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ORIGINAL ARTICLE

Cloning and expression of *Clostridium perfringens* type D vaccine strain epsilon toxin gene in *E. coli* as a recombinant vaccine candidate

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ABSTRACT

Background and Objectives: *Clostridium perfringens,* a Gram-positive obligate anaerobic bacterium, is able to form resistant spores which are widely distributed in the environment. *C. perfringens* is subdivided into five types A to E based on its four major alpha, beta, epsilon and iota toxins. The aim of the present study was cloning and expression of *C. perfringens* type D vaccine strain epsilon toxin gene.

Materials and Methods: Genomic DNA was extracted and the epsilon toxin gene was amplified using *Pfu* DNA polymerase. The PCR product was cloned into pJET1.2/blunt cloning vector. The recombinant vector (pJETε) was sequenced using universal primers. At the next step epsilon toxin gene was subcloned into pET22b(+) expression vector and transformed into *E. coli* Rosetta (DE3) host strain.

Results: The recombinant protein has been expressed in *E. coli* Rosetta (DE3) cells after subcloning of *C. perfringens etx* gene (1008 bp) into the expression vector.

Conclusion: We concluded that *E. coli* Rosetta strain was suitable for the expression of recombinant *C. perfringens* epsilon toxin protein from pET22ε expression vector. This recombinant cell can be used for further research on recombinant vaccine development.

Keywords: *C. perfringens,* Epsilon toxin gene, Vaccine strain, Cloning, Expression

INTRODUCTION

Clostridium perfringens, which was previously known as *Clostridium welchii,* is a Gram-positive, anaerobic, non-motile rod-shaped bacterium and is able to form environmentally resistant spores (1). This ubiquitous microorganism could be found in a variety of habitats such as soil, water and lower mammals and human intestine. The bacterium can be found widely all over the world due to its genomic variation. It produces numerous toxins which may cause various diseases such as diarrhea, necrotizing bowel disease and gas gangrene in humans as well as diarrhea, necrotic enteritis and fatal enterotoxemia

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in animals. The bacterium produces 17 toxins from which epsilon is the major one (2).

C. perfringens has a circular chromosome of approximately 3.6 Mbp with a low G+C content of approximately 25% (3, 4). Its genome includes extra chromosomal genetic elements in the form of plasmids and phages encoding mobile genes that vary in size and composition (5, 6). *C. perfringens* is a model for genetic engineering studies due to its striking physiological features (7) and several studies on the cloning and expression of its toxin genes have been reported.

Epsilon toxin is the third most potent clostridial toxin after botulinum and tetanus neurotoxins (8) and is active in a wide range of animal species and plays a central role in enterotoxemia of sheep and lambs (9). In 1992, epsilon toxin gene of *C. perfringens* type D had been cloned and expressed in *E. coli*. Studies have shown that the *etx* gene has 987bp and is located between base nucleotide 188 (start codon) and the stop codon at position 1174 (10). Type D epsilon toxin gene of *C. perfringens* has been cloned and expressed and its N terminal 20 amino acids sequence has been determined (11). The recombinant toxin has been strongly recognized by anti-native epsilon antibodies. The antibodies against this recombinant toxin recognized the native epsilon toxin and neutralized its action in mice models (12). The recombinant type B and the native type D toxins have similar molecular weights and isoelectric points and both of them react with the specific monoclonal antibodies. In 2010, *etx* gene was cloned and the potency of *E. coli* expressed recombinant epsilon toxoid was evaluated through administration to the goats, sheep and cattle. The results have shown adequate immunization of ruminants against enterotoxemia (13).

Numerous items can modify the current manufacturing procedure of clostridial vaccines and their efficiency but the biological process is often economically permanent. Industrial production and purification of toxins produced by *C. perfringens* is very difficult. Thus, heterologous expression systems are good alternative tools for production of these toxins. In the present study, expression of epsilon toxin in *E. coli* Rosetta(DE3) host strain has been reported.

