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Construction, expression, purification and characterization of secretin domain of PilQ and triple PilA-related disulfide loop peptides fusion protein from *Pseudomonas aeruginosa*

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ABSTRACT

Objective(s): Infection with *Pseudomonas aeruginosa* has been a long-standing obstacle for clinical therapy due to the complexity of the genetics and pathogenesis, as well for widespread resistance to antibiotics, thus attaching great importance to explore effective vaccines for prevention and treatment. This paper focuses on the introduction of novel *Pseudomonas aeruginosa* type IV pili (T4P)-based fusion protein containing the secretin domain of PilQ and tandem PilA-related peptides.

Materials and Methods: We surveyed the expression of the $PilQ_{380-705}$-PilA fusion protein in-frame with pET26b vector in which a rigid linker was used between two polypeptides and flexible linkers were inserted between the three tandem repeats and each *pilA* domains. The transformants were expressed in *Escherichia coli* BL21. The reactivity of specific antisera to the fusion protein was assessed by ELISA. The biological activities of this candidate vaccine were evaluated by western blotting, opsonophagocytosis, and twitching inhibition assays.

Results: The fusion protein was purified in high yield by osmotic shock method using HisTrap affinity column. The protein was confirmed by immunoblot analysis. The checkerboard titration showed that the optimal dilution of the antibody to react with antigen is 1:128. Results of opsonophagocytosis assay revealed that the antibodies elevated to the fusion protein promoted phagocytosis of the PAO1 and 6266E strains, so that the twitching immobilization test confirmed these results.

Conclusion: Due to excellent killing activity mediated by opsonic antibodies and efficient immobilization of the strains, it seems that $PilQ_{380-705}$-PilA fusion protein could be a reliable candidate vaccine against *P. aeruginosa* infection.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative non-lactose fermenting oxidase-positive bacterium that causes serious infections in patients with disrupted epithelial barriers and/or compromised immune systems. As a versatile opportunist, *P. aeruginosa* is capable of causing acute infection, particularly in patients with mechanical ventilation and those with cystic fibrosis (CF) (1). The bacterium is the common causes leading to hospital-acquired infections (2). *P. aeruginosa* can affect and infect almost any part of the human, but normally targets surface-exposed epithelial cells, such as skin, airways, and eyes. *P. aeruginosa* infections are typically treated with antimicrobial agents which are ineffective against chronic infections. The treatment of the infected individuals has been complicated by the extraordinary capacity of this bacterium to develop intrinsic and acquired antimicrobial resistance to almost all antibiotics (3). The pathogenesis of *P. aeruginosa* infections is multifactorial and includes a complex of virulence factors; hence, it has made vaccine development difficult. Attachment is an initial and a critical step for the establishment of infection that involves bacterial adhesins and host receptors. One of the most essential adhesins in *P. aeruginosa* is pili (4).

Type IV pili (T4P) is the most common type of

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bacterial pili and is thin, long, flexible, and retractable protein filament. T4P is polarly localized, filamentous surface appendages present at the cell surface of a broad range of pathogenic and environmental bacterial species (5). This adhesive cell surface structure is the prominent virulence factor that is critical for initiation of the infection by mediating attachment to host cells, where non-piliated strains have been reported to show a 90% decrease in their ability to bind human pneumocytes (6), and also mutant strains that are unable to produce T4P are attenuated in virulence (7, 8). Furthermore, another study revealed that non-piliated strains caused 28%-96% fewer cases of *P. aeruginosa* pneumonia in comparison with piliated strains in the mouse model (9). T4P has been classified into two different subtypes, type IVa pilus (T4aP) and type IVb pilus (T4bP), based on differences in the architecture of the assembly systems and the structure of the major pilin subunit. T4aP is found in a wide range of bacterial species such as *P. aeruginosa*, whereas T4bP is identified mostly in enteric pathogens (5).

