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Original Article

Evaluation and Comparison of *Clostridium* Epsilon-Alpha Fusion Gene Expression Using Different Commercial Expression Vector

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Abstract

Clostridium perfringens and *Clostridium septicum* are gram-positive, anaerobic, spore-forming rods and pathogens for humans and livestock, which are widespread in nature as well as human and animal digestive systems. *C. perfringens* produces numerous different exoproteins, which are various systems of action. The major *C. perfringens* toxins include alpha, beta, epsilon, and iota. *C. perfringens* are classified into five groups (A-E) on the basis of the production of these lethal toxins. Furthermore, toxins secreted from *C. septicum* include alpha, beta, delta, and gamma. Epsilon and alpha toxins of *C. perfringens* and *C. septicum* are the major causes of enterotoxemia and braxy in sheep and goats, respectively. The production of recombinant immunogenic proteins of these bacteria using suitable expression vectors and expression prokaryotic hosts can be a convenient method for the reduction of the costs and production time of clostridial anaerobic vaccines. In the present study, recombinant *Escherichia coli* strain TOP10 containing pJETεα was used for the evaluation of *C. perfringens* type D and *C. septicum* epsilon-alpha fusion protein using different commercial vectors. After the extraction of pJETεα from the recombinant cell, it was digested by *Nde*I and *Xho*I restriction enzymes and subcloned into pET22b (+), pET26b (+), and pGEM-B1 expression vectors in *E. coli*/Rosetta and *E. coli*/BL21 (DE3). The expression of recombinant fusion toxin was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting in three different temperatures, various isopropyl β-D-1-thiogalactopyranoside (IPTG) gradients, and different times using pGEMεα, pET22εα, and pET26εα vectors in *E. coli*/Rosetta and *E. coli*/BL21 (DE3). According to the obtained results, recombinant *E. coli*/Rosetta/pET22εα showed better expression at a temperature of 37°C after 6 h of induction by IPTG.

Keywords: *C. perfringens*, *C. septicum*, Epsilon-alpha, Fusion protein, Expression

Évaluation et Comparaison de l'expression du Gène de Fusion *Clostridium* Epsilon-Alpha en Utilisant Différents Vecteurs D'expression Commerciaux

Résumé: Clostridium perfringens et Clostridium septicum sont des bâtonnets et agents pathogènes à gram positif, anaérobie et sporulés pour les humains et le bétail, qui sont répandus dans la nature ainsi que dans les systèmes digestifs humains et animaux. C. perfringens produit de nombreuses exoprotéines différentes, qui sont différents systèmes d'action. Les principales toxines de C. perfringens comprennent alpha, bêta, epsilon et iota. C. perfringens sont classés en cinq groupes (A-E) sur la base de la production de ces toxines mortelles. De plus, les toxines sécrétées par *C. septicum* comprennent les toxines alpha, bêta, delta et gamma. Les toxines Epsilon et

alpha de *C. perfringens* et *C. septicum* sont les principales causes d'entérotoxémie et de braxy chez les moutons et les chèvres, respectivement. La production de protéines immunogènes recombinantes de ces bactéries en utilisant des vecteurs d'expression appropriés et des hôtes procaryotes d'expression peut être une méthode pratique pour la réduction des coûts et du temps de production des vaccins anaérobies clostridiens. Dans la présente étude, la souche *d'Escherichia coli* TOP10 recombinante contenant pJETεα a été utilisée pour l'évaluation de la protéine de fusion C. perfringens type *D* et *C. septicum* epsilon-alpha en utilisant différents vecteurs commerciaux. Après l'extraction de pJETεα de la cellule recombinante, il a été digéré par les enzymes de restriction *Ndel* et *Xhol* et sous-cloné dans les vecteurs d'expression pET22b (+), pET26b (+) et pGEM-B1 dans *E. coli* / *Rosetta* et *E. coli* / BL21 (DE3). L'expression de la toxine de fusion recombinante a été évaluée par électrophorèse sur gel de polyacrylamide-dodécyl sulfate de sodium et Western blot à trois températures différentes, divers gradients d'isopropyl β-D-1-thiogalactopyranoside (IPTG) et à différents moments en utilisant les vecteurs pGEMεα, pET22εα et pET26εα dans *E. coli* / Rosetta et *E. coli* / BL21 (DE3). Selon les résultats obtenus, *E. coli* / Rosetta / pET22εα recombinante a montré une meilleure expression à une température de 37° C après 6 h d'induction par IPTG.
Mots-clés: *C. perfringens*, *C. septicum*, Epsilon-alpha, Protéine de fusion, Expression

