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Full Length Article

Expression of α-β1-β2-ε Soluble Fusion Protein of *Clostridium perfringens* in *Escherichia coli* and its Immunogenicity in Mice

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Abstract

Currently, there is an urgent need to develop genetically engineered multivalent subunit vaccines or DNA vaccines to provide protection against infection of multiple types of *Clostridium perfringens* bacteria. Toward this end, in this study, a set of molecular biotechnologies were used to efficient express and obtain soluble fusion protein of alpha-beta1-beta2-epsilon-toxin (α-β1-β2-ε-toxin) derived from *C. perfringens* in *Escherichia coli.* A soluble fusion protein of α-β1-β2-ε-toxin was obtained for the first time with efficient expression in *Escherichia coli* system by conducting codon optimization, removing the signal peptide, selecting sequences of higher hydrophilicity and antigenicity, removing the lethal gene and optimizing the expression conditions. Noticeable rise of antibody level was detected in the serum of mice after immunization with the expressed protein. The survival rate of the immunized mice during the 7-day challenge period was 100% in the cases of *C. perfringens* types A, B, C and D.

Key words: *Clostridium perfringens*; Subunit vaccine; Immunogenicity; Expression; Purification; Soluble protein

Introduction

Clostridium perfringens is an important human and veterinary pathogen. It is highly associated with the diseases including braxy, lamb dysentery, hemorrhagic enteritis in cattle and sheep and ovine/bovine enterotoxemia, which cause great economic loss in livestock husbandry system (Glenn and Miskimins, 2005; Kalender *et al*., 2009; Lebrun *et al*., 2010; Muylaert *et al*., 2010; Savic *et al*., 2012). It is also one of the major pathogens associated with the human diseases such as food poisoning and traumatic gas gangrene (Lucey *et al*., 2004). It is widely distributed in soil, sewage, food, feed and feces and intestinal tracts of humans and animals (Mueller *et al*., 2010; Wang *et al*., 2011). Alpha, beta1, beta2 and epsilon toxins are the major exotoxins secreted by *C. perfringens*. The *C. perfringens* species can be divided into five serotypes, A, B, C, D and E, based on the spectrum of the produced toxins (Miyamoto *et al*., 2008; Morris, 2009). At present, immunization against *C. perfringens*-associated diseases is mainly done with traditional inactivated monovalent/multivalent vaccines or toxoid vaccines (Ewoldt and Anderson, 2005). Although these traditional vaccines are able to induce a certain level of protective antibody in animals, there exist concerns regarding the problems found during the use of these vaccines, such as safety and stability issues and the significant side effects after immunization (Chandran *et al*., 2010). These problems are the major obstacle to the application of these vaccines. In contrast, genetic engineering subunit multivalent vaccine has various advantages, for instance, it contains effective immune components that are necessary in producing protective immune response, while eliminating the components unrelated to immunity (*e.g*., components associated with pyrogen, stress, allergen). Thus, it has better safety and stability compared to traditional vaccine. In recent years, scientists have made great efforts on genetic engineering subunit vaccine against *C. perfringens*. For example, chickens were well protected from infection of salmonella and three serotypes of *C. perfringens* after immunization with *C. perfringens* gene engineering subunit vaccine using salmonella as a carrier (Wilde *et al*., 2019). It was reported that the toxin gene of *C. perfringens* was edited, optimized and used for foreign gene expression. The result showed that the expressed protein provided protection for pigs from diarrhea and necrotizing enteritis caused by *C. perfringens* (Hamza *et al*., 2018; Huang *et al*., 2019).

To inhibit and control of *C. perfringens*-associated diseases, there is an urgent need to develop genetically engineered multivalent subunit vaccines or nucleic-acid vaccines to provide protection against infection by multiple types of *C. perfringens* bacteria (Lebrun, 2007; Lobato *et al*., 2010). For the purpose of development of novel multivalent subunit vaccine against different types of *C. perfringens*, in this study we developed α-β1-β2-ε soluble fusion protein of *C. perfringens* using genetic engineering approach. The fusion protein was evaluated and demonstrated to serve as an efficient vaccine for *C. perfringens* prevention and control.

