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Protective Properties of Nontoxic Recombinant Exotoxin A (Domain I-II) Against *Pseudomonas aeruginosa* Infection

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Background: Antibiotic resistance and the need for long-term treatments especially for chronic infections necessitate the development of a vaccine against *Pseudomonas aeruginosa* infection.
Objectives: In this study, recombinant exotoxin A (domains I and II), (ExoA I-II) protein was expressed, purified and its immunological characteristics were evaluated in a mouse model.
Materials and Methods: The genomic DNA was extracted from *P. aeruginosa* strain PAO1. The DNA encoding for domains I and II of exotoxin A was amplified by PCR and cloned into the pET22b expression vector. The construct was then transformed into *E. coli* BL21 and the protein expression was evaluated by the SDS-PAGE method. The Ni-NTA affinity chromatography was used for recombinant protein purification. Mice were then immunized subcutaneously on day 0, 21, 42 and 72 with exotoxin A (Domains I, II). Antibody production was evaluated by the ELISA method. The immunized and control group mice were exposed to an approximate $2 \times LD50$ (7.5×10^7 CFU) of clinical strain of mucoid *P. aeruginosa*.
Results: Sequencing of the cloned gene showed that the sequence of ExoA I-II gene was in accordance with ExoA I-II from *P. aeruginosa* PAO1. SDS-PAGE analysis indicated the expression of recombinant protein with a molecular weight of 45 KDa. Vaccination with ExoA I-II produced a significant amount of specific IgG antibodies in mice. Also immunization of mice with ExoA I-II increased survival times against intra-peritoneal challenge with an approximate 7.5×10^7 CFU ($2 \times LD50$) of clinical strain of *P. aeruginosa*.
Conclusions: Results of this study suggested that recombinant ExoA I-II is a highly immunogenic protein which can be used as a new vaccine candidate against *P. aeruginosa*.

Keywords: Exotoxin A; *Pseudomonas Aeruginosa*; Recombinant Vaccine; Vaccine Candidate

1. Background

Pseudomonas aeuginosa (*P. aeruginosa*) is an important pathogen in immunocompromised patients, such as patients suffering from acute immune deficiency (AIDS), cancer, burn wounds and cystic fibrosis (CF). Infections caused by *P. aeruginosa* are often difficult to eradicate because it requires minimal nutrition and can tolerate a wide range of temperatures (1). This bacterium is commonly isolated from nosocomial infections, especially from ventilator-associated pneumonia. *P. aeruginosa* is also a major cause of pulmonary infection in cystic fibrosis patients and bacteremia due to this bacterium has high mortality (2, 3).

On the other hand, *P. aeruginosa* has the ability to easily acquire antimicrobial resistance. Emergence of multiple drug resistant *P. aeruginosa* has become asignificant problem in clinical settings due to limited therapeutic options (4, 5). Therefore, alternative ways to treat and prevent *Pseudomonas* infections are necessary. Different approaches have been used for the development of vaccines against *P. aeruginosa* infections (6). Various antigenic and virulence factors, including lipopolysaccharide, flagellin, Pili, polysaccharides with high molecular weight, alginate and outer membrane proteins have been investigated as vaccine candidates (7-10), but early trials produced disappointing results.

Exotoxin A is a major virulence factor that is produced by most clinical strains of *Pseudomonas aeruginosa*. Exotoxin A is a potent cytotoxic agent that is lethal for a variety of animals, including subhuman primates. It has been shown that exotoxin A deficient mutants exhibita virulence 20 times less than the wild type strain in the mouse models (11). The mechanism of exotoxin A activity in mammalian cells has been studied in detail. The toxin binds and enters into the cells by receptor-mediated

Implication for health policy/practice/research/medical education:
This study has implications on development of vaccines against *P. aeruginosa* infections.

endocytosis and then translocates to the cell cytoplasm, where it inactivates elongation factor 2 (EF-2) and inhibits protein synthesis. Exotoxin A also has the ability to inhibit host response to infection. In adult mice, the 50% mean lethal dose (LD50) of intravenously injected exotoxin A was approximately 1 μg. mouse $^{-1}$. The role of exotoxin A in *P. aeruginosa* infection of burn wound has been examined in the burned mouse model, which closely resembles *P. aeruginosa* infection in human burns. It was shown that the survival of burned mice infected with 2xLD50 doses of a toxigenic strain of *P. aeruginosa* (PA103) was enhanced upon the intravenous injection of anti-exotoxin A serum. In addition, anti-exotoxin A serum reduced the number of viable *P. aeruginosa* microorganisms within the blood and livers of burned/infected mice (12). These findings suggest that exotoxin A is an important antigenic protein that has been considered as a promising vaccine candidate for pseudomonas infections. Exotoxin A consists of three domains: domain I (1-252aa, 365-404aa), domain II (253-364aa), and domain III (405-613aa), which is a toxic moiety (11). Domain I is responsible for attachment of Exo A to the cells and domain II is a transporter that enters exotoxin in to the cells.