Introduction

Clostridia are gram-positive rods, anaerobic in their metabolism with central or subterminal heat resistant spores, and widespread in the environment, which are observed in soil, water, sewage, feces, and intestinal tract of humans and animals. Some of these bacteria are responsible for severe diseases in humans and animals, due to the production of highly potent protein toxins (Rood and Cole, 1991; Cordoba et al., 2001; Popoff and Bouvet, 2009; Bakhshi et al., 2016). *C. perfringens* and *C. septicum* are among the most important pathogen bacteria of *Clostridium* genus, causing illness in domestic animals (Hatheway, 1990; Mainil, 2006).

C. perfringens is responsible for a wide range of diseases, including food poisoning, gas gangrene, necrotizing enteritis, enterotoxemia, and intestinal tract infections in the livestock (Songer, 1996; Rood et al., 2018). *C. perfringens* produces numerous different exoproteins, which are various systems of action. The major *Clostridium* toxins include alpha, beta, epsilon, and iota. *C. perfringens* is classified into five groups (A-E) on the basis of the production of these lethal toxins (McDonel, 1980; Li et al., 2013). Epsilon toxin secreted by *C. perfringens* types D and B is responsible for a rapidly fatal enterotoxemia in sheep, goats, and cattle. It is a member of the pore-forming toxins coded by *etx* gene and located on large plasmids (Sayeed et al., 2007; Uzal et al., 2010; Popoff, 2011).

Epsilon toxin is the most potent toxin of *C. perfringens*. It is secreted into the prototoxin form (32.9 kDa) in the intestinal tract of the infected animals that is activated by proteolytic enzymes, such as trypsin, α-chymotrypsin, and λ-protease. Epsilon toxin LD_{50} is 50-320 ng/kg in mice depending on the type of protease used (trypsin/chymotrypsin: 50 ng/kg) (Alves et al., 2014; Ferreira et al., 2016).

C. septicum is a resident bacterium of human and animal microflora (Pilehchian Langroudi, 2015). It can produce several toxins (i.e., alpha, beta, gamma, and delta); however, alpha toxin is the major toxin and necessary for its virulence (Langroudi, 2015). This bacterium can be responsible for spontaneous myonecrosis and gas gangrene in humans and animals, braxy in cattle and goats, and heavy losses in the livestock industry. Alpha toxin is also a prototoxin (46.55 KDa) at the time of synthesis and requires cleavage by proteases for activation (Knapp et al., 2010; Langroudi, 2015). The study and evaluation of different expression vectors are required to select suitable vectors for high-level protein production.

Recently, important studies have been carried out on the fusion protein, cloning, expression, and immunogenicity of recombinant *Clostridium* toxins for

the selection of suitable vaccine candidates. In 2006, in a study carried out by Jia-Ning *C. perfringens* α-β fusion gene was cloned in plasmid pZCPAB and transformed into *E. coli*/BL21(DE3); then, its expression was evaluated (Bai et al., 2006). Pilehchian Langroudi et al. designed *C. perfringens* types B and D ε-β fusion toxin and it was cloned in *E. coli*; subsequently, they have reported successful studies on the expression and immunogenicity of fusion toxin (Pilehchian Langroudi et al., 2011; Langroudi et al., 2013).

Previously, the authors of the present study designed a new construct containing *C. perfringens* epsilon toxin and *C. septicum* alpha toxin genes fused by a linker using bioinformatics approach and it was ligated into pJET1.2/blunt cloning vector; then, pJETεα was cloned into *E. coli*/TOP10. The present study used recombinant *E. coli* strain TOP10 containing pJETεα for the extraction of epsilon-alpha fusion gene and investigation of the expression of its fusion protein using different commercial expression vectors.

