Cloning, Expression and Characterization of Recombinant Exotoxin A-Flagellin Fusion Protein as a New Vaccine Candidate against *Pseudomonas aeruginosa* Infections

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ABSTRACT

Background: Infections due to *Pseudomonas aeruginosa* are among the leading causes of morbidity and mortality in patients who suffer from impaired immune responses and chronic diseases such as cystic fibrosis. At present, aggressive antibiotic therapy is the only choice for management of *P. aeruginosa* infections, but emergence of highly resistant strains necessitated the development of novel alternative therapeutics including an effective vaccine. Several *P. aeruginosa* antigens have been tested for vaccine development, including lipopolysaccharide alone, polysaccharides alginate, extracellular proteins, exotoxin A (exo A) and killed whole cell. However, none of them are currently available clinically. **Methods:** In this research, recombinant exoA-flagellin (fliC) fusion protein as a cocktail antigen was expressed and purified and its antigenic characteristics were evaluated. **Results:** Expression of recombinant fusion protein by *E. coli* using pET22b vector resulted in production of exoA-fliC fusion protein in high concentration. Based on Western-blotting results, recombinant fusion protein showed a good antigenic interaction with sera from patients with various *P. aeruginosa* infections. **Conclusion:** These results suggested that recombinant exoA-fliC fusion protein can be produced in the laboratory, and tested as a candidate vaccine in *P. aeruginosa* infections. *Iran. Biomed. J. 17 (1): 1-7, 2013*

INTRODUCTION

*P*seudomonas aeruginosa is a Gram-negative opportunistic pathogen that commonly infects patients with impaired immune response. *P. aeruginosa* is associated with severe infections in patients with burns, cystic fibrosis and subjects receiving immunosuppressive drugs. The bacterium is frequently isolated from wounds, sputum, blood and other clinical samples from nosocomial infections. *P. aeruginosa* septicemia has a high mortality especially in burn patients [1].

P. aeruginosa has several virulence factors that play important roles in the pathogenesis of the bacterium. Surface factors, such as pilli, flagellum and polysaccharide layer of lipopolysaccharide are involved in attachment and colonization of the bacterium. Proteins secreted by this bacterium also have an important role in the distribution and tissue damage [1, 2]. This bacterium has different chromosomal and plasmid-mediated resistance genes involved in the resistance of the bacterium to antimicrobial agents. Even newly developed antibiotics have failed to reduce the mortality rate associated with this organism. Low permeability of outer membrane and different efflux pumps are also among common mechanisms of drug resistance in *P. aeruginosa* [3]. Hence, due to high prevalence of antimicrobial resistance, the immunoprophylaxis and immunotherapy might be an effective method for treatment and control of *P. aeruginosa* infections. It has been shown that neutralization of bacterial virulence factors can result in prevention and reduction of mortalities due to *P. aeruginosa* infections [4].

For this purpose, different antigenic and virulence factors, such as outer membrane proteins, toxins, flagella, pilli and high molecular weight poly-

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Table 1. Primer sequences were used in amplification of exotoxin A (exoA) and flagellin (fliC) gene fragments.

ExoA gene	Forward	GGATCCCCGAGGAAGCCTTCGAC	*Bam* HI
	Reverse	GTGTTGACGGTCAAGGCCATGCCGTCGCCGAGGAACTC	
FliC gene	Forward	GAGTTCCTCGGCGACGGCATGGCCTTGACCGTCAACACCAAC	
	Reverse	GTTCAGCGACTCTGCGCTCATCTCCTCGAG	*Xho* I

saccharides have been evaluated as vaccine candidates [5-8]. *P. aeruginosa* exotoxin A (exoA) plays an important role in virulence of the bacterium and it has been shown that the exoA-deficient mutants exhibit virulence 20 times less than the wild type strain in the mouse models [9].