T4P plays an important role in many processes including bacterial locomotion known as twitching motility, aggregation, infection by pilus-specific bacteriophage, DNA uptake, attachment to biotic and abiotic surfaces, host cell invasion and biofilm formation (10). The pilus fiber is composed of the hundreds copies of PilA (or pilin, the major structural subunit) that is encoded by an operon that positively controlled by the *algR* regulator (11). The pilin (PilA) can be divided into three different regions: a highly conserved hydrophobic N-terminal α-helix region; a hypervariable central region; and a semi-conserved C-terminal region containing β-strands. The receptor binding domain (RBD) of the pilin is a suitable candidate for peptide vaccines (12). The RBD contains a disulphide-bonded loop (DSL) that structurally is highly conserved among type IV pilins of all species of *P. aeruginosa*, although the size of the DSL (from 12 to 31 amino acids in *P. aeruginosa*) and its sequence is varied among pilin alleles. The DSL of the pilin subunit was shown by the monoclonal antibody preventing attachment to epithelial cell receptors, this finding suggests that PilA itself acts as main structural subunit as well as adhesion (8, 13). Finally, type IV pili from all strains of *P. aeruginosa* has a common receptor; however, the sequence diversity presents a significant obstacle to the development of broadly protective RBD-based vaccines targeting the type IV pili (12).

Pili is rapidly extended and retracted via the most influential molecular machine that organized with four subcomplexes: the cytoplasmic motor subcomplex (consisting of PilBTUCD), the inner membrane alignment subcomplex (PilMNOP), the outer membrane secretin pore subcomplex (PilQ and PilF), and the pilus itself (PilA) (14). There are significant functional and structural similarities between the pilus assembly apparatus and type II secretion system (15). T4P across the outer membrane via a large oligomeric channel made of a single protein. The PilQ (77 kDa; ORF PA5040) is encoded by the highly conserved *pilMNOPQ* operon (16), as a member of the secretin family (10). PilQ is essential for T4P biogenesis. The secretin domain of the PilQ is more highly conserved at its C-terminus. This region embedded in the outer membrane facilitates the passage of folded proteins, filamentous phage particles, DNA, and other macromolecules pass through the outer membrane (17).

In the present study, we designed a chimeric fusion plasmid containing the $pilQ_{380\text{-}705}$ gene, which codes the immunologic domains of PilQ secretin (the C-terminal domain of PilQ) flanked by *pilA* region coding the three peptides of DSL (in triplicate forms). To the best of our knowledge, for the fist time, we report the purification, characterization and biological activities of a novel fusion protein ($PilQ_{380\text{-}705}$-PilA) from *P. aeruginosa*.

Materials and Methods

Strains, plasmids, enzymes, reagents and growth media

Escherichia coli (*E. coli*) strains BL21 (DE3) and Top10F, as expression and preservation hosts, were preserved in our laboratory. The *P. aeruginosa* laboratory strain PAO1 and 6266E (a clinical piliated strain that kindly obtained by Shahid Beheshti University of Medical Sciences, Tehran, Iran) were used. The fusion construct pET26b/$pilQ_{380\text{-}705}$-*pilA* (recombinant secreted expression vector) synthesized by Biomatik Corporation (Cambridge, Ont., Canada). All enzymes for DNA manipulations were obtained from NEB (USA). The anti-His (C-Term)-HRP monoclonal antibody was obtained from Invitrogen (USA). Ni^{2+}-NTA agarose was purchased from Qiagen (USA). The strains were cultured in LB broth or on agar (Merck, Germany) at 37 °C with or without 30 μg kanamycin/ml (Bioscience, Canada).

Construction of the expression vector

The fusion gene was inserted into the *E. coli* expression vector pET26b, in frame with the PelB signal peptide, a T7 promoter, kanamycin-resistant gene and the C-terminal six-His-tagged sequence. The $pilQ_{380\text{-}705}$ gene containing a BamHI site located at the 5′ end, and an EcoRI site located at the 3′ end. The *pilA* region was located between HindIII site (at the 5′ end) and XhoI site (at the 3′ end). Therefore, the rigid linker flanked by EcoRI at the 3′ end of the encoding region for $pilQ_{380\text{-}705}$ gene and HindIII at the 5′ end of the encoding region for *pilA*. As shown in Figure 1 and Table 1, the secretin domain of PilQ ($PilQ_{380\text{-}705}$) was fused to a tandem form of PilA via a rigid linker $(A(EAAAK)_2ALEA(EAAAK)_2A$, called L1).