Material and Methods

Materials. Plasmid pGEM-B1 was purchased from Bioneer Company in South Korea. Plasmid pET22b (+) and plasmid pET26b (+) were prepared by Novagen (USA). *Taq* polymerase, deoxynucleotide triphosphate, deoxyribonucleic acid (DNA) size markers of 100 and 1000 bp, prestained protein ladder, sodium dodecyl sulfate, agarose, tris base, acrylamide, and bis-acrylamide were obtained from CinnaGen (Iran). *Pfu* DNA polymerase, T4 DNA ligase, plasmid DNA purification kit, geneJET gel extraction kit, and *Nde*I and *Xho*I restriction enzymes endonuclease were prepared by Fermentas (Thermo Scientific™ Germany). Nickel-nitrilotriacetic acid (Ni-NTA) Agarose was prepared by Qiagen (USA). Sheep primary antibody and conjugate anti-sheep Horseradish peroxidase were purchased from DAKO Company (Glostrup, Denmark). Bacterial strains, namely *C. perfringens* type D, *C. septicum*, and *E. coli* strains Rosetta and BL/21 (DE3), were obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran.

Construction of Epsilon-Alpha Fusion Gene. In previous studies, pJETεα and recombinant *E. coli*/TOP10/pJETεα were produced. Briefly, *C. perfringens* and *C. septicum* were cultured in brain heart infusion broth, and genomic DNA extraction was performed by the phenol-chloroform method using suitable primers (epsilon toxin gene forward primer: 5′TGGGAACTTCGATACAAGCA3′, epsilon gene toxin reverse primer: 5′TGAACCTCCTATTTTGTATCCCA3′, alpha toxin gene forward primer: 5′GAGCATATGTCAAAAAAATCTT3′, alpha toxin gene reverse primer: 5′CCCTCGAGTATATTATTAATTA3′, epsilon alpha fusion gene primers: forward 5′AATCATATGAAAAAAAATCTTGTAAAAAGT 3′, reverse 5′TTTCGCCGCCGCTTCCGCTTTTATTCCTGGTG CCTTAAT 3′). Epsilon toxin gene (HQ179546.1) and alpha toxin gene (JN793989) were retrieved from GenBank, and epsilon-alpha fusion was constructed by fusion polymerase chain reaction (PCR). Then, it was cloned into pJET1.2/blunt and recombination *E. coli*/TOP10/pJETεα was produced.

Subcloning of ε-α Fusion Gene. The recombinant vector pJETεα was extracted by plasmid DNA purification kit (Fermentas) according to the manufacturer's instructions from recombinant *E. coli*/TOP10/pJETεα and digested by restriction enzymes *Nde*I and *Xho*I. In addition, the epsilon-alpha fusion gene was obtained using geneJET gel extraction kit from 1% agarose gel electrophoresis. The extracted epsilon-alpha fusion gene was sent to Bioneer Company for sequencing. The pGEM-B1, pET22b (+), and pET26b (+) were digested by *Nde*I and *Xho*I for the generation of sticky ends. The ε-α fusion gene was ligated into the pGEM-B1, pET22b (+), and pET26 (+) vectors by T4 DNA ligase, and the expression recombinant vectors carrying the ε-α fusion gene of *C.*

perfringens type D and *C. septicum* were constructed. *E. coli*/Rosetta and *E. coli*/BL21 (DE3) were selected as expressional hosts. There were grown in Lysogeny broth (LB) medium with 50 µg/ml ampicillin. Then, the competent cells were made by temperature shock and $CaCl_2$, and the recombinant vectors, including pGEMεα, pET22εα, and pET26εα, were transformed into the cells. The recombinant cells were confirmed by antibiotic screening in LB agar/ampicillin, colony PCR, extraction of recombinant plasmids, and sequencing of recombinant vectors by Bioneer Company for the conformation of ligation and transformation.

Expression of ε-α Fusion Protein. The recombination bacteria were inoculated in LB broth containing a selective antibiotic (ampicillin 50 µg/ml) and grown at 37°C to OD_{600} of 0.65 to 0.75. To study the expression of fusion proteins was used from different isopropyl β-D-1-thiogalactopyranoside (IPTG) (range: 0.1-0.5 mM) concentrations and different growth temperatures (25, 31, and 37°C) at different times (1, 3, 6, and 18 h). Bacterial growth was continued for 18 h; subsequently, recombinant bacteria pellet was collected by centrifugation and stored at -20°C to carry out sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