MATERIALS AND METHODS

Bacterial strains and cultivation. *C. perfringens* type D vaccine strain (CWD CN409), *E. coli* TOP10F' and Rosetta (DE3) (14) strain were purchased from Razi institute. *C. perfringens* type D vaccine strain was cultured anaerobically in the liver extract medium using anoxomat chambers (Mart® microbiology, Netherlands) at 37 °C, pH 7.5 for 18 hours. Routine microbial control tests were done to confirm the identity of bacterial isolates. *E. coli* strains were cultured as previously described (15).

DNA extraction and gene amplification. Genomic DNA was extracted by phenol-chloroform method (16). Primers were designed for amplification of epsilon toxin encoding gene from *C. perfringens* genomic DNA. Complete nucleotide sequence of *etx* gene was retrieved from the gene deposited in GenBank under accession number HQ179546. *etx* gene was amplified using *Pfu* DNA polymerase and specific forward (5′-aatcatatgaaaaaaaatcttgtaaaaagt-3′) and reverse primers (5′-aatctcgagttttattcctggtgccttaat-3′) including *NdeI* and *XhoI* restriction sites, respectively.

Cloning of *etx* gene. Linearized pJET1.2/blunt vector was ligated with amplified *etx* gene in order to develop the recombinant cloning vector, pJETε. Screening of recombinant *E. coli* TOP10ε clones was done via colony PCR using universal sequencing primers. The empty cloning vector was used as the negative control of PCR. Identity of recombinant cloning vector, pJETε, was confirmed thorough restriction digestion with *NdeI* and *XhoI* enzymes (Fermentas). Sequencing analysis of the purified recombinant vector was also performed and BLAST and phylogenetic analysis was accomplished.

Subcloning of *etx* gene. The expression vector, pET22b (+), was digested with *NdeI* and *XhoI* and ligated with *etx* gene digested from the cloning vector. Screening of recombinant expression vector was done via colony PCR of individual colonies. Empty pET22b vector was used as negative control of PCR. Sequencing of the expression vector was also performed.

Expression of recombinant epsilon toxin. Recombinant *E. coli* cells were inoculated into LB broth medium and incubated at 37 °C to reach 0.6-0.7 OD_{600}. Final 0.5 mM IPTG concentration was used

to induce the expression of recombinant protein and bacterial incubation was continued for 18 hours. To optimize the expression of recombinant protein, different concentrations of IPTG (0.5, 1 and 1.5mM), different incubation temperatures (25, 31 and 37 °C) and times (3, 6 and 18 h.) were examined. Protein expression was analyzed by SDS-PAGE.

Purification of epsilon protein. To purify the recombinant epsilon protein which had a C-terminal His-tag, the bacterial pellet was re-suspended in lysis buffer and the cells were lysed by sonication on ice. The cell lysate was centrifuged at 13, 680(× g) and the clarified supernatant was loaded on Ni-NTA resin at the flow rate of 1 ml/min. The column was washed with 5 volumes of washing buffer and finally the protein was eluted by adding elution buffer (17). The purified protein was analyzed using SDS-PAGE and western blotting. The protein concentration was measured using a standard procedure (18).

RESULTS

Cloning of *etx* gene. Genomic DNA of *C. perfringens* type D was extracted and used for PCR amplification of *etx* gene (approximately 927 bp) (Fig. 1). Colony PCR on TOP10ε colonies containing the recombinant pJETε cloning vector using *etx* forward and reverse primers confirmed the presence of a 927 bp DNA fragment via 1% agarose gel electrophoresis (Fig. 2). Identity confirmation was done through restriction digestion with *NdeI* and *XhoI* (Fig. 3).

Subcloning of *etx* gene. Double digested *etx* gene was purified using agarose gel extraction kit according to the manufacturer and its sequence was confirmed through sequencing. Subcloning of the gene into pET22b (+) expression vector was done and the recombinant vector was transformed into *E. coli* Rosetta (DE3) strain. Screening of colonies was performed via *etx* gene specific colony PCR and sequencing analysis.

Protein expression. IPTG induction of protein expression was tested through SDS-PAGE analysis showing the expression of recombinant protein 3 h after induction (Fig. 4). Different concentrations of IPTG had no significant effect on protein expression (Fig. 5). The effect of different temperatures and incubation times, after IPTG induction, on protein expression was significant (Figs. 6 and 7).