Table 1. The amino acid sequence that used to design the *pilQ*$_{380-705}$-*pilA* fusion gene on the pET26b plasmid

linkers and tandems	Amino acid sequence	References
pilA 1	ACKSTQDPMFTPKGCDN	12
pilA 2	CNITKTPTAWKPNYAPANC	(data not shown)
pilA 3	CAISGSPANWKANYAPANC	(data not shown)
L3	$(GGGGS)_3$	this study
L2	GGGGS	this study
L1	$A(EAAAK)_2ALEA(EAAAK)_2A$	this study

The tandem *pilA* region contains three domains, *pilA* 1-3, that each domain contains three tandem repeats. The *pilA* 1 gene, which includes three tandemly repeated copies encodes a 17-residue sequence of PilA 1 peptide (12). The *pilA* 2 and *pilA* 3 genes also contain three tandemly repeated copies of the 19-residue sequence that encode the PilA 2 and PilA 3, respectively (data not shown). The glycine/serine as a flexible linker was used between the three monomer sequences of *pilA* (GGGGS, called L2) and between each domain ($(GGGGS)_3$, called L3). The flexible linkers, comprised of glycine and serine, were used to avoid the interference resulting from the direct ligation between epitopes or subsequent generation of new epitopes. In the designation of the construct, we have inserted a nucleotide G before the start codon ATG immediately after the BamHI site (ggatccGATG) of the pET26b vector, resulting in the correct framing of the gene of the insert.

After the fusion plasmid was transformed into *E. coli* Top10F competent cells, transformants were selected on LB plates (1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar, pH 7.5) supplemented with 30 µg kanamycin ml. The recombinant plasmid pET26b/*pilQ*$_{380-705}$-*pilA* was verified by restriction enzyme digestion. The fusion vector was treated with the restriction endonucleases BamHI, EcoRI, HindIII and XhoI (Jena Bioscience Kit, Germany) according to manufacture instruction. The digested fragments were separated by 1.2% (w/v) agarose gel electrophoresis.

Expression, and purification of the fusion protein

The expression and purification of the engineered fusion protein were performed as previously described for human growth hormone (18), with slight modification. *E. coli* BL21 (DE3) was transformed with the fusion construct pET26b/*pilQ*$_{380-705}$-*pilA*. A single colony was grown overnight in Luria-Bertani (LB) medium supplemented with kanamycin (30 µg/ml). One liter of LB medium with kanamycin was inoculated at 37 °C with 10 ml of overnight culture. Upon reaching an OD_{600} of 0.8, the T7 promoter of fusion vector was induced by IPTG (Sigma, USA) to a final concentration of 1 mM. After an incubation period of 4 hr, cells were harvested at 8000 g for 30 min (7 g of wet weight cell pellet) and the periplasmic *E. coli* fraction was extracted via osmotic shock procedure.

The harvested cells were suspended in 25 ml hypertonic solution [30 mM Tris, 20% w/v sucrose, 0.5 mM ETDA, pH 8] and incubated for 30 min at 4 ºC. Cells were centrifuged and the supernatant collected. Cells were re-suspended in 25 ml hypotonic solution [5 mM $MgSO_4$] and incubated for 30 min at 4 ºC followed by an additional centrifugation. The supernatant from the hypotonic solution was combined with the supernatant from the hypertonic solution, centrifuged to remove debris, and finally dialyzed against phosphate buffered saline (PBS, pH 7.4) at 4 °C for 48 hr. The periplasmic solution containing soluble fusion protein was clarified over a 0.45 µm filter and purified by Ni^{2+}-affinity chromatography as follows. The HisTrap affinity column contains Ni-NTA agarose (Qiagen) pre-equilibrated with 20 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8. The clarified osmotic shock fluid was loaded onto the HisTrap affinity column and washed with equilibration buffer for six column volumes. Bound protein was eluted with three column volumes of 20 mM Tris, 300 mM NaCl, 500 mM imidazole, pH 8. Fractions with an absorbance at 280 nm greater than 0.05 were pooled, concentrated and buffer exchanged into PBS, and subject to analysis by 12% SDS–PAGE. The protein concentration was quantitatively measured by using a NanoDrop 2000c (Thermo Scientific, USA) and Bradford protein assay using standard albumin (Sigma, USA) and finally aliquoted in 1 mg/ml vials.