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Materials and Methods

Experimental Animals

A total of two hundred, 56-day-old female BALB/C mice (18–22 g in weight) were randomly assigned into two groups: one group with 160 mice was divided into 4 subgroups with 40 mice in each, which were immunized respectively with the four expressed proteins followed by challenging with the toxin; the other group, served as control, was divided into 4 subgroups with 10 mice in each, which were immunized with PBS and underwent same challenging. Vectors: the pET32a expression vectors were purchased from Novagen Inc., Madison, WI, USA. Competent cells: BL21 (DE3) competent cells were purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China. Enzymes and reagents: restriction enzymes *Bam*HI and *Xho*I, 2000 DNA Marker, T4 DNA ligase, SDS, IPTG, and *Taq* PCR Master Mix were provided by Takara Biotechnology Co., Ltd., Dalian, China. Agarose, DNA Rapid Purification and Recovery Kit, DNA Extraction Kit, and Plasmid Rapid Isolation Kit were all purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China. The pre-stained protein maker was purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China. The His-tag protein purification kit (10 mL) and the size exclusion chromatography columns (Superdex^{TM} 2000) were purchased from GE Healthcare Bio-Sciences, Pittsburgh, PA, USA. The horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich Co. LLC., Beijing, China. Bacterial strains: *C. perfringens* virulent strains, *i.e.*, type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), were produced by China Institute of Veterinary Drug Control. Gene synthesis: sequencing and synthesizing of the fusion protein target gene was completed by BGI, Shenzhen, China.

Synthesis of the Alpha- beta1-beta2-epsilon Fusion Gene

Toxin alpha (amino acids Trp^{29} to Lys^{258}, GenBank Accession No: ABA55036.1) was fused to toxin beta1 (amino acids Asn^{28} to Ile^{336}, GenBank Accession No: AJI77140.1) and toxin beta2 (amino acids Ala^{31} to Tyr^{389}, GenBank Accession No: AAW66350.1) and toxin epsilon (amino acids Lys^{33} to Lys^{328}, GenBank Accession No: Q02307.1). The signal peptide of the fusion protein was removed and the codon was optimized. The genes α-β1-β2-ε was obtained by chemical synthesis.

Construction and Transformation of Recombinant Expression Vectors

The synthetic α-β1-β2-ε gene was used as the template and was amplified by overlapping polymerase chain reaction (PCR) using the forward primer F1 (5′-ATGTGGGATGGAAAAATTGATGGA-3′) and the reverse primer R1 (5′-TCATTTGATGCCCGGTGCTTTGA-3′). The target plasmid was subsequently transformed into *E. coli* competent cells BL21 (DE3). Cultures of the *E. coli* strain BL21(DE3) were uniformly smeared on Luria-broth (LB) plates (supplemented with ampicillin) and grown at 37°C for 16 h. Single colonies were then picked and grown overnight with shaking. The plasmid DNA was isolated and double digested with the enzymes *Bam*HI and *Xho*I.

Analysis and Identification of the Recombinant Protein Expression

The Pet32a-α-β1-β2-ε strain was grown in LB liquid medium (supplemented with 50 μg/mL Ampicillin) at 37°C and incubated in a shaker (Thermo MaxQ™ 6000) at 200 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. After collecting 1 mL bacterial fluid as an un-induced reference, the protein expression was induced by addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to the rest of the culture. The induced protein expression for the strain was conducted by using 1 m*M* of IPTG at 16°C and incubated for over 16 h. The bacterial fluids with and without being induced with IPTG were then collected for expression analysis. The bacterial fluids (1 mL) were collected and transferred into an appropriately marked centrifuge tube (1.5). The bacterial fluid was centrifuged at 8,000 rpm and 4°C for 30 min. The bacterial precipitate was then obtained after the removal of the supernatant. The cells were then suspended in phosphated -bufferd saline (PBS, 1 mL) and centrifuged at 8,000 rpm for 5 min, followed by the removal of the supernatant. The protein precipitate was washed and re-suspended in PBS (200 μL) and then sonicated until clearness. The attained fluid containing all bacterial proteins was centrifuged at 16,000 rpm and 4°C for 30 min. The supernatant and protein precipitate were collected. The protein precipitate was re-suspended in PBS (50 μL) then washed and precipitated. The all-bacterial-protein fluid, protein supernatant, and protein precipitate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer (10 μL, 5 times) and completely mixed. Fractions of 6 μL were collected for SDS-PAGE analysis.