Purification of ε-α Fusion Protein. The ε-α fusion protein, which carries a histidine tag at carboxy terminal, was purified by Ni-NTA resin. The recombination cell pellet was suspended in lysis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, and imidazole 10 mM; pH 8), and the cells were disrupted with sonication on ice (8 pulses of 45 sec with 30-sec intervals). The cell lysate was centrifuged at 13,680 g for 20 min at 4°C, and the supernatant was passed through a Ni-NTA resin column at a flow rate of 1 ml/min. The extracted fraction from the column was washed with 5 volumes of washing buffer containing imidazole. Finally, the recombinant proteins were eluted with elution buffer (NaH_2PO_4 50 mM, NaCl 300 mM, and imidazole 250 mM; pH 8), and the purified protein was stored at 80°C for analysis by SDS-PAGE and western blotting.

Results

Subcloning of ε-α Fusion Gene. In previous studies, *C. perfringens* type D epsilon toxin gene and *C. septicum* alpha toxin gene were fused and contained pJETεα. According to the findings of sequence analysis, the length of the fusion gene is 2,358 bp, where nucleotides 1 to 984 are related to epsilon toxin gene with its signal peptide, nucleotides 985 to 1,020 form a linker sequence optimizing for *E. coli*, and nucleotides 1,021 to 2,358 belong to alpha toxin without its signal peptide. The *Nde*I and *Xho*I restriction sites and their flanking regions at the 3' end of epsilon and 5' end of alpha toxin genes are also present. This sequence was deposited in GenBank under the accession number of KU726861 (Kamalirousta and Pilehchian). The digested ε-α fusion gene was recovered by agarose gel extraction kit according to the instructions of the company and its sequence was confirmed by DNA sequencing. Subcloning fusion gene into expression vectors was conducted, and the vectors were transformed into *E. coli*/Rosetta and *E. coli*/BL21 (DE3). Recombinant colonies containing ε-α fusion gene were screened by growth in LB/ampicillin agar, colony PCR (empty vectors considered negative control) (Figure 1), and analysis of restriction enzymes digestion mapping in 1% agarose (Figure 2).

Fusion Protein Expression. The induction of fusion toxin expression by different concentrations of IPTG was indicated, with no significant change in the level of the recombinant protein expression (Figure 3); however, the roles of temperature and time were significant. Accordingly, at a temperature of 37°C and 6 h after induction, maximum protein expression occurred. In addition, protein expression at 31°C was better than that reported for 25°C. *E. coli*/Rosetta had more efficiency in the level of ε-α fusion protein

expression than *E. coli*/BL (DE3) (Figure 4).

Purification of ε-α Fusion Protein. The ε-α fusion protein containing a histidine tag at carboxyl terminus was purified through Ni-NTA resin and became visible as an approximately 75-kDa protein on SDS-PAGE and western blotting (Figure 5).