2. Objectives

In this study, we reported on the expression and purification of ExoA I-II as a nontoxic antigen. We also evaluated the protective activity of anti-ExoA I-II antibodies against *P. aeruginosa* infection.

3. Materials and Methods

3.1. Cloning, Expression and Purification of Nontoxic ExoA I-II

Genomic DNA of *P. aeruginosa* strain PAO1 was extracted by phenol-chloroform extraction and ethanol precipitation method as described elsewhere (13). The ExoA I-II gene was amplified by polymerase chain reaction (PCR) using specific forward, 5'-GGATCCCCGAGGAAGCCTTCGAC-3' and reverse 5'-GCCGTCGCCGAGGAACTCCTCGAG-3' primers containing*BamhI* and *XhoI* restriction sites, respectively. The amplification conditions were as follows; initial denaturation at 94°C (4min) and 31 cycles consisting of 94 ° C (1 min), 65 ° C (1 min), 72 ° C (1 min) and a final extension at 72 ° C (5 min). PCR product of ExoA-I,II was gel purified by a purification kit (Macherey Nagel) and analyzed by electrophoresis.

Cloning of the ExoA-I, II gene was carried out by ligation of the PCR product into the PTZ57R vector using T-A cloning method according to the manufacturer's instructions (Fermentas). The ligate was then transformed into the *E. coli* DH5α and screening was performed by PCR and restriction analysis. Positive clones of PTZ-ExoA I-II were sequenced (MWG-Germany) for analysis of the sequence integrity.

For recombinant expression of ExoA I-II in *E. coli*, the insert was removed from pTZ-ExoA vector by digestion with *BamHI* and *XhoI* enzymes and sub-cloned into the pET22b expression vector. Then, the pET22b-ExoA construct was transformed into *E. coli* BL21, and the protein expression was assessed using 12% (w/v) sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE). An unstained protein molecular weight marker (14.4 kDa to 116 kDa) was used for monitoring the progress of SDS-PAGE. Ni-NTA affinity chromatography method (Qiagen, Chatsworth, CA, USA) was used for His tagged fusion protein purification. The purified recombinant protein was dialyzed against PBS, pH 7.4 for removal of imidazole. SDS-PAGE was used to analyze the purity of the protein and product concentration was assessed by Bradford method with bovine serum albumin as the standard (Sigma, Product Number B6916).

Western blotting was used to evaluate the immunological properties of recombinant ExoA I-II protein. Briefly, the recombinant protein was separated on SDS-PAGE gel using pre-stained protein ladder molecular weight marker (10 KDa to 170 KDa) for monitoring the progress of SDS-PAGE and was then transferred to PVDF membranes by semi-dry blotting. Next, the reactivity of recombinant protein with antibody to *P. aeruginosa* native exoA (Sigma Product Number P2318) was analyzed. HRP-conjugated anti-rabbit IgG and DAB substrate were used for reaction detection. Finally, lipopolysaccharide (LPS) contamination of purified recombinant protein was assessed by Limulus amebocyte lysate (LAL) assay method.

3.2. Active Immunization of Mice

6-8 weeks old female BALB/c mice were purchased from the Pasture Institute (Tehran-Iran). All animals were housed under pathogen-free conditions at the Tabriz University of Medical Sciences animal house (Tabriz-Iran). Two groups of 6-8 week old female BALB/c mice (6 mice per group) were subcutaneously immunized with 20μg of recombinant ExoA I-II with complete Freund's adjuvant (test group) or PBS (control group) on days 0, 21 and 42 with incomplete Freund's adjuvant and day 72 without adjuvant. This research was approved by the Research Ethics Committee ofthe Faculty of Medical sciences, Tarbiat Modares University.