Optimization of the Temperature and Duration for the Induced Expression

Cultures of the pET32a-α-β1-β2-ε strain were grown in LB liquid medium supplemented with 50 μg/mL Ampicillin (obtained by adding Ampicillin in LB liquid medium until the concentration of Ampicillin reaches 50 μg/mL). The PET32a-α-β1-β2-ε strain cultures were incubated at 37°C in a shaker (Thermo MaxQ™ 6000) at 200 rpm until the OD_{600} value (taking the LB liquid medium with 50 μg/mL ampicillin as reference) reached 0.6. The expression

experiments were conducted with 1 m*M* of IPTG at 16°C over 8, 16 or 24 h.

Optimization of the IPTG Concentration

The recombinant pET32a-α-β1-β2-ε strain constructed was cultured at 16°C and induced for 16 h at the presence of IPTG with concentration of 0.1, 0.3, 0.5, 0.75, 1 or 1.5 m*M*. The pellet harvested after culture at different concentration of IPTG described above was analyzed by running on SDS-PAGE, and the optimal condition for protein expression was determined.

Purification of α-β1-β2-ε Proteins

Nickel affinity chromatography followed by size-exclusion chromatography was implemented for purification of the His-tagged toxins. The entire-bacterial-protein supernatants were filtered by 0.22-μm filters and then applied to a HiTrap™ Chelating HP 5-mL column prepacked with a pre-charged Ni^{2+} column that was equilibrated with buffer 1 (20 m*M* Tris, 150 m*M* NaCl, water (solvent) and pH 8.0). Nickel affinity chromatography was performed using Fast Performance Liquid Chromatography (ÄKTA FPLC, GE Healthcare). After the protein was loaded onto the Ni^{2+}-chelating affinity column, it was washed with 10 bed volumes of buffer 1 and 10 bed volumes of buffer 2 (20 m*M* Tris, 150 m*M* NaCl, 50 m*M* imidazole, water (solvent) and pH 8.0). The protein peak was monitored on ÄKTA FPLC. The recombinant protein on Ni-NTA was then eluted with buffer 3 (20 m*M* Tris, 150 m*M* NaCl, 300 m*M* imidazole, water (solvent) and pH 8.0). The eluted samples of the target protein peak were collected using ÄKTA FPLC and used as target protein samples after nickel affinity chromatography. The target protein samples were applied to Superdex 200-pg column for further purification. Buffer 1 was used as the running buffer for the column. Most imidazole was removed from the target protein samples after size-exclusion chromatography. The purified target protein samples were collected from the column by monitoring the protein elution profile. The protein (soluble target protein) concentration in the purified target protein samples was quantitatively acquired by NanoDrop™ 2000 UV-Vis Spectrophotometer (ND 2000). The protein concentration in the all-bacterial-protein fluid was also obtained by using ND 2000 and thus we determined the total bacterial protein concentration.

Preparation of the *C. perfringens* Vaccine

A α-β1-β2-ε protein solution (with concentration of 1000 μg/mL) was obtained for immunization test by dissolving the purified α-β1-β2-ε protein in sterile PBS. The α-β1-β2-ε protein solution was mixed with complete Freund's adjuvant (volume ratio 1:1) and followed by the process of emulsification. The resultant oil-emulsion vaccine was used as the first vaccine candidate. The resultant oil-emulsion vaccine, obtained by mixing the α-β1-β2-ε protein solution with incomplete Freund's adjuvant (1:1) followed by emulsification, was used as the second vaccine candidate. The control group (PBS) was also used for immunization with complete or incomplete Freund's adjuvant (volume ratio 1:1) followed by emulsification, was used as the first or the second vaccine candidate.

Recovery and Culture of *C. perfringens* Strains

The *C. perfringens* virulent strains used in this study, *i.e.*, type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), were provided by China Institute of Veterinary Drug Control. The anaerobic beef-liver broth (400 μL) was used to triturate the cells contained in the ampules, resulting in homogenized suspensions of the frozen bacteria. The bacterial suspensions were then added to the anaerobic beef-liver broth (containing beef extract peptone) at the volume ratio of 1:100. The ampules were sealed with 1 to 2-centimeter-thick liquid paraffin to prevent the contact with air. The prepared ampules of bacteria were placed in an anaerobic chamber and incubated at 37°C for 16–24 h. The growth of the bacteria was examined thereafter. After the process of smearing, staining, and microscopic examination and after one to two generations of propagation, the recovered *C. perfringens* was then ready for use. Fractions of the recovered *C. perfringens* were stored in 30% (v/v) glycerol-saline solution at -80°C.