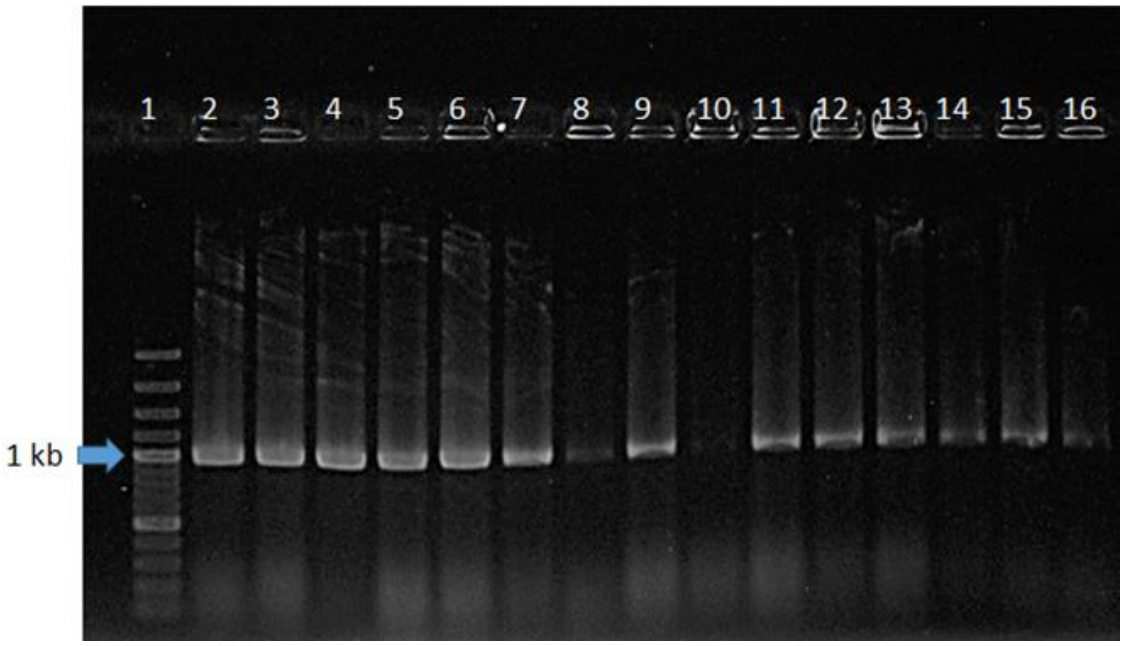

Figure 1. Polymerase chain reaction (PCR) analysis of recombinant cell colonies using epsilon forward and reverse primers; lane 1: 100 bp plus deoxyribonucleic acid size marker; lanes 2-3: colony PCR of *E. coli*/Rosetta/pET22εα; lanes 4-5: *E. coli*/Rosetta/pET26εα; lanes 6-7: *E. coli*/Rosetta/pGEMεα; lanes 8-10: *E. coli*/BL21/pET22εα; lanes 11-13: *E. coli*/BL21/pET26εα; lanes 14-16: *E. coli*/BL21/pGEMεα

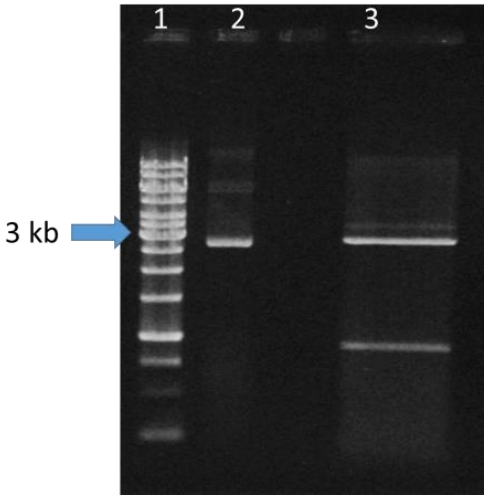

Figure 2. Restriction digestion of pET22εα recombinant cloning vector; lane 1: 1 kb deoxyribonucleic acid size marker; lane 2: undigested pET22εα; lane 3: digested pET22εα

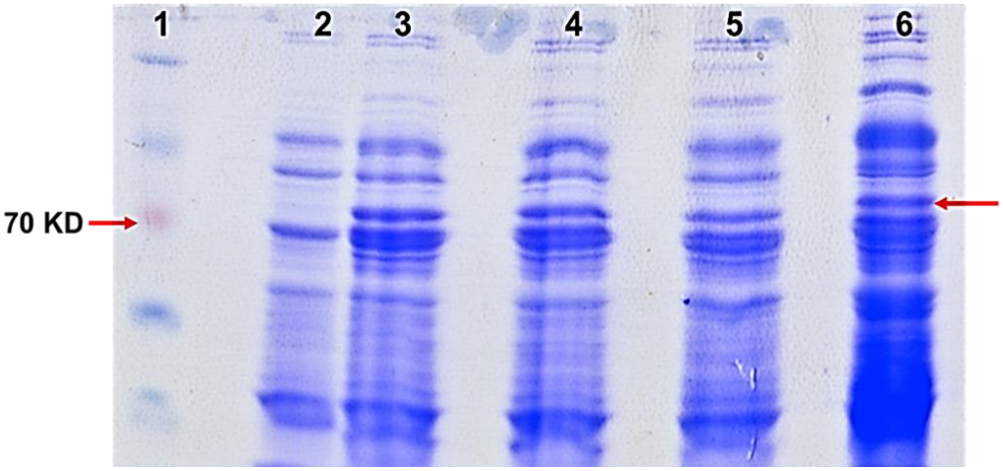

Figure 3. Effect of isopropyl β-D-1-thiogalactopyranoside (IPTG) and gradients on fusion protein expression; lane 1: Pageruler Prestained Protein Ladder #SM0671; lane 2: recombinant *E. coli*/Rosetta/pET22εα lysate before induction; lanes 3-6: recombinant *E. coli*/Rosetta/pET22εα lysate 6 h after induction with 0.1, 0.2, 0.3, 0.4, and 0.5 mM IPTG at 37°C