3.3. Bleeding From Animals

One week after the last injection, sera were collected from mice in test and control groups, and stored at -20 °C until analysis. Housing of one mouse from the test group and one mouse from the control group was continued for a further four months. Four months after the last injection, these mice were bled to check decreasing or stability of anti-exotoxin A level. After bleeding, 20 μg of recombinant ExoA I-II (without adjuvant), as a booster, was injected

to the test group and equal volumes of PBS to the control group mice, and 1 week after this injection, both mice were bled again. The sera were stored at -20 °C until analysis.

3.4. Enzyme-Linked Immunosorbent Assay (ELISA)

ExoA I-II specific IgG antibodies in sera were determined using an indirect ELISA. The 96 well microtiter plate were coated with ExoA I-II at a concentration of 5 μg.ml $^{-1}$ in 0.1 M carbonate/bicarbonate, pH 9.6 and incubated for 45 min at 37 °C. Plates were washed two times with 250 μL of wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked with PBS-T (2% bovine serum albumin in PBS containing 0.05% Tween 20) for 45 min at 37 °C. Plates were washed two times with 250 μL of wash buffer. Sera samples diluted to 1:10000 were added (100 μL per well) and incubated for 45 min at 37 °C. Plates were washed five times with 250 μL of wash buffer; followed by incubation with peroxidase conjugated anti-mouse antibody diluted to 1:5000 as a secondary antibody, for 45 min at 37 °C. Plates were washed five times with 250 μL of wash buffer. Peroxidase activity was detected with 3, 3´, 5, 5´-tetramethyl-benzidine (TMB), after 15 min of incubation in the dark, stopped with 1 M H_2SO_4 and the absorbance was measured at 450 nm.

3.5. Protection Assay

At first, 24 female BALB/c mice were divided into five groups (6 mice per group) for determination of bacterial infection LD 50. Mice were intraperitoneally injected with different dilutions of the clinical strain of mucoid *P. aeruginosa* in PBS (5×10^7, 7.5×10^7, 1×10^8, and 12.5×10^8 CFU). The mice were followed for 10 days, mortality was recorded, and LD50 was determined according to the Reed and Muench method (14). Two weeks after the final immunization, the immunized and control groups' mice were challenged with intraperitoneal injection of 7.5×10^7 CFU clinical strain of mucoid *P. aeruginosa*. The survival rate was recorded for 10 days.

3.6. Statistical Analysis

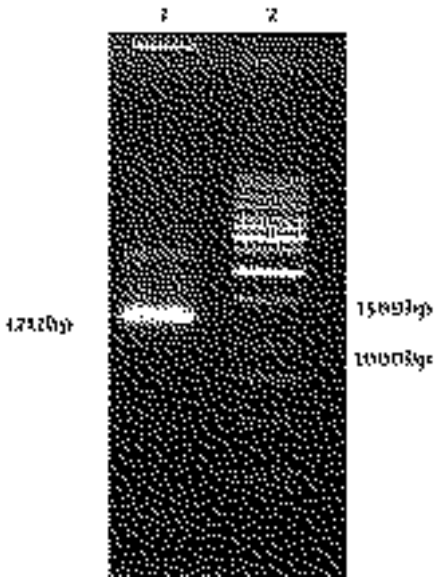
Statistical analysis was performed using the Stata software. The differences between the anti-ExoA I-II specific IgG-test group and control group were analyzed by student's t-test. Differences were considered to be statistically significant with a P-value of $P \leq 0.05$.

4. Results

4.1. Cloning, Expression, Purification and Characterization of ExoA I-II

Amplification of ExoA I-II from *P. aeruginosa* PAo1 resulted in a PCR product of 1212 bp that was in accordance with the expected product size (Figure 1).

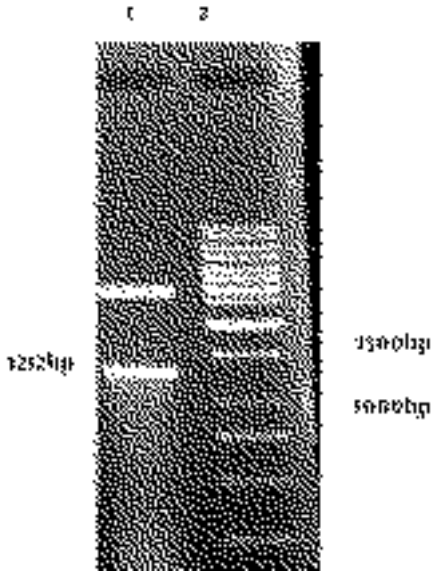
Figure 1. Electrophoresis of PCR Product on Agarose Gel (1%)



Lane 1: ExoA I-II, Lane 2: 1kb DNA size Marker.