***C. perfringens* Toxin Challenge**

The effect of α-β1-β2-ε proteins was examined against *C. perfringens* strains type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), respectively. One hundred female Kunming mice (18–22 g in weight) were randomly assigned to two groups: one group with 80 mice for toxin challenge and the other group with 20 control mice for PBS reference. The first-time, second-time and third-time immunizations were conducted on the mice from the toxin-challenge group. The first-time immunization was done with the first vaccine candidate. The second and third-time immunizations were done with the second vaccine candidate. The immunized mice were administered with the dose level of 0.2 mL per mouse (α-β1-β2-ε protein 100 μg per mouse) each time. The control mice from PBS− reference group were injected with PBS in the presence of complete or incomplete Freund's adjuvant (volume ratio 1:1) with the level of 0.2 mL per mouse for the three times of immunization. Blood was collected from the mice before the first-time immunization. Serum was isolated from the collected blood for use as negative control. The second-time immunization was conducted 14 days after the first-time immunization, and third-time immunization was performed 14 days after the second-time immunization. All mice from

both groups were injected with varied types of *C. perfringens* two weeks after the third-time immunization, respectively. The specific level of injection is: 1.5×10^9 cfu for type A (C57-10), 2×10^9 cfu for type B (C58-5), 1.5×10^8 cfu for type C (C59-4), and 1.8×10^9 cfu for type D (C60-11).

Measurement of Antibody Level in the Immunized Mice

Twenty 49-day-old female Kunming mice were immunized with α-β1-β2-ε protein, and titer of antibody against the target protein was detected at 7 days post-immunization, and afterward it was continuously monitored for 84 days with an interval of about 7 days. Serum was isolated from the collected blood and was stored at -80°C for observation of the antibody level variation. Antibody titer was measured by indirect ELISA examination. The specific procedure of indirect ELISA is described in the followings. The ELISA plate was coated with the purified α-β1-β2-ε proteins. The concentration of the coating protein and the dilution of the serum were optimized based on checkerboard square matrix titration. The optimized concentration of the BSA blocking reagent was determined by applying BSA of varied concentrations. The working concentration and reaction time of the HRP-conjugated IgG were also optimized. The criterion: the optimized reaction condition corresponds to the highest P/N value (OD_{450} ratio of the positive to negative controls). Based on the described indirect ELISA procedure, the antibody titer in the serum of the mice was measured for the period of 0–12 weeks after the first-time immunization.

Ethical Approval and Informed Consent

The animal experiments were conducted in accordance with the guidelines of the China Animal Disease Control Center (CADC) on the Review of Welfare and Ethics of Laboratory Animals approved by the China Animal Disease Control Center (CADC) Administration Office of Laboratory Animals (CADCAOLA). All the animal procedures were conducted under the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of China Animal Disease Control Center (CADC). All methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all individual participants included in the study.

Results

Analysis and Identification of the Protein Expression

The soluble α-β1-β2-ε target protein (140 kD) was observed in the all bacterial-protein fluid and supernatant of pET32a-α-β1-β2-ε induced with IPTG. Insoluble fraction was not found in protein sample isolated from strain pET32a-α-β1-β2-ε upon induction, as demonstrated in Fig. 1.

Purification of α-β1-β2- ε Proteins

It was shown that the α-β1-β2-ε target proteins based on pET32a-α-β1-β2-ε were mostly soluble and existed in the broken-cell supernatant fluid. The control band of the soluble expression of the protein was clear. The amount of impurities in the supernatant was relatively low. With optimization of the nickel affinity chromatography procedure (ÄKTA FPLC), a higher level of purity was achieved in the soluble target protein band. Most imidazole was removed from the target protein samples after further purification with size-exclusion chromatography. The purified soluble protein was employed in the preparation of diagnostic antigen, monoclonal antibody, or genetically engineered subunit vaccine.

The results showed that the amount of the expressed α-β1-β2-ε protein gradually increased with time as the pET32a-α-β1-β2-ε strain cultures were induced at 37°C for 1–4 h. There was a drop of the amount of the expressed protein when induced for 5 h. The amount of the expressed α-β1-β2-ε protein increased in dependent of the decrease of temperature. The maximal amount of α-β1-β2-ε protein reached under the condition of 16°C for 16 h. There was a slight decrease in the expressed α-β1-β2-ε protein while the duration was extended to 24 h. Therefore, it was experimentally confirmed that the optimal condition for the induced expression on pET32a-α-β1-β2-ε was 16°C for 16 h.