Endotoxin (LPS) removal

Endotoxins (or LPS, lipopolysaccaride) were removed from the fusion protein using ε-poly-L-lysine-agarose (Pierce High Capacity Endotoxin Removal Spin Column, 0.5 ml, #88274; Thermo Fisher Scientific, Inc., USA). Endotoxin binding to the resin occurs at pH 6-8; therefore, the resin was equilibrated with an endotoxin-free buffer containing 10-50 mM Tris-HCl buffer containing 0.1-0.2 M NaCl (pH 7). The sample was added and incubated at 4-22 °C with gentle end-over-end mixing for one hour. The tube was centrifuged (500 × g for 1 min) and finally the sample was collected at the bottom of the column. The Limulus amebocyte lysate assay (Lonza, USA) was performed according to the manufacturer's directions to detect the presence of any remaining LPS (lipopolysaccaride or endotoxin) in the samples.

SDS-PAGE electrophoresis and Immunoblot analysis

The resulting bacterial pellet or purified protein were separated by SDS-PAGE, according to Laemmli (19). The samples were directly resuspended in an appropriate volume of sample buffer. The discontinuous gel consisted of a 5% stacking gel and a 12% resolving gel which was run on a vertical

electrophoresis unit (Mini PPROTEAN 3 cell, Bio-Rad). The fusion protein characterized in this study contains a carboxy terminal poly-histidine tag. For detection of the presence of recombinant protein, the cell-lysed supernatant, and the purified samples were electrophoresed and then transferred onto PDVF membrane (Hi-bond Amersham Biosciences, USA) at 25 V for overnight by using a Mini-PROTEIN tetra cell (Bio-Rad, USA). The membrane was then blocked for 2 hr in 5% (w/v) skim milk. After blocking, the membrane was transferred to a tray containing the anti-His (C-term)-HRP Antibody (Invitrogen, USA) diluted 1:5000 in blocking buffer and then incubated for 1-2 hr with gentle agitation. The membrane was then washed 5 times in TBST (Tris buffer saline contain 0.1% Tween-20) for 5 minutes each. Finally, it was developed by adding 3, 3′-diaminobenzidine (DAB) (Sigma, USA) solution allowing it to incubate until bands were seen. The reaction was stopped by rising with water.

Preparation and purification of polyclonal anti r-fusion protein IgG

The female New Zealand white rabbits (Pasteur Institute of Iran, Karaj, Iran) were immunized with 400 µg of the r-fusion protein administered subcutaneously and boosted twice with 200 µg with two-week intervals. The rabbits were anesthetized intramuscularly with an injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). Blood samples were collected prior to immunization and 2 weeks after each immunization. Sera were collected from the retracted clot, clarified by centrifugation (3500 g) and then aliquoted (1 ml) and stored at -20 °C. When sufficient r-fusion antibodies were prepared, the rich fractions pooled and the specific IgGs (except IgG3) purified by using protein A/G agarose (Invitrogen, USA) according to the manufacturer's instructions. Protein concentration in IgG fractions was quantitatively measured using a Bradford protein assay and NanoDrop (2000c spectrophotometer, Thermo Scientific, USA). Anti r-fusion IgG and non-immune IgG were aliquoted at a concentration of 1–2 mg/ml and finally stored at -20 °C until use. All protocols were approved by the Ethics Committee of the Pasteur Institute of Iran.