Sub-cloning of theinsert (ExoA I-II) into the expression vector, pET-22b wasverified by digestion with *BamhI* and *XhoI* restriction enzymes (Figure 2) and the integrityof ExoA I-II in the construct was confirmed by DNA sequencing.

Figure 2. Electrophoresis of Recombinant Vector pET22b-ExoA I-II Digested by *BamhI* and *XhoI*

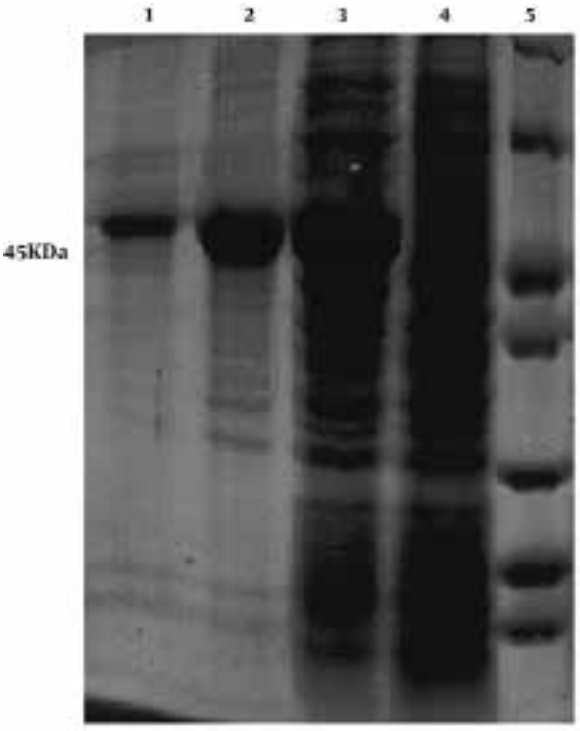


Lane1: pET22b-ExoA I-II digested with *BamhI* and Xho I, Lane 2: 1kb DNA size marker.

Analysis for expression of recombinant ExoA I-II-(His) 6-tag protein revealed that the protein was highly ex-

pressed in *E. coli*, which appeared as a 45 KDa protein in SDS-PAGE. Both supernatant and the pellet of cell lysates were tested for the presence of recombinant protein. The majority of the expressed protein was detected in inclusion bodies. Figure 3 shows the commassie blue-stained SDS-PAGE gels of *E. coli* lysates before and after induction, and the purified recombinant ExoA I-II protein.

Figure 3. SDS-PAGE (12%) Analysis of the Expression of ExoA I-II in *E. coli*



Lane 1, 2: purified recombinant protein, 3: pellet of IPTG induced bacteria, Lane 4: pellet of un-induced bacteria, Lane 5: standard protein size marker (KDa)

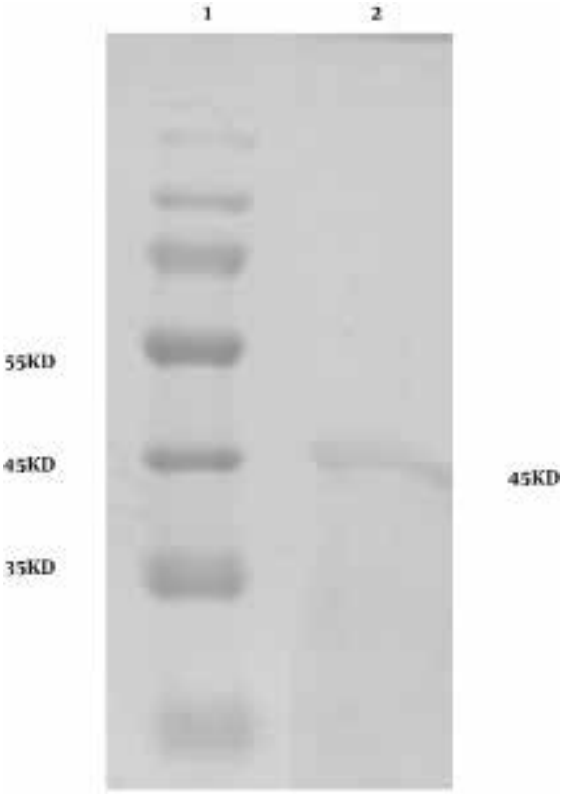
Western blot analysis of the purified protein showed that ExoA I-II could be recognized by an antibody against native Exotoxin A antibody (Figure 4).