Optimization of IPTG Concentration

The results showed that the amount of the expressed protein varied with the IPTG concentration. The amount of the expressed α-β1-β2-ε protein was proportional to the IPTG concentration when it varied between 0.1 – 1.5 m*M*. There was a decrease in the amount of the expressed protein when the IPTG concentration was 1.5 m*M*. This might be due to toxicity of IPTG. As such, the optimal IPTG concentration was determined as 1 m*M* for the induced expression.

Result of Toxin Challenge Test

The toxin-challenge result indicated that certain levels of protection were developed in the α-β1-β2-ε immunized mice against *C. perfringens* type A to D, respectively. The survival rate of the immunized mice during the 7-day challenge period was 100% in the cases of *C. perfringens* types A, B, C and D. All mice in the PBS-reference groups died. A chart is provided in Table 1 demonstrating the survival rate in the immunized group under four types toxin challenge.

The Level of the Antibody Induced Against the Candidate Vaccine

The purified α-β1-β2-ε protein was used as the diagnostic antigen coated to the ELISA plate. Measurement was done

for the immune antigen of the mice or the antibody titer in the serum after the toxin challenge. It was found that remarkable sensitivity and specificity can be achieved in the measurement by employing the α-β1-β2-ε protein as the diagnostic antigen. The maximum P/N value was achieved with 5 μg/mL of α-β1-β2-ε protein and serum dilution being 1:200. Therefore, it was determined that the optimal coating-antigen concentration was 5 μg/mL and the optimal serum dilution was 1:200. By setting the concentration of HRP-conjugated goat anti-mouse IgG as 1:20000 at 37°C for 1 hour, an optimal OD_{450} value was achieved 8 min after the addition of TMB solution. The antibody titer in the serum of 20 mice, which were in a separate group, intended only for monitoring the kinetic of immune response, was measured for a 12-week period after immunization. A remarkable rise in the level of antibody titer was observed in the mice immunized with the α-β1-β2-ε fusion toxin protein. Specifically, the level of antibody titer rapidly increased 7 days after immunization, and reached the peak 6–7 weeks after immunization. The highest level was kept until a slight decrease was noticed 8 weeks after immunization, as demonstrated in Fig. 2.

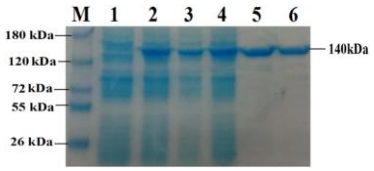

Fig. 1: SDS-PAGE analysis of the purified protein
M: Protein Marker (molecular weight, up to down: 180 KD, 120 KD, 72 KD, 55 KD, 26 KD); Lane 1: Protein sample isolated from strain pET32a upon induction (negative control); 2:Protein sample isolated from strain pET32a-α-β1-β2-ε upon induction (soluble and insoluble fraction); 3:Protein sample isolated from strain pET32a-α-β1-β2-ε conducted with 1 m*M* of IPTG at 37 °C and incubated for 4 hours (soluble fraction); 4: Protein sample isolated from strain pET32a-α-β1-β2-ε conducted with 1 m*M* of IPTG at 16°C and incubated for 16 hours (soluble fraction); 5: α-β1-β2-ε protein (soluble fraction) purified by nickel column; 6: α-β1-β2-ε protein (soluble fraction) purified by column molecular sieve

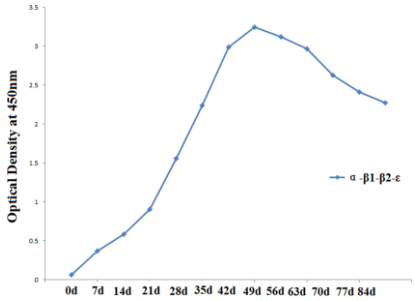

Fig. 2: Kinetic titration of antibody of the immunized mice
Twenty 56-day-old female BALB/C mice were immunized with α-β1-β2-ε protein, and titer of antibody against the target protein was detected at 7 days post-immunization, and afterward it was continuously monitored for 84 days with an interval of about 7 days

Discussion

In this study, we reported the construction of PET32a-α-β1-β2-ε expressing the multi-toxin fusion protein of *C. perfringens*. We demonstrated the efficient expression and purification of the soluble target protein, after a series of efforts, including codon optimization, removing signal peptide and lethal gene, selecting sequences of higher hydrophilicity and antigenicity, as well as optimizing the expression conditions. Dramatically increased level of antibody was detected in the serum of mice immunized with the expressed protein. The survival rate of the immunized mice was 100% challenged with type A, B, C and D of *C. perfringens*, suggesting the fusion protein could potentially be used as an ideal antigen for development of vaccine against *C. perfringens*.