ELISA quantification assay

In order to determine the biological activity of the candidate protein, an optimized indirect quantitative ELISA was done. Briefly, 96 well microtiter plates (Greiner, Germany) was coated with serial dilutions of the purified fusion protein in bicarbonate buffer (15 mM NaCO3, 35 mM $NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. The plate was then rinsed three times with PBST (PBS containing 0.05% Tween-20) and incubated with blocking solution (PBS containing 5% (w/v) skimmed milk) at 37 °C for 2 hr. After washing three times with PBST, a serial dilution (from 1:32 to 1:12800) from rabbit anti-PilA-PilQ primary antibody (100 µl/well) in blocking buffer prepared. Following 2 hr incubation at 37 °C, plates were rinsed three times with PBST, and 1:10000-diluted HRP-conjugated anti-rabbit IgG (Sigma, USA) was added to each well and incubated for 2 hr at 37 °C. After washing 5 times, the chromogenic substrate 3, 3, 5, 5′-tetramethyl benzidine (Sigma, USA) was added (100 µl/well) and the plate was incubated to develop color. The plate was cooled to room temperature before the absorbencies were measured in an ELISA plate reader (Awareness Stat Fax 2100, USA) at 450 nm.

Opsonophagocytic killing assay

The opsonophagocytosis assay was performed according to the method of Faezi *et al* (20). Briefly, bacterial suspensions (PAO1 and 6266E strains) were prepared at an approximate concentration of 2×10^9 CFUs/ml in 1% bovine serum albumin (BSA). Mouse macrophages were used at a final concentration of 2×10^7 CFUs/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). A 3-week-old baby rabbit (Pasture Institute, Karaj, Iran) was bled and prepared serum was used as a complement source (final concentration, 4%). Three different concentration (0.1, 0.2 and 0.3 µg) of specific anti r-fusion antibodies were used. For the opsonophagocytic assay, the bacteria (2×10^9 cells per well) were first incubated with an equal volume of heat-inactivated (at 56 ºC for 30 min) specific polyclonal IgG at 22 ºC for 60 min and then washed twice with BSA (1% (w/v)) for elimination of excessive antibodies. After suspending with 200 µl of 1% BSA, 100 µl of mouse macrophages was mixed with 100 µl complement in sterile 48-well microfuge plate (Greiner bio-one, Germany) and then incubated in a shaker at 37 ºC for 90 min. Shortly thereafter (time 0) and after 90 min, 25 µl of the mixture was removed, diluted in saline and finally plated for bacterial enumeration. Normal rabbit serum (NRS) (1:4 dilution) was used as pre-immune serum (control IgG). The opsonic killing activity of immune sera was compared to pre-immune sera statistically. Omitted antibodies, complement, or macrophage substituting with 100 µl of BSA were components of the control tubes. This experiment was performed in duplicate for each quantity. The following formula was used for calculation of the percentage of killed bacteria in different groups, and the results were compared together.

Opsonophagocytosis (%) = [1- (CFU of immune serum at 90 min / CFU of pre-immune serum at 90 min)] ×100

Twitching inhibition test

To verify the functionality of the specific polyclonal antibodies, the twitching inhibition assay was carried out according to Castric *et al* (21) as follows. Different

concentrations (0.1, 0.2 and 0.3 µg) of specific rabbit anti r-fusion protein IgG (filter-sterilized) were added to LB broth (containing 1% agar), which was poured into a 15 × 90 mm plastic Petri dish. After solidification, the plate was dried for 6 hr at room temperature. A single colony of the *P. aeruginosa* strains to be tested was stab-inoculated with a toothpick to the bottom of the plates. After an 18 hr incubation at 37 °C, the diameter zone of growth of different strains obtained at the interstitial surface of the agar and the plate was measured. For each assay, triplicate plates were examined.

Statistical analysis

Data were analyzed by ANOVA or Kruskal–Wallis test, depending on the assay. Differences were considered significant at *P* less than 0.05. Statistical analysis was performed using the software GraphPad Prism version 6.0 for Windows, (GraphPad Software, San Diego, CA, USA).