Analysis for reactivity of recombinant protein with sera from patients with *P. aeruginosa* infections showed that all sera contained antibody against exotoxin A and highly reacted with purified recombinant ExoA I-II protein (Figure 5).

4.2. Antibody Responses

Monitoring of antibody production in immunized and control groupmice after four doses of immunization with recombinant ExoA I-II protein showed significant ($P < 0.0001$) amounts of specific antibody production (Table 1). No specific antibodies were detected in mice in the negative control group that were injected with PBS.

Figure 4. Western Blotting Analysis of the Recombinant ExoA I-II Protein Probed with Polyclonal Anti-Exototina (sigma)



Lane 1: pre-stained protein size marker (KDa), Lane2: ExoA I-II

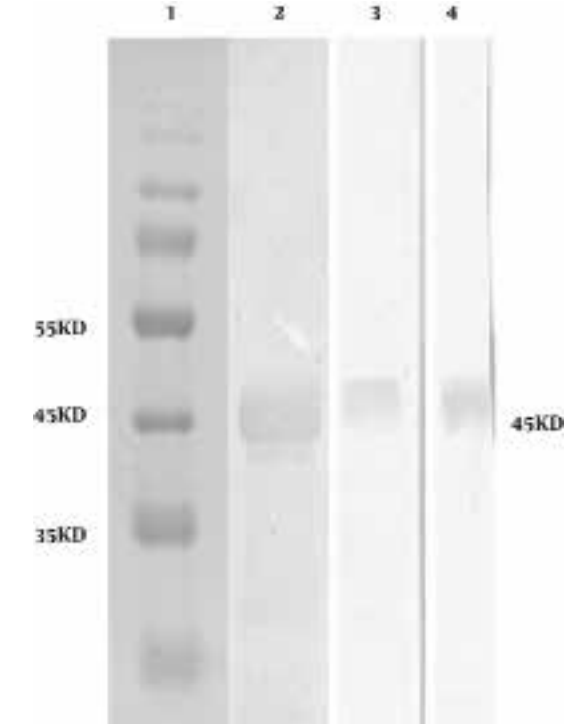
4.3. Protection Assay Results

Analysis of survival rate from bacterial challenge test of immunized and control group mice showed a partial protection, and compared to control group, 40% of mice immunized with recombinant ExoA I-II survived after a challenge with 7.5 X 10^{7} CFU (2XLD50) of clinical strain of mucoid *P. aeruginosa* (survival rate 40%) whereas 80% mice in control groups died one day after the challenge (Figure 6).

Table 1. Antibody Responses of Mice Immunized with Recombinant ExoA I-II Protein of *P. aeruginosa* in Sera Diluted to 1:10000

Bleeding Time	ODA45
1 week after the last injection	1.255
four months after the last injection	
Before booster injection	0.319
1 week after a booster injection	0.625

Figure 5. Western-Blotingof the Recombinant ExoA I-II Protein Probed with the Sera from Patients with *Pseudomonas aeruginosa Infection*



Lane1: re-stained protein size marker and Lanes 2-4, reaction of purified recombinant fusion protein with the sera from patients with *Pseudomonas aeruginosa* infection.

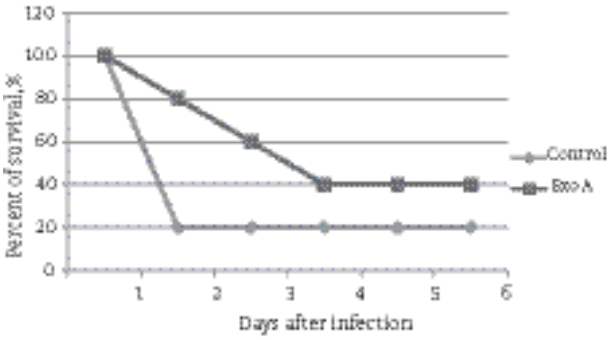


Figure 6. Survival Rate of Immunized and Control Group Mice after Challenge with 7.5×10^7 CFU Clinical Strain of *P. aeruginosa*