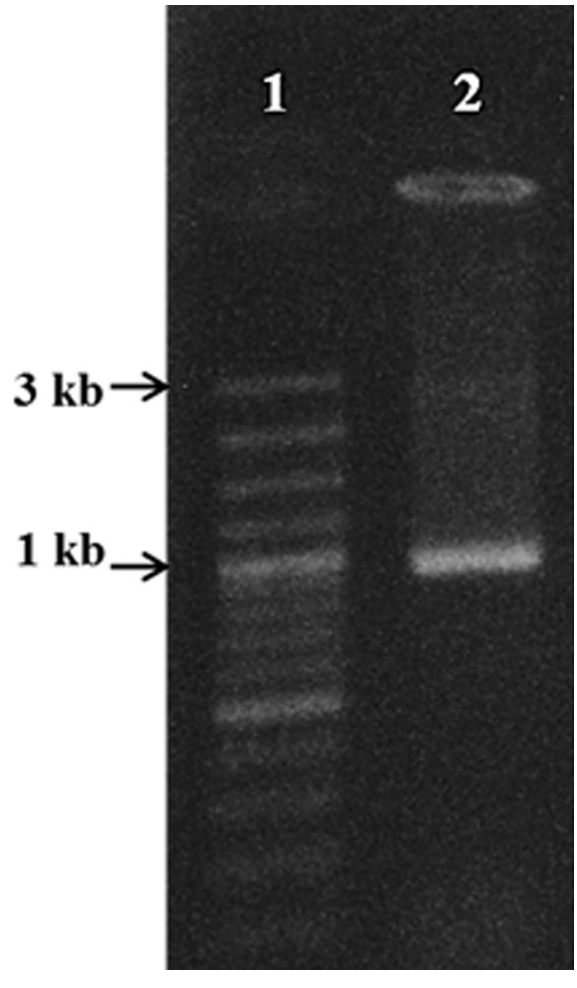

Fig. 1. PCR amplification of *C. perfringens* type D epsilon toxin gene. Lane 1:100 bpDNA ladder; Lane 2: epsilon toxin gene.

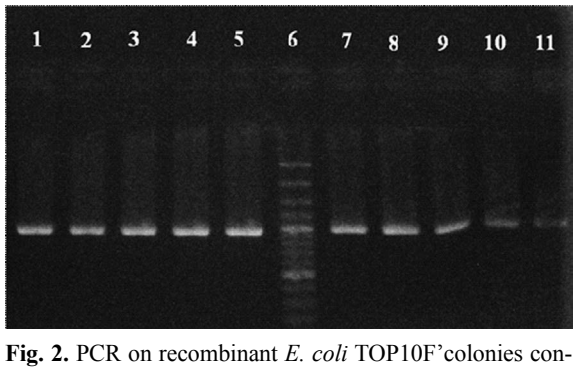

Fig. 2. PCR on recombinant *E. coli* TOP10F'colonies containing pJETε. Lanes 1-11: PCR products (927 bp); Lane 6:100 bpDNA ladder.