Results

Construction of plasmid for periplasmic expression of fusion protein

To overcome problems related to cytoplasmic expression of PilQ$_{380\text{-}705}$-PilA fusion protein, the pET26b plasmid was constructed for periplasmic expression of the protein. The coding sequence of *pilQ* secretin gene (*pilQ*$_{380\text{-}705}$) in associated with tandemly repeated of three *pilA* genes without stop codon was constructed in the pET26b expression vector. The *pilQ* secretin gene was flanked by BamHI and EcoRI restriction sites at the 5′ end and 3′ end respectively. The HindIII and XhoI restriction sites were introduced upstream and downstream of the tandem *pilA* genes, respectively. Therefore, the coding sequence was preceded by a pelB signal sequence at the N-terminal region and a six His-tag at the C-terminal of the fusion gene. On the analogy of this, the fusion construct of pET26b/*pilQ*$_{380\text{-}705}$-*pilA* was constructed as shown in Figure 1. The fusion construct transformed into *E. coli* Top10F cells and selected on LB containing kanamycin (30 µg/ml). Transformants were characterized by enzymatic digestion. The recombinant plasmid, pET26b/*pilQ*$_{380\text{-}705}$-*pilA*, was extracted and its orientation confirmed by digestion with four restriction enzymes that mentioned above. The target fragments with the expected sizes have been shown in Figure 2. Sequence analysis of recombinant pET26b/*pilQ*$_{380\text{-}705}$-*pilA* confirmed that there are no amplification errors and that construction was accurate.

Over-expression of the PilQ$_{380\text{-}705}$-PilA

To construct an over-expression system, the coding sequence of pilQ$_{380\text{-}705}$-pilA, whose theoretical molecular size is approximately 64 kDa, was constructed into expression vector pET26b to express a C-terminal His-tagged fusion protein under the control of strong promoter T7 in *E. coli.* The recombinant plasmid, pET26b/pilQ$_{380\text{-}705}$-pilA, were transformed into *E. coli* BL21 (DE3). This strain carries the T7 RNA polymerase gene under control of the chromosomal lacUV5 promoter. IPTG addition induces expression of T7 RNA polymerase resulting in transcription of the fusion gene under control of the T7 promoter in cells harboring the pET26b/*pilQ*$_{380\text{-}705}$-*pilA* vector. By the Sec-dependent transport pathway, the N-terminal pelB secretion signal

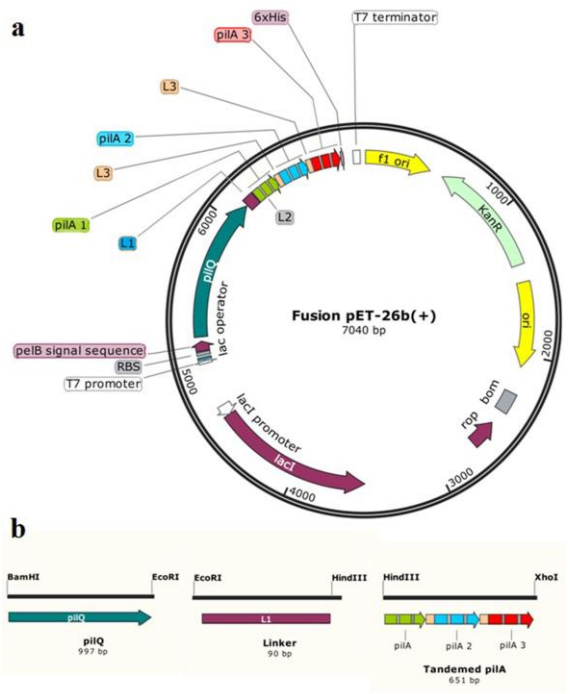

Figure 1. Panel (a); schematic representation of the recombinant pET26b/*pilQ*$_{380\text{-}705}$-*pilA* plasmid. Panel (b); details of the chimera located downstream of the *pilQ* gene and the relevant restriction sites