5. Discussion

P.aeruginosa is an aerobic, gram-negative opportunistic pathogen, responsible for nosocomial infections especially in Intensive Care Units (ICU) and burn wards. Infections caused by *P. aeruginosa* are usually difficult to eradicate because of its diverse virulence factors, inherent resistance to many antibiotics and ability to acquire resistance to different antibiotics. Thus, an effective immune prophylactic/therapy method is desirable to supplement conventional antibiotic therapy for treatment of *P. aeruginosa* infections (15). Exotoxin A secreted by *P. aeruginosa* is a toxic pathogenic factor, which inhibits protein synthesis by host cells. Exotoxin A is extremely lethal, causes histopathological changes within the liver, induces apoptosis of the hepatocytes and enhances the expression of different pro-inflammatory cytokines (12). Research has shown that these damaging effects are neutralized in the presence of anti-exotoxin A antibodies that are essential for protection against *P. aeruginosa* infections (12, 16, 17). Native exotoxin A is toxic and its modification into toxoid form by different methods and its immunologic properties have been reported in several *in vitro* and *in vivo* studies (7, 12, 16, 17). In this research, we reported a new recombinant nontoxic form of exotoxin A. We constructed a nontoxic exotoxin A (ExoA I-II) by deletion of the enzymatic domain from the carboxyl terminus of toxin. This nontoxic exotoxin A retains most of its antigenicity and can induce protective immunity. Some studies have used the native Exotoxin A purified from bacterial culture for investigation but it has been shown that purification of native exotoxin A from culture medium is very difficult and proteases secreted into the culture medium decreases the yield of toxin (18). Therefore, production of recombinant ExoA I-II is preferred for preparation of ExoA I-II with high quality and quantity. In this study ExoA I-II gene was isolated from *P. aeruginosa* PAO1, then cloned in to the pET22b vector and overexpressed in *E. coli* as inclusion bodies aggregates. pET22b vector carries six histidine residues in the c-terminal of the protein. The His-tag facilitates purification of recombinant protein by Ni^{2+}-sepharos resin. In our study, highly purified recombinant protein was obtained after purification. Purified recombinant ExoA I-II effectively refolded using dialysis against urea gradients and PBS without protein aggregation or precipitation.

Reaction of sera from patients infected with *P. aeruginosa* with recombinant ExoA I-II demonstrated the presence of a high titer of anti-exotoxin A antibody in all of these sera. This finding is consistent with the results reported by cross et al. (19). They reported, that, there are distinct differences in the titers of anti-exotoxin A antibody in sera obtained from dead and surviving patients after *P. aeruginosa* infection (19). These observations thus indicate that anti-exotoxin A antibody might be one of the most effective factors in protecting humans from life-threatening *P. aeruginosa* infection. Thus, to avoid the pathogenesis of *Pseudomonas* infection, development of a vaccine against exotoxin A might be an appropriate approach. These results also indicate that this non-toxic exotoxin A protein may be used as a serodiagnostic antigen for rapid diagnosis of *P. aeruginosa* infections. Analysis of sera from mice immunized with recombinant non-toxic exotoxin A revealed that the protein induced production of a significant level of IgG antibodies. We also found stability in the level of antibody in the immunized group for four

months, which was doubled by a booster injection (Table 1). This finding verified the highly immunologic property of non-toxic exotoxin A proteins. Several *in vitro* and *in vivo* studies showed that the antibodies induced by non-toxic exotoxin A can neutralize the exotoxin A cytotoxicity (7, 12). Therefore non-toxic exotoxin A can be used in active and passive immunization for neutralization of pathological effects of exotoxin A. The protective efficacy of *P. aeruginosa* exotoxin A is a subject of controversy. Manafi A et al. reported a 93.8% protection in burned mice following active immunization with a toxoid of exotoxin A (7). Denis-Mize and colleges also reported an increase in survival rate and protection properties in mice immunized by toxoid of exotoxin A (19). However, Palovskiset al. found that the survival rate did not increase significantly following active immunization with a toxoid of exotoxin A and infection with *P. aeruginosa* in burned mice (20), while Chen et al. reported less protection for burned mice immunized with nontoxic exotoxin A (15). In our results, recombinant ExoA I-II showed a protective efficacy of 40% against challenge with 7.5×10^{7} CFU (2XLD50) of clinical strain of mucoid *P. aeruginos* . Immunization with recombinant ExoA I-II also increased the survival time in immunized mice compared to mice in the control group (Figure 6). The variation in protection efficiency may be related to differences in the challenge strain and method, immunization doses and routes, infection site and etc, but the increase in survival time is a common finding that has been reported by most studies. Therefore, it is suggested that use of exotoxin A in combination with other antigens in vaccine preparations would be beneficial. In conclusion, the recombinant non-toxic exotoxin A protein described in the present study is a useful antigen to include in vaccine preparation for induction of an efficient immune response.

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Authors' Contributions

All author had participated equally in the present study.

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The authors declare that they have no competing interests to disclose.

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