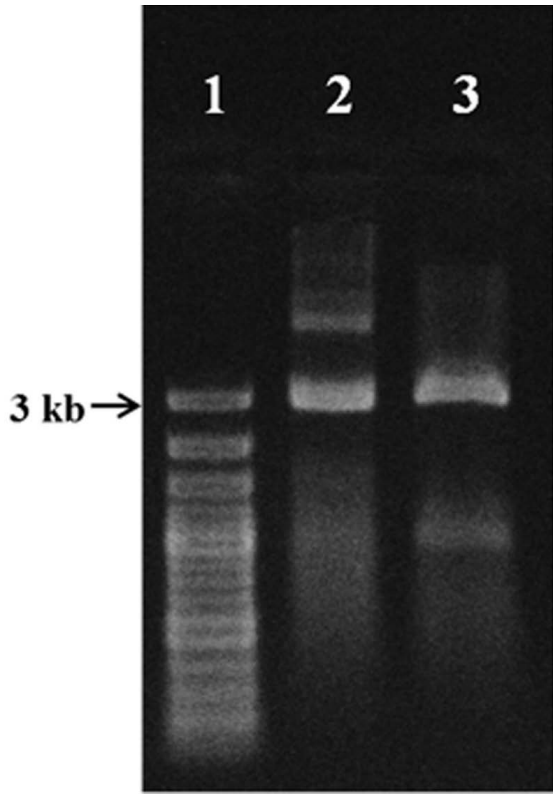

Fig. 3. Restriction digestion of recombinant pJETε cloning vector. Lane1: 100 bpDNA ladder; Lane 2: undigested vector; Lane 3: digested pJETε.

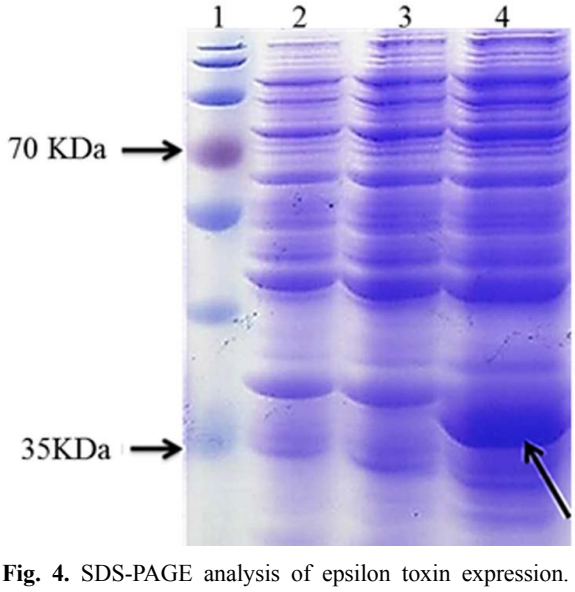

Fig. 4. SDS-PAGE analysis of epsilon toxin expression. Lane 1: Protein molecular weight marker; Lane 2: Negative control (*E. coli* RosettaDE3 strain containing empty pET22b+); Lane 3& 4: Recombinant *E. coli* strain before and after 3 h induction; Arrow indicates the recombinant epsilon toxin band.

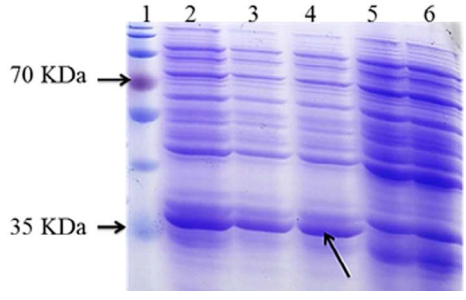

Fig. 5. Optimization of recombinant epsilon toxin expression by different IPTG concentrations. Lane1: Protein molecular weight marker; Lanes 2-4: Bacterial lysate 3h after induction with 0.5, 0.1 and 1.5 mM IPTG at 37 °C; Lane 5: Bacterial lysate before induction; Lane 6: Negative control lysate (*E. coli* Rosetta containing empty pET22b+); Arrow shows recombinant epsilon toxin band.

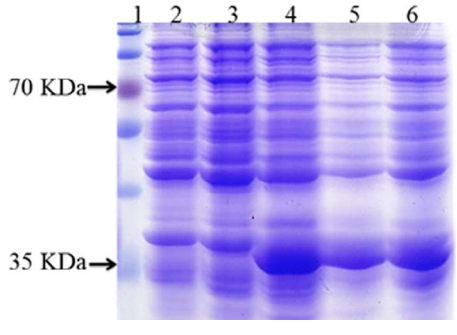

Fig. 6. Different temperatures for the expression of recombinant epsilon toxin. Lane 1: Protein molecular weight marker; Lanes 2-4: Bacterial lysate at 25, 31 and 37 °C, 3 h after induction with 0.5mM IPTG; Lane 5: Bacterial lysate before induction; Lane 6: Negative control (*E. coli* Rosetta strain containing empty pET22b+); Arrow shows recombinant epsilon toxin band.