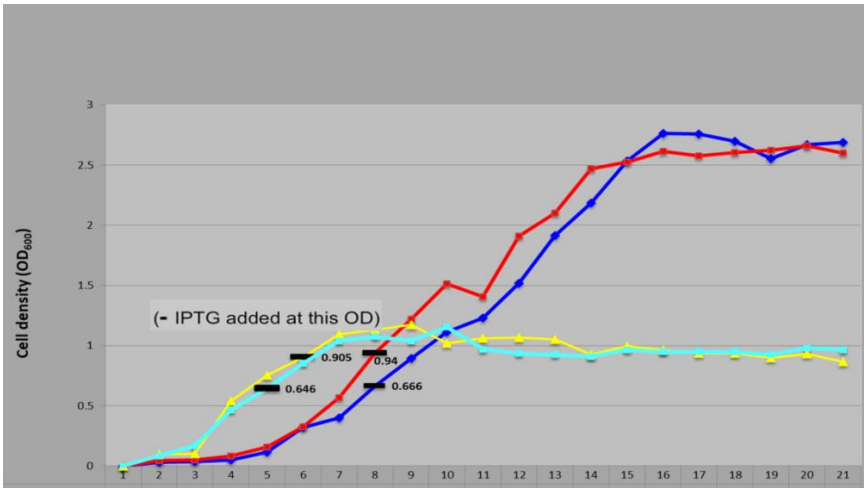

Figure 4. Growth curves of recombinant *E. coli* comparison to negative control in Lysogeny broth medium containing ampicillin; recombinant host strain: Rosetta; plasmid: pET22b (+); recombinant gene: epsilon-alpha fusion gene; negative control host strain: Rosetta; plasmid: pET22b (+)

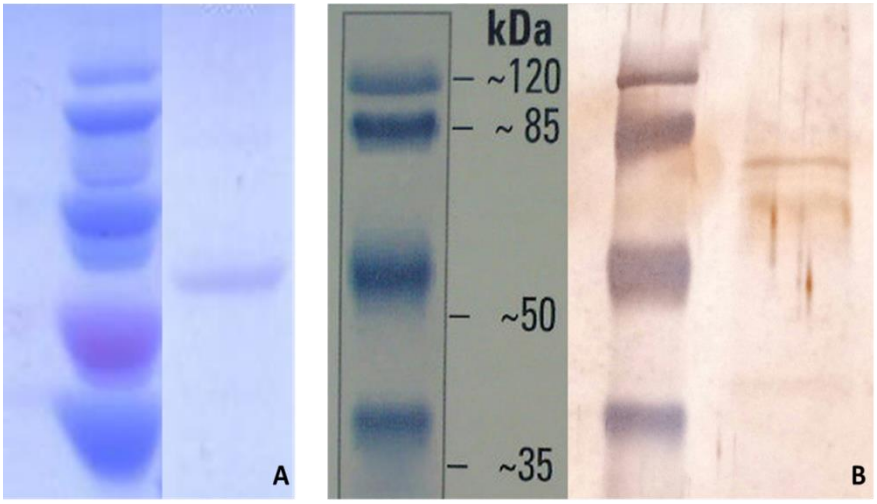

Figure 5. Purification of ε-α fusion protein by nickel-nitrilotriacetic acid resin and western blotting

Discussion

Clostridia are the producers of powerful toxins and exoproteins. These exotoxins are important factors in their pathogenicity and virulence. *C. perfringens* type D epsilon toxin and *C. septicum* alpha toxin play a major role in enterotoxemia and braxy (Kennedy et al., 2005; Uzal et al., 2014). The use of clostridial toxins for the production of effective vaccines to prevent livestock diseases caused by clostridial pathogens is a common and very effective method in vaccine manufacture (Knight et al., 1990). Proper cloning and expression systems are required to express and produce high-quality recombinant proteins.