The mechanism of exoA activity in mammalian cells has been studied in detail [9-11]. The toxin binds and enters into the cells by receptor-mediated endocytosis and then translocates to the cell cytoplasm, where it inactivates elongation factor 2 and inhibits protein synthesis. Also, exoA has ability to inhibit the host response to infection [9]. ExoA is an antigenic protein that induces humoral immune responses in animal models. Based on the results of different studies, these antibodies are highly protective. Therefore, exoA has been considered as a promising vaccine candidate for pseudomonas infections [10-12].

The bacterial flagellum binds to epithelial cells via asialo-GM1 receptors and induces inflammatory responses through activation of kappa B nuclear factor by interaction with the toll-like receptor-5 and -2 [13]. The bacterial flagella are strong immunogenic factors and active or passive immunization with flagellar antigens induces antibody production. These antibodies inhibit bacterial distribution, thus prevent systemic infection in a mouse model of burn and pulmonary infections [13]. Studies have shown that monoclonal antibodies against *P. aeruginosa* flagellin (fliC) are very protective against *P. aeruginosa* infections in various animal models. Therefore, this antigen has been also suggested as a vaccine candidate for *P. aeruginosa* [14-16].

It has been demonstrated that immunization with a single antigen cannot induce protective immune response in an optimal level, whereas vaccines containing cocktail of different antigens are capable of generating higher level of immune responses. Different cocktails or fusion proteins have been attempted against pseudomonas infections, such as exoA-pillin [10], exoA-oprF-oprI [12], fliC$_{a,b}$-oprF-oprI [7], but there is no any report on preparation of exoA-fliC-combined vaccine for pseudomonas infection.

In the present study, we describe the preparation of recombinant exoA and fliC protein as a new vaccine candidate against pseudomonas infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* strains PAO1 and 8821M were used for DNA extraction and amplification of exoA and fliC genes, respectively. *E. coli* strains DH5α and BL21 (Novagen, USA), plasmids pTZ57R (Fermentas, Lithuania) and pET22b (Novagen, USA) were used in cloning and recombinant expression.

Primer design. Primers for exoA gene (domains I-II, the binding and translocator domains) were designed based on exoA gene sequence of *P. aeruginosa* PAO1. Primers for fliC were designed to amplify N-terminal 170 amino acids from fliC gene sequence of *P. aeruginosa* 8821M. Therefore, 1212 bp N-terminal nucleotides of exoA gene and 510 bp N-terminal nucleotides of fliC gene were selected for primer design (Table 1).

DNA extraction and PCR amplification of fliC and exoA genes. Genomic DNA was extracted with phenol-chloroform method as described elsewhere [17]. ExoA and fliC genes were amplified separately by PCR in 25-μl volumes. The amplification conditions for exoA were initial denaturation at 94°C (4 min) 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). The program for fliC were 94°C (4 min), 31 cycles consisting of 94°C (1 min), 58°C (1 min), 72°C (1 min) and an additional extension time at 72°C (5 min). PCR products related to fliC and exoA were gel purified separately by a purification kit (Macherey Nagel, Germany) and analyzed by electrophoresis.

Preparation and amplification of exoA-fliC fusion gene by overlapping PCR method. Overlapping PCR was used for construction of exoA-fliC fusion gene [18]. Briefly, 5 μl of exoA and fliC PCR products was annealed and used as template in a PCR reaction containing exoA forward and fliC reverse primers. The gene amplification conditions were 95°C (4 min), 30 cycles consisting of 94°C (1 min), 60°C (60 s), 72°C (1.5 min) and an additional extension time at 72°C (10 min). The PCR product was electrophoresed on 1% agarose gel and analyzed by gel document. The PCR
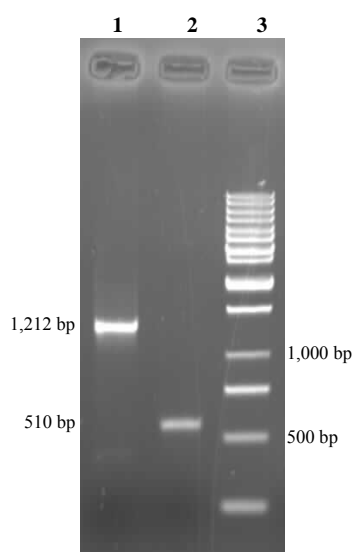
Fig. 1. Electrophoresis of PCR product on agarose gel (1%). Lane 1, exoA, lane 2, fliC and lane 3, DNA size marker (1 kb).

band related to fusion gene was cut and recovered by using a purification kit (MN, Macherey Nagel, Germany).