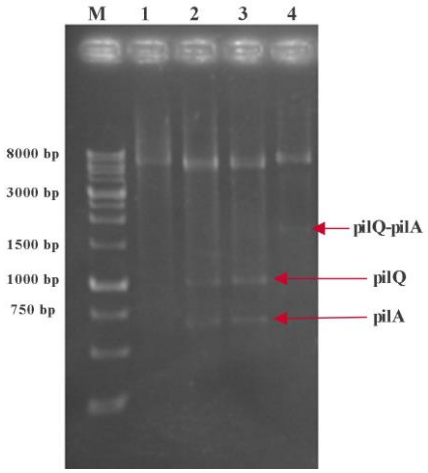

Figure 2. Agarose gel electrophoresis analysis of recombinant pET26b/*pilQ*$_{380\text{-}705}$-*pilA* with restriction enzyme digestion. (Lane M); DNA marker (1 kb), (Lane 1); BamHI mono digested recombinant vector with BamHI buffers (≈ 7080 bp band for pET26b/*pilQ*$_{380\text{-}705}$-*pilA*), (Lane 2 and 3); Quadruple digested recombinant vector with BamHI and XhoI buffers, respectively. Three expected fragments were observed on the gel (red arrow, ≈ 5360, 981 and 649 bp bands), (Lane 4); BamHI/XhoI double digested of pET26b/*pilQ*$_{380\text{-}705}$-*pilA* plasmid with BamHI buffers (red arrow, ≈ 5360 and 1720 bp bands)

Table 2. Evaluation of twitching inhibition of *P. aeruginosa* strains PA01 and 6266E with different concentrations of antibodies raised against the r-fusion protein. The twitching plates (LB plate containing 1% agar) were rehydrated with the rabbit anti r-fusion protein IgG and normal rabbit serum (NRS). The mean diameter of bacterial spreading was measured according to millimeter (mean ± SD) after 18 hr incubation at 37 °C

P. aeruginosa strains	serum samples			
	Rabbit anti r-fusion protein IgG			Normal rabbit serum (NRS)
	0.1 µg	0.2 µg	0.3 µg	
PAO1	17.6±1.453	16.9±1.899	13.2±1.566 ***	28.1 ± 2.984
6266E	18.9±1.279	16.1±1.731	14.9±1.471 &	29.7 ± 2.461

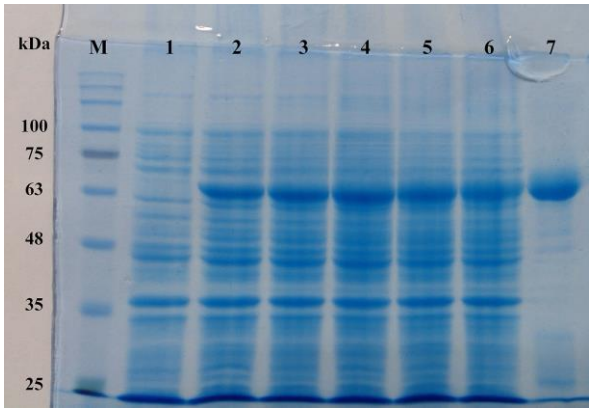

Figure 3. SDS-PAGE analysis of the expression of PilQ$_{380\text{-}705}$-PilA fusion protein in *E. coli*. The total proteins of the BL21harboring pET26b/*pilQ*$_{380\text{-}705}$-*pilA* plasmid was harvested and loaded on 12% (v/v) SDS-PAGE after for 5 hr induction with or without IPTG. (Lane M) denote molecular weight marker proteins; (lane 1) total cell lysate of non-induced bacteria; (lanes 2-6) 1-5 hr after induction with IPTG, respectively; (lane 7) purified fusion protein after HisTrap Chelating and Ni-affinity chromatography (≈ 62 kDa)

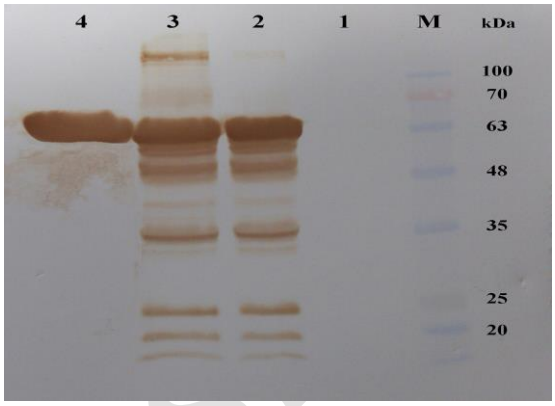

Figure 4. Western blot analysis of the expressed PilQ$_{380\text{-}705}$-PilA-6His tