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OPEN PEER REVIEW

Molecular Cloning of a New Bi-Functional Fusion Protein of *Clostridium perfringens* Type A Alpha and *Clostridium septicum* Alpha Toxin Genes in *E. coli*

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1 Original submission

3 comments · 7 Jan 2020 – 17 Jun 2020

Referee 1 Reviewer

MINOR REVISION 7 Jan 2020

Molecular cloning of a new bifunctional fusion protein of Clostridium perfringens type A alpha and Clostridium septicum alpha toxin genes in E. coli.
Reviewer Comments
1. Add the manufacturer and country of origin of the materials used in this research to the materials and methods section.
2. All primer sequences in the text should be revised to ensure their accuracy.
3. Discuss the significant results of this study in more detail in the discussion section.
4. Some of the references do not follow the journal guidelines. Carefully revise all the references.
5. Minor spacing problems exist between the words in some parts of the text.
6. Adjust the paper according to the journal guidelines.

Referee 2 Reviewer

MINOR REVISION 9 Feb 2020

Abstract
The abstract doesn't contain the important points of your study.
Please prepare your keywords according to the MeSH system.

Introduction
This sentence was garbled:
"…..… alpha toxins and linked together via a????????????????? The purpose of this study was to design a new fusion structural model consisting of cpa and csa genes ….."
It is not correct to write the following words here:
"In silico analysis used for the chimeric fusion protein structure Glycine linker fragment using fusion PCR(overlap-extension PCR)(12).."
Methods

The type of study was not indicated.
Please provide the sizes of the primers in base pairs and the related references.
What were your controls?
The software and tests used for statistical analysis and the comparisons performed were not properly described.
Results
The figures and tables did not contain legends and titles, scales, and definitions of abbreviations.
The figures and tables were not consistent with the main text regarding content and numbering.
Discussion
The limitations of the study and suggestions were not addressed at the end of the discussion.

The acknowledgment and funding sections were not presented at the end of the article.
Almost 50% of the references are of the old reference type. Please change them and use the new reference type.

Editor-in-Chief MAJOR REVISION 17 Jun 2020

Major revision

2

Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Reviewers' Comments:

Reviewer 1:

Q1. Add manufacturer and country of materials used in this research in materials and methods section.

R1. Amplification of *cpa* and *csa* genes were performed independently in a 25µl reaction mix containing 1x PCR buffer (with 1.5mM $MgSo_4$) (**Thermo Fisher Scientific, Lithuania**), 200µM dNTPs mix (**Sinaclon, Iran**), 0.5µM each primer (**Sinaclon, Iran**),1unit *Pfu DNA polymerase* (**Thermo Fisher Scientific, Lithuania**), 100ng template DNA and sterile DNase free water (**Sinaclon, Iran**) in 0.2 ml thin layer micro tube.

The blunt-ended PCR products in size of about 1/2 kb were Purified using Gel Extraction Kit (**Vivantis, Malaysia**) according to the manufacturer, s recommendations(21, 22).

Briefly: 1µl pUC57(**Bio Basic, Canada**), 13µl purified fusion gene, 1µl T4 ligase DNA (**Thermo Fisher Scientific, Lithuania**), 6µl 5X reaction buffer (**Bio Basic, Canada**) and 4 µl sterile DNase free water (**Sinaclon, Iran**) were mixed in a 0.2 ml micro tube and incubated one night at 16°C.

Cells were cultured on a LB agar plate containing 50µgr/ml ampicillin(**Bio Basic, Canada**) and incubated at 37°C for one night as described previously(23).

Plasmid extraction was performed by Plasmid Extraction Kit (**Vivantis, Malaysia**) at the next step and then plasmid digestion carried out using *Nde1* and *Xho1* restriction enzymes (**Thermo Fisher Scientific, Lithuania**).

Additionally, the sequencing of extracted recombinant plasmids was performed to confirm ligation and transformation in **Bio Basic Company, Canada**(19).

Q2. All primer sequences in the text should be revised, to ensure their accuracy.