Cloning and sequencing. Cloning of fusion gene was carried out by ligation of PCR product into the pTZ57R vector using TA cloning method according to the manufacturer's instructions (Fermentas, Lithuania). The ligation was transformed into the *E. coli* DH5a, and screening was performed by PCR and restriction analysis. Positive clones of pTZ-exoA-fliC were sequenced (Eurofins MWG Operon, Germany) for analysis of the sequence integrity.

Subcloning and fusion protein expression. For recombinant expression of exoA-fliC fusion protein in *E. coli*, the insert was removed from pTZ-exoA-fliC vector by digestion with *Xho*I enzymes and subcloned into the pET22b expression vector. Then, the pET22b-exoA-fliC construct was transformed into *E. coli* BL21, cultured in LB broth containing ampicillin and incubated at 37°C and 15 rpm/min shaking. In an optical density equal to 0.5 (600 nm), the culture was induced by adding 1 mM IPTG and it was continued for further four hours. The cells were harvested by centrifugation at 6,000 ×g for 3 minutes and the protein expression was assessed using SDS-PAGE.

Preparation of cellular lysate and analysis of solubility of expressed protein. For expressed protein solubility analysis, *E. coli* BL21 containing pET22b-exoA-fliC vector was cultured in 10-ml LB broth supplemented with ampicillin. After 18 hours, it was transferred to 100-ml LB broth containing ampicillin and incubated at 37°C and 250 rpm shaking. In optical density eua = 0.5 (600 nm) 1 mM IPTG was added to

the culture and the incubation was continued for two hours in 37°C and 250 rpm shaking. Then, bacterial pellet was collected by centrifugation at 7000 ×g for 10 min, dissolved in 3 ml of lysis buffer and sonicated on ice (20 s, 80%, 10 periods). After cell lysis, it was centrifuged at 8000 ×g for 10 min and the supernatant and pellet were analyzed by SDS-PAGE for determining the solubility of expressed protein.

Protein purification from inclusion body. Ni-NTA affinity chromatography method (Qiagen, Chatsworth, CA, USA) was used for His-tagged fusion protein purification according to the manufacturer's instructions. Purification was performed under denaturing condition using 8 M urea for denaturation. The sonication pellet (inclusion body) was washed 3× with PBS + 1% Triton X-100 and dissolved in 4-6 ml of 8 M urea lysis buffer. The supernatant was then mixed with 2 ml resin, incubated at room temperature for 30 min and transferred to chromatography column. The contamination proteins were washed away from the column using wash buffer (20 mM sodium phosphate, 300 mM NaCl, 50 mM imidazol, pH 8.0). The protein was then collected from column by using elution buffer (20 mM sodium phosphate, 300 mM NaCl and 250 mM imidazol, pH 8.0). For removing imidazol, the purified recombinant protein was dialyzed against PBS, pH 7.4. The purity of protein was analyzed by SDS-PAGE and protein concentration was assessed by Bradford's method.

Western-blotting. Western-blotting was used to evaluate the immunological properties of recombinant exoA-fliC fusion protein. For this purpose, the recombinant protein was separated on SDS-PAGE gel and transferred to PVDF membranes by semi-dry blotting. Then, the reactivity of recombinant protein with antibody to *P. aeruginosa* native exoA (Sigma Product Number P2318) and sera (1:2000) from patients with *P. aeruginosa* infections were analyzed. The blood samples of patients with systemic and local *P. aeruginosa* infections were obtained from Sina Hospital (Tabriz). The *P. aeruginosa* infections in these patients were nosocomial and multi-resistant drug infections. HRP-conjugated anti-rabbit IgG (1:5000), HRP-conjugated anti-human IgG (1:5000) and DAB substrate were used for reaction detection.