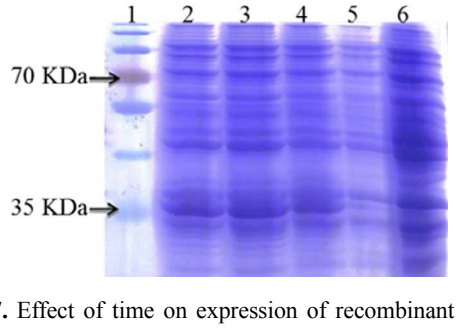

Fig. 7. Effect of time on expression of recombinant epsilon toxin. Lane1; Protein molecular weight marker; Lanes 2-4: Bacterial lysate after 3, 6 and 18h after induction with 0.5 mM IPTG; Lane 5: Bacterial lysate before induction; Lane6: Negative control (*E. coli* Rosetta containing empty pET22b+).

Purification of r-protein. The recombinant protein was purified through affinity chromatography using Ni-NTA resin. Purified protein appeared as an approximately 35 kDa protein on SDS-PAGE and western blot using sheep primary and anti-sheep HRP conjugated secondary antibody (Fig. 8).

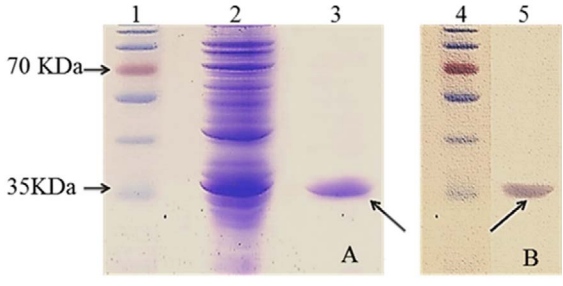

Fig. 8. Purification of recombinant epsilon toxin by Ni-NTA resin and Western blotting analysis. (A) SDS-PAGE analysis of purified protein; Lane 1: Protein molecular weight marker; Lane 2: Bacterial lysate 3hours after induction; Lane 3: Purified epsilon toxin; (B) Western blot analysis of purified protein; Lane 4: Protein molecular weight marker; Lane 5: Western blot of the purified recombinant epsilon toxin.

DISCUSSION

In spite of using traditional methods in production of effective vaccines, large scale production of such vaccines from different *Clostridium* species needs high safety conditions and precise quality control steps. Therefore, production of these candidate vaccines in a safe host is beneficial (19). Recombinant DNA technology has enabled researchers to produce vaccines for diseases which could not be treated using the traditional methods such as infections caused by *C. perfringens* species (19). *C. perfringens* is a potent pathogen because of its ability to generate several toxins. In this study pJET1.2 blunt vector was used due to its advantages over the other available TA cloning vectors. Blunt ends of this vector avoided self-ligated vector products (16, 17). In this study, the recombinant vector was used in DNA sequencing, protein expression or gene expression functional analysis. In a previous study, Souza and the colleagues cloned *etx* gene in pET11a expression vector and expressed the recombinant epsilon toxin as inclusion bodies which were successfully used in animal immunization (12). Toxicity of the fusion epsilon-beta gene of *C. perfringens* types D and B revealed that toxigenesis of vaccine strains is one of the factors playing an important role in the effectiveness of the designed vaccines (17). This finding made us to select *C. perfringens* vaccine strain (CWD CN409) for the present study.

In 2010, a vaccine candidate containing detoxified recombinant epsilon toxin and aluminum hydroxide as the adjuvant was developed and co-administered with sheep pox vaccine to sheep and it was shown that sheep were being protected against both sheep pox and enterotoxemia (20).

In the present study, experimental optimizations revealed that different concentrations of IPTG have no obvious effect on the level of protein expression. Our findings revealed that different temperatures and incubation times had significant effects on the expression of epsilon toxin protein as optimal thermal condition was 37 °C and the highest yield of the recombinant protein was achieved 6h after IPTG induction.

CONCLUSION

This study enabled us to express recombinant vaccine candidates indicating the possibility of prevention of Clostridial diseases through manufacturing vaccine candidates under safer conditions and lesser costs.

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