The expression and purification approaches for major *C. perfringens* exotoxin proteins are accompanied with relatively complex procedures. The expression products are usually in the form of insoluble inclusion bodies. So far expression of soluble protein has rarely been found in literatures. The construction of soluble-vector expression and the optimization for efficient expression of soluble protein was a key topic in this field. Although great efforts were made by researchers on expression and purification of major *C. perfringens* exotoxin proteins, all toxin proteins reported are inactive inclusion bodies (Mathur *et al*., 2010; Pilehchian *et al*., 2013; Gong *et al*., 2015). Highly efficient expression and purification of soluble and bioactive exotoxin proteins have not been reported. Lobato *et al*. (2010) tried to fuse the *C. perfringens* epsilon and beta toxin genes, and partly obtained the expression of fusion proteins with similar biological activity of the native proteins. The detoxified epsilon-beta fusion toxoid protected up to 90% of immunized mice against experimental challenge. Zeng *et al*. (2011) prepared vaccines by adding adjuvant to a number of recombinant fusion toxins that were inactivated with formaldehyde. These developed vaccines, together with goat (sheep) pox vaccines, were employed to immunize goats (sheep). The results confirmed that the immunized animals were protected against both goat (sheep) pox and enterotoxaemia (Chandran *et al*., 2010; Zeng *et al*., 2011). Pilehchian *et al*. cloned epsilon-toxin gene and transformed the recombinant plasmid into E. *coli* competent cells (Pilehchian *et al*., 2013). The recombinant epsilon toxin was inactivated, mixed with adjuvant, and then employed to immunize livestock ruminants. Increase of the antibody titer was observed in the animals after boosting immunization. It was measured that the recombinant toxin was able to provide a certain level of immune protection against *C. perfringens* (Mathur *et al*., 2010; Pilehchian *et al*., 2013). Gong *et al*. constructed a recombinant plasmid encoding the fusion genes of α-, β2- and ε-toxins. However, the α-β1-β2-ε proteins expressed with the recombinant plasmids were inclusion bodies. In addition, they did not test the immunogenicity of the recombinant protein in animal experiment (Gong *et al*., 2015).

The expression and purification approaches for major *C. perfringens* exotoxin proteins are accompanied with relatively complex procedures. In this study, an efficient

Table 1: The survival rate after challenge

Strain type for challenge	Number of mice in immunization group	Number of mice in control group	Survival rate (survived/immunized)	
			Immunization group	Control group
A	40	10	100% (40/40)	0% (0/10)
B	40	10	100% (40/40)	0% (0/10)
C	40	10	100% (40/40)	0% (0/10)
D	40	10	100% (40/40)	0% (0/10)

expression of soluble antigen in E. *coli* was successfully achieved through a series of efforts, *e.g*., conducting codon optimization, removing signal peptide and optimizing the expression conditions. We constructed the expression vector PET32a-α-β1-β2-ε for the multi-toxin fusion protein. Efficient expression and purification of the soluble target protein was successfully achieved. The expressed multi-toxin fusion protein exhibited remarkable immunogenicity. The success in efficient expression of active proteins in E. *coli* provides a solid foundation for further development of genetically engineered subunit vaccines. The genetically engineered subunit vaccine candidate described in this study has advantages, such as high safety, purity, stability, and high yield. In the meanwhile, multiple types of toxins can be combined together through genetic engineering to construct new fusion toxin proteins for vaccine development (Langroudi *et al*., 2011; Pilehchian *et al*., 2013).

Conclusion

We successfully obtained a soluble fusion protein of α-β1-β2-ε-toxin for the first time with efficient expression in E. *coli*. The expressed multi-toxin fusion protein exhibited remarkable immunogenicity. Survival rate of the immunized mice during the 7 day challenge period was 100% in the cases of *C. perfringens* types A, B, C and D. The expressed protein can be further utilized in the development of genetically engineered trivalent subunit vaccines against animal diseases caused by *C. perfringens* infections, *e.g*., braxy, ovine enterotoxemia, lamb dysenterygas and sheep struck. It may have promising application potentials and prospects.

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