RESULTS

PCR amplification of P. aeruginosa exoA and fliC genes. PCR amplification of exoA (domains I and II) gene from *P. aeruginosa* strain PAO1 and fliC gene from *P. aeruginosa* strain 8821M resulted in a PCR products of 1212 bp and 510 bp, respectively, that was in expected product sizes (Fig. 1).
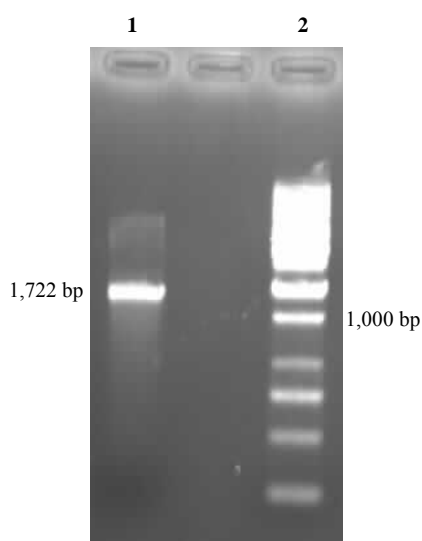
Fig. 2. Electrophoresis of overlapping PCR product on agarose gel (1%). Lane 1, exotoxinA-fliC and Lane 2, DNA size marker (1 kb).

Preparation of exoA-fliC fusion gene by overlapping PCR. PCR amplification of exoA-fliC fusion gene using exoA forward and fliC reverse primers resulted in a fragment of 1722 bp (Fig. 2). Sequencing fusion gene after cloning into pTZ57 vector confirmed the integrity and in-frame fusion of exoA-fliC genes.

Recombinant expression of exoA-flagellin fusion protein. For high-level expression of exoA-fliC fusion protein in *E. coli*, the insert was removed from pTZ-57 cloning vector and subcloned into the pET22b expression vector along with c-terminal 6-His tag (Fig. 3). Induction of *E. coli* BL21 with IPTG transformed with expression cassette resulted in a high level
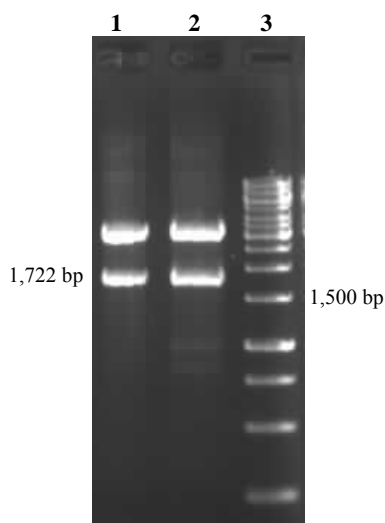

Fig. 3. Electrophoresis of digestion product. Lane 1 and 2, recombinant vector pET22b-exo-fliC digested whit *Bam*hI and *Xho*I; Lane 3, DNA size marker (1 kb).

expression of recombinant protein as appeared in SDS-PAGE analysis of the lysate of induced bacteria. Figure 4 shows the Coomassie blue stained SDS-PAGE gels of *E. coli* culture before and after induction. Recombinant exoA-fliC fusion protein was expressed as a 63-kDa protein in high concentration. Purification of His-tagged fusion protein by Ni-NTA affinity chromatography resulted in a highly pure protein which was appeared as a single band in SDS-PAGE analysis of purified protein (Fig. 4).