The pJET1.2/blunt is a suitable vector for cloning blunt-end PCR products amplified by *Pfu* polymerase and other proofreading DNA polymerases. This vector is linearized with blunt-ends, which is inhibiting self-ligated vector products. The pJET1.2/blunt vector contains a lethal gene in multiple cloning site and ampR gene as selective markers. Using recombinant DNA techniques and protein fusion can construct a chimeric structure containing two or more functional and important genes, and then the chimeric structure insert the suitable host cells for cloning and replication.

Protein fusion technology is an appropriate, functional, and efficient strategy to provide an economic justification for the expression and production of proteins. The expression levels of recombinant proteins are high in bacteria, and bacterial expression systems will be suitable if there is no need for post-translational modifications. Among many expressive present systems, *E. coli* is quite obvious and has the best features (Dertzbaugh, 1998). Due to the prokaryotic nature of epsilon and alpha toxins and no necessity of the post-translational modifications, each of these toxins was expressed in different strains of *E. coli* and other hosts, such as *Bacillus subtilis*, *Lactococcus lactis*, and *Streptococcus pneumoniae*. Furthermore, the secretion of toxins out of the cell has been reported.

Currently, fusion strategy is commonly used in production and purification processes (Nilsson et al.,

1997). Previously, the expression of *C. perfringens* alpha-beta fusion protein and its immunologic investigation methods were reported (Bai et al., 2006). The expression and immunogenicity of alpha-beta2-beta1 *C. perfringens* recombination fusion protein in *E. coli* were studied and the results showed that the protein was well expressed and provided good immunity to animals (Zeng et al., 2011).

A study demonstrated the expression of clostridium beta toxin in *E. coli*/BL21 (DE3) and *E. coli*/Rosetta strains. *E. coli*/Rosetta could increase the expression of toxin (Bakhshi et al., 2016). Furthermore, in another study, *C. perfringens* epsilon-beta recombinant fusion protein was expressed in *E. coli*/Rosetta by recombinant pET22εβ and its immunogenic properties was studied in mice (Langroudi et al., 2013). The purpose of the aforementioned studies was to achieve prokaryotic systems easier than *clostridium* to produce toxins and then provoke more and better immune responses against these toxins.

According to the objectives of the present study, the chimeric gene was digested, purified, and ligated in expressional plasmids, namely pET22b (+), pET26b (+), and pGEM-B1. Afterward, recombinant plasmids were transformed into expressional hosts, including *E. coli*/Rosetta and *E. coli*/BL21 (DE3). The results of the SDS-PAGE and western blotting showed that the epsilon-alpha fusion protein at the *E. coli*/Rosetta was well expressed, but not observed in *E. coli*/BL21 (DE3). *E. coli*/Rosetta has appropriate transfer ribonucleic acid (tRNA) for rare codons (AGG, CCC, CUA, AGA, AUA, and CGG) and transcriptional RNA polymerase for T7 promoter (Steen et al., 1986; Rosano and Ceccarelli, 2014). Due to the presence of rare codons (AUA, AGG, AGA, CUA, and GGA) in the fusion gene sequence, *E. coli*/Rosetta is suitable for expression.

The expression of epsilon-alpha fusion protein in different IPTG gradients did not change significantly; nevertheless, it demonstrated significant changes in thermal gradients and different times; accordingly, the optimum temperature for expression was observed at 37°C and started 1 h following the induction of protein expression and continued until 22 h. The highest level of expression was at the 6^{th} h of induction. The obtained results of this study are consistent with the findings of similar studies conducted by Goswami et al. (1996) and Langroudi et al. (2013). The use of recombinant fusion protein as a source of vaccine production requires further investigation of the vaccine parameters, such as the level of toxin, needed or unnecessary adjuvant, type and level of adjuvant, and amount of vaccine injection for at least IU/ml.

Conclusion

The results of the current study showed that *E. coli*/Rosetta and pET22b(+) are suitable for *Clostridium* epsilon-alpha fusion gene expression and can be used for further studies on the preparation of recombinant fusion vaccine.

Authors' Contribution

Study concept and design: R. P. L.
Acquisition of data: H. R. S.
Analysis and interpretation of data: S. A. and A. H.
Drafting of the manuscript: H. R. S.
Critical revision of the manuscript for important intellectual content: R. P. L.
Statistical analysis: S. A. and A. H.
Administrative, technical, and material support: Razi Vaccine and Serum Research Institute

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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