R2. The designed primers (5′→3′) used in the study (flanking regions, restriction enzyme sites, primer and linker) (19-21):
cpa fp: TGGCATATGAAAAGAAAGATTTGTAAG=27

cpa rp: GCCCTCGAGTTTTATATTATAAGTTGA=27

csa fp: GAGCATATGTCAAAAAAATCTTTTGCT=27

csa rp: GCCCTCGAGACGTTTTCCTCTTCTCTT'=27

linker+ *cpa* rp: TTTCGCCGCCGCTTCCGCTTTTATATTATAAGTTGA=36

Linker+ csa fp:

GCGGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGCGCTTACAAATCTTGA

AGAG=54

cpa fp for nested PCR: AGACTTTTGCAGAGGAAAGA=20

csa rp for nested PCR: TATCTTTTGCACGATACCCA=20

pUC57 fp: GCCATTCGCCATTCAGG=17
pUC57 rp: TAGCTCACTCATTAGGCAC=19(according to the manufacturer's recommendations).

Q3. Discuss more on significant results of this study in discussion section.

R3. Our results of *in silico* analysis (C-score = -2.68 and TM-score = 0.41 ± 0.14) indicates a model of correct topology. Because C-score is typically in the range of (-5 to 2) and TM-score is more than 0.17. Our results of Ramachandran plot (amino acid residues in favored and allowed regions = 88.4%) show geometrical structure of alpha-alpha fusion protein is confirming as a natural like protein. Because the percent of amino acid residues is more than 85% in favored and allowed regions (17, 18).

In other study by Langroudi and coworkers in 2011 epsilon–beta fusion gene is synthetized by fusion PCR method and the synthetic fusion gene confirmed by nested PCR (21).

The resulting recombinant cell (*E. coli* TOP10/ pUC57/αα) was confirmed by colony PCR that produced 5kb and 2.3kb DNA fragments by using pUC57 forward and reverse primers and *cpa* forward and *csa* reverse primers respectively, and also negative control PCR product showed one 2.7kb bond(Fig.4B).

Similar to our study, by Kamalirousta and coworkers in 2018 epsilon–alpha fusion gene cloned into pGEM-B1 plasmid vector and then the recombinant vector was transformed into *E. coli* TOP10. The recombinant cell was confirmed by colony PCR, enzyme digestion and sequencing analysis (24). One of the limitations of this study was the complexity and difficulty of connecting these two genes by PCR fusion method. It is suggested that this technique be used for other *Clostridium* toxins and for the fusion of more than two genes.

Q4. Some of the references are not based on the journal guidelines. Revise carefully all the references.

R4. The all references adjust to base on the journal guidelines. e.g. Freedman JC, Theoret JR, Wisniewski JA, Uzal FA, Rood JI, McClane BA. *Clostridium perfringens* type A-E toxin plasmids. *Research in Microbiology*. 2015;**166**(4):264-79. doi: 10.1016/j.resmic.2014.09.004.

Q5. Minor spacing problems exist between the words in some parts of the text.

R5. Minor spacing problems to be removed that exist between the words in some parts of the text. e.g**.** The *cpa* gene is located on a chromosome and is 1197bp in length, and translated into a 398 amino acids holoprotein with an N-terminal signal sequence of 28 amino acids, which when cleaved results in a mature protein with a molecular weight of 43kDa(6, 7).

Q6. Adjust paper according to journal guidelines.

R6. The paper adjusts according to journal guidelines

Reviewer 2:

Abstract

Q1. The abstract doesn't contain the important points of your study.

R1. A notable finding of the our research was a new approach for fusion of *C.perfringens* type A alpha and *C.septicum* alpha toxin genes based on fusion PCR strategy.

Q2. Please prepare your keywords according to the MeSH system.

R2. Cloning, *Clostridium perfringens* type A, *Clostridium septicum*, α-α Fusion Gene.

Introduction

Q1. This sentence was disturbed:
……. alpha toxins and linked together via a????????????????? The purpose of this study was to design a new fusion structural model consisting of cpa and csa genes ….
It is not correct to write the following words right here:
"In silico analysis used for the chimeric fusion protein structure Glycine linker fragment using fusion PCR(overlap-extension PCR)(12).."

R1. alpha toxins and linked together via a Glycine linker fragment using fusion PCR(12). The purpose of this study was molecular cloning of a new bifunctional fusion protein of *Clostridium perfringens* type A alpha and *Clostridium septicum* alpha toxin genes in *E. coli.*

Methods

Q1. The type of study was not indicated.

R1. This was an experimental study, mentioned in results section.

Q2. Please write the size of the primers base pairs and references related to them.

R2. The designed primers (5′→3′) used in the study (flanking regions, restriction enzyme, primer and linker) (19-21):
cpa fp: TGGCATATGAAAAGAAAGATTTGTAAG=27

cpa rp: GCCCTCGAGTTTTATATTATAAGTTGA=27

csa fp: GAGCATATGTCAAAAAAATCTTTTGCT=27

csa rp: GCCCTCGAGACGTTTTCCTCTTCTCTT'=27

linker+ *cpa* rp: TTTCGCCGCCGCTTCCGCTTTTATATTATAAGTTGA=36

Linker+ csa fp:

GCGGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGCGCTTACAAATCTTGA

AGAG=54

cpa fp for nested PCR: AGACTTTTGCAGAGGAAAGA=20

csa rp for nested PCR: TATCTTTTGCACGATACCCA=20

pUC57 fp: GCCATTCGCCATTCAGG=17
pUC57 rp: TAGCTCACTCATTAGGCAC=19(according to the manufacturer's recommendations).

Q3. What were your controls?

R3. For negative control pUC57 non-recombinant plasmid vector (pUC57without alpha-alpha fusion gene) was used.

Q4. The software and tests used for statistical analysis, and the comparisons done properly were not introduced.

R4. One *E. coli* strain TOP10 competent but not transformed colony also cultured on the same plate.
Statistical analysis: *In silico* analysis in the study were performed by online and Swiss-pdb Viewer(spdv) 4.10 software. Graphical illustrations were constructed using Microsoft PowerPoint2016 and UCSF chimera software.

Results

Q1. The figures and tables did not contain legends and titles, scales, and definition of abbreviations.

R1. e.g. Figure3. 1% agarose gel electrophoresis of PCR product(A), fusion PCR product(B) and nested PCR product(C).

(A) Lane 1: 100 bp DNA size marker, lane 2: *C. perfringens* type A alpha toxin gene, lane 3: *C.septicum* alpha toxin gene;

(B) Lane 1: 1kb DNA size marker, lane 2: *C. perfringens* alpha -*C. septicum* alpha(α-α) fusion toxin gene;

(C) Lane 1: Nested PCR product for confirmation fusion gene, lane 2: 100bp DNA size marker.

Q2. The figures and tables were not compatible with the main text regarding contents and numbering.

R2. In text; So, the tertiary structure of fusion protein construction was confirmed and visualized with UCSF Chimera tool (Fig. 2).

In figures: Figure 2. Informative 3D Visualization of predicted alpha-alpha fusion protein tertiary structure by I-TASSER.
Amino acids1-28: Signal peptide(red); Amino acids 29-398: Activated alpha peptide *C. perfringense* (green); Amino acids 399-410: Linker fragment(blue); Amino acids 411-777: Activated alpha peptide *C.septicum* (purple).

Discussion

Q1. The limitations of the study and suggestions were not addressed at the end of discussion.

R1. One of the limitations of this study was the complexity and difficulty of connecting these two genes by PCR fusion method.
It is suggested that this technique be used for other *Clostridium* toxins and for the fusion of more than two genes.

Q2. Acknowledgment, funding sections were not presented at the end of the article.

R2. Acknowledgment: This article has been extracted from the thesis written by Mr. Ali haghroosta in School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran (Registration No: 435). The authors wish to express their warm thanks to all the scientific members of Department of Microbiology, School of Medical, Shahid Beheshti University of Medical Sciences, and also all the scientific members, and staffs of Anaerobic Bacterial Vaccines Production Department of Razi Vaccine and Serum Research Institute.

Funding/Support: This study is done using financial and scientific support of Shahid Beheshti University of Medical Sciences as well as Razi Vaccine and Serum Research Institute

Q3. Almost 50% of references are the old reference type. Please change them and use the new reference type.

R3. New reference substituted to the old reference.

2. Freedman JC, Theoret JR, Wisniewski JA, Uzal FA, Rood JI, McClane BA. *Clostridium perfringens* type A-E toxin plasmids. *Research in Microbiology*. 2015;**166**(4):264-79. doi: 10.1016/j.resmic.2014.09.004.

3. Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, et al. Toxin Plasmids of *Clostridium perfringens*. *Microbiology and Molecular Biology Reviews*. 2013;**77**(2):208–233.

4. Sakurai J, Nagahama M, Oda M. *Clostridium perfringens* alpha toxin: characterization and mode of action. *J Biochem*. 2004;**136**:569–574.

5. Neeson BN, Clark GC, Atkins HS, Lingard B, Titball RW. Analysis of protection afforded by a *Clostridium perfringens* alpha-toxoid against heterologous clostridial phospholipases C. *Microb Pathog*. 2007;**43**:161–165.

6. Cavalcanti MTH, Porto T, Porto ALF, Brandi IV, Lima Filho JL, Pessoa Junior A. Large scale purification of *Clostridium perfringens* toxins: a review. *Brazilian Journal of Pharmaceutical Sciences*. 2004;**40**(2):151-164.

7. Uppalapati SR, Kingston JJ, Qureshi IA, Murali HS, Batra HV. *In Silico, in Vitro* and *in Vivo* analysis of binding affinity between N and C-domains of *Clostridium perfringens*

alpha toxin. *PLoS ONE*. 2013;**8**(12):e82024. doi: 10.1371/journal.pone.0082024.

8. Nanjappa S, Shah S, Pabbathi S. *Clostridium septicum* gas gangrene in colon cancer: importance of early diagnosis. *Hindawi Publishing Corporation.* 2015;Article ID 694247:1-3. http://dx.doi.org/10.1155/2015/694247.

9. Kennedy CL, Lyras D, Cordner LM, melton-Witt J, Emmins JJ, Tweten RK, et al. Pore-forming activity of alpha-toxin is essential for *Clostridium septicum*-mediated myonecrosis. *Infection and Immunity*. 2009;**77**(3):943-951.

16. Chen JE, Huang CC, Ferrin TE. RRDistMaps: a UCSF Chimera tool for viewing and comparing protein distance maps. *Bioinformatics*. 2015;**31**(9):1484-6.

23. Chen X, Guo P, Xie Z, Shen P. A convenient and rapid method for genetic transformation of *E. coli* with plasmids. Antonie van Leeuwenhoek. 2001;**80**(3):297-300.

24. Kamalirousta M, Pilehchian R. Development of a new bifunctional fusion protein of vaccine strains *Clostridium perfringens* type D and *Clostridium septicum* epsilon-alpha toxin genes. *Microbiology Research Journal International*. 2018;**23(**2):1–9.

Associate Editor's Comments:

Associate Editor 1:
Q1. please update introduction, discussion and references according to new references.

R1.
Update introduction:
The virulence of this organism is largely dependent to its ~ 17 toxins. However, *C. perfringens* strains on the base of production of alpha, beta, epsilon, and iota major toxins have been divided into 5 types A, B, C, D and E(2). All types of *C.perfringens* strains can produce alpha toxin(CPA), the predominant product in *C. perfringens* type A and is stated as a zinc metallophospholipase C that has phospholipase C, lecithinase and sphingomyelinase activities that result in haemolysis and necrosis. Crystallographic studies of the CPA structure show that it has two biologically active domains (3,4): an N-terminal domain that contains the single active site of the enzyme and a C–terminal domain that is essential for both toxic and cytolytic activity. N-terminal and C–terminal domains are immunogenic, but only C–terminal domain stimulates a protective immune response (3,5). It is well established that both CPA and teta toxin (perfringolysin O) are responsible for *C. perfringens*-mediated myonecrotic infection

Update discussion:
Our results of *in silico* analysis (C-score = -2.68 and TM-score = 0.41 ± 0.14) indicates a model of correct topology. Because C-score is typically in the range of (-5 to 2) and TM-score is more than 0.17. Our results of Ramachandran plot (amino acid residues in favored and allowed regions = 88.4%) show geometrical structure of alpha-alpha fusion protein is confirming as a natural like protein. Because the percent of amino acid residues is more than 85% in favored and allowed regions (17, 18).

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One of the limitations of this study was the complexity and difficulty of connecting these two genes by PCR fusion method.

It is suggested that this technique be used for other *Clostridium* toxins and for the fusion of more than two genes.

Update references:

new references: 2,3,4,5,6,7,8,9,16,23 and 24

2. Freedman JC, Theoret JR, Wisniewski JA, Uzal FA, Rood JI, McClane BA. *Clostridium perfringens* type A-E toxin plasmids. *Research in Microbiology*. 2015;**166**(4):264-79. doi: 10.1016/j.resmic.2014.09.004.

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2 Revision 1

1 comment · 19 Jul 2020

Editor-in-Chief

ACCEPT 19 Jul 2020

Accept