Western-blotting results. Western-blotting analysis showed that recombinant exoA-fliC was reacted with antibody against native exoA antibody (Fig. 5). This finding verified the correct conformational structure of recombinant protein produced in *E. coli*. Analysis for reactivity of recombinant fusion protein to sera from patients with *P. aeruginosa* infections showed that all sera contained antibody against exoA-fliC and they were highly reacted to the recombinant protein (Fig. 6).
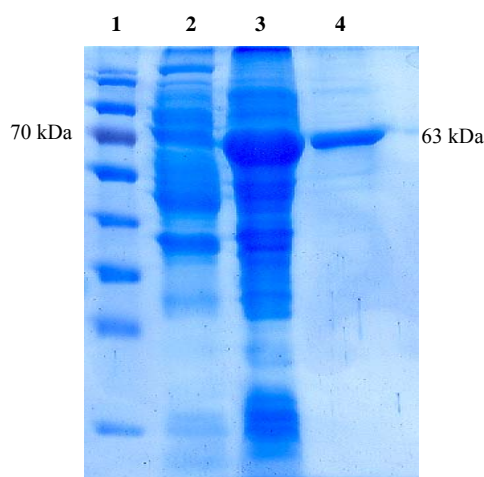

Fig. 4. Analysis of expressed and purified recombinant fusion protein on SDS-PAGE (12%). Lane 1, standard protein size marker; lane 2, pellet of un-induced bacteria; lane 3, pellet of IPTG-induced bacteria and lane 4, purified-recombinant fusion protein.

DISCUSSION

P. aeruginosa is the second-leading cause of nosocomial infections that is frequently resistant to most of commonly used antibiotics [19]. Therefore, the management of *P. aeruginosa* infections is a major problem in clinical practice. Nowadays, development of alternative methods for treatment of *P. aeruginosa* infections is very necessary [20]. Various new approaches have been investigated to combat pseudomonas infection. Among them, immunotherapy and vaccine development seem to be promising therapeutic
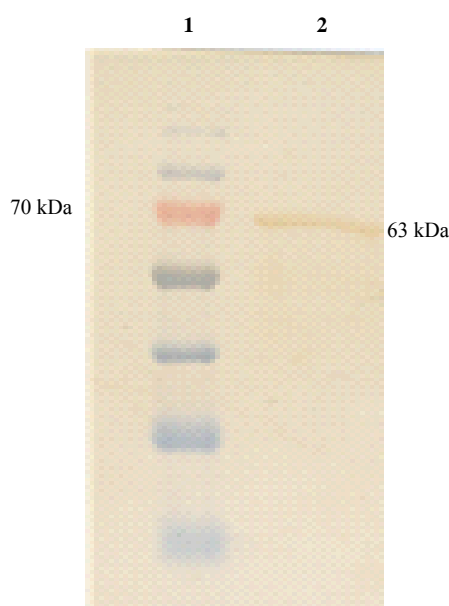
Fig. 5. Western-blot of recombinant fusion protein probed with polyclonal anti-exotoxin antibody (Sigma, UK). Lane 1, pre-stained protein size marker and lane 2, purified fusion protein.

and preventive tools [4]. Several different antigens have been evaluated for their immunogenicity and protective response in mouse models [21, 22], but it has shown that none of these tested antigens alone were able to induce sufficient protective response. Therefore, it has been postulated that effective immune response induction may need the use of a cocktail of different antigens [9, 21, 22].

Enhanced clearance of *P. aeruginosa* following immunization of mice with different cocktail antigens, including LPS-exoA [23], exoA-oprF-oprI [12], exoA-alginate [24], fliC$_{a,b}$-oprF-oprI [7] and exoA-Elastase-alkaline protease [25] have been reported by different investigators. These studies proposed exoA and fliC of *P. aeruginosa* as important vaccine candidates [13, 26].

We report here the development of a recombinant exoA-fliC fusion protein as a new vaccine candidate against *P. aeruginosa* infections. A study has used native exoA and fliC purified from bacterial culture; however, it has shown that purification of native exoA from culture medium is very difficult and proteases secreted into the culture medium decrease the yield of product [27]. Therefore, production of recombinant exoA is preferred for preparation of exoA with high quality and quantity.

On the other hand, the native exoA is toxic and its modification into toxoid form by different methods may alter its conformation, which this can affect immunological properties of the protein. However, in the recombinant approach, it is possible to genetically

manipulate the structure of exoA and select desired domains and epitopes. For example, it has been shown that deletion of Glu-553 in the toxic domain of exoA abolishes the toxicity of pseudomonas exoA [28]. In our study, non-toxic exoA with suitable immunogenic properties was obtained by deletion of enzymatic domain.

In the case of fliC, it has been shown that efficiency of purification of native fliC from bacteria is low and production of fliC by recombinant technology is a preferred method [29]. In the recombinant method, not only production of large amounts of fliC is possible but also selection of conserved and immunogenic domains and deleting of unnecessary domains are feasible. In our study, a 510-bp fragment of fliC gene (encoding 170 conserved N-terminal amino acids of fliC) and a 1212-bp fragment of exoA gene (encoding 404 N-terminal amino acids of exoA as non-toxic domain) were selected and used for recombinant expression and protein production.

In this research, exoA-fliC fusion gene was prepared by overlapping PCR method and showed that this method is rapid, easy and cost-effective for preparation of fusion protein. This finding is consistent with the previous reports about advantages of overlapping PCR for production of fusion genes [18, 30].

Expression of recombinant fusion protein by *E. coli* using pET22b vector resulted in production of exoA-
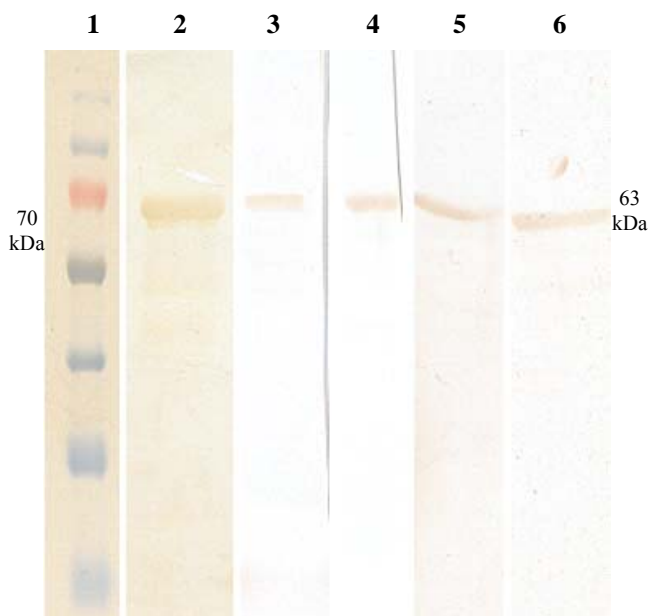

Fig. 6. Western-blot of recombinant fusion protein probed with the sera from patients with *Pseudomonas aeruginosa* infection. Lane1, pre-stained protein size marker and Lanes 2-6, reaction of purified recombinant fusion protein with the sera from patients with *Pseudomonas aeruginosa* infection.

fliC fusion protein in high concentration, that it is appeared as an approximately 63-kDa band in SDS-PAGE. Western-blot analysis showed the reactivity of exoA-fliC fusion protein with antibody against native exoA, that confirms the correct structure of recombinant fusion protein. This finding indicated that this system is a suitable expression system for production of recombinant exoA-fliC fusion protein, that is consistent with several previous studies [17, 27-29].

Sera from patients infected with *P. aeruginosa* recognized the recombinant fusion protein in 1:2000 dilution. This finding verified the highly antigenic properties of the selected proteins. These results showed that exoA and fliC of *P. aeruginosa* are immunogenic during infection and all patients' sera reacted with recombinant protein. These results also indicate that this fusion protein may be used as a serodiagnostic antigen for rapid diagnosis of *P. aeruginosa* infections.

In conclusion, the results of this study suggest that the exoA-fliC fusion protein prepared in this study is a highly antigenic molecule that can be considered as a new vaccine candidate for further evaluations in animal models.

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