. Knapp O, et al. Identification of the channel-forming domain of *Clostridium perfringens* Epsilon-toxin (ETX). Biochim Biophys Acta. 2009;1788(12):2584-93.
9. Bokori-Brown M, et al. Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. FEBS J. 2011;278(23):4589-601.
10. Popoff MR. Epsilon toxin: A fascinating pore-forming toxin. FEBS J. 2011;278(23): 4602-15.
11. Tweten RK. *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. Vet Microbiol. 2001;82(1):1-9.

12. Melton JA, et al. The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. J Biol Chem. 2004;279(14):14315-22.
13. Knapp O, et al. *Clostridium septicum* alpha-toxin forms pores and induces rapid cell necrosis. Toxicon. 2010;55(1):61-72.
14. Kelley LA, et al. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015;10(6):845-58.
15. Bozorgkhoo Z, PL, Khaki P, Jabbari AR, Moradi Bidhendi S, Moosawi Shoshtari M. Molecular cloning of *Clostridium septicum* vaccine strain alpha toxin gene in *E. coli.* Archives of Razi Institute. 2014;69(1):15-20.
16. Pilehchian Langroudi R, et al. Fusion of *Clostridium perfringens* type D and B epsilon and beta toxin genes and its cloning in E. coli. Archives of Razi Institute. 2011;66(1):1-10.
17. Hunter SE, et al. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect Immun. 1992; 60(1):102-10.
18. Melton-Witt JA, Bentsen LM, Tweten RK. Identification of functional domains of *Clostridium septicum* alpha toxin. Biochemistry. 2006;45(48):14347-54.
19. Arai R, et al. Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein Eng. 2001;14(8):529-32.
20. Steinthorsdottir V, et al. Expression and purification of *Clostridium perfringens* beta-toxin glutathione S-transferase fusion protein. FEMS Microbiol Lett. 1995;130(2-3):273-8.
21. Uppalapati SR, et al. Generation and characterization of an inter-generic bivalent alpha domain fusion protein alphaCS from *Clostridium perfringens* and Staphylococcus aureus for concurrent diagnosis and therapeutic applications. J Appl Microbiol. 2012;113(2):448-58.
22. Pilehchian Langroudi R, Shamsara M, Aghaiypourk. Expression of *Clostridium perfringens* epsilon-beta fusion toxin gene in *E. coli* and its immunologic studies in mouse. Vaccine. 2013; 31(32):3295-3299.
23. Das S, et al. Generation and characterization of recombinant bivalent fusion protein r-Cpib for immunotherapy against *Clostridium perfringens* beta and iota toxemia. Mol Immunol. 2016;70:140-8.



*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://www.sciencedomain.org/review-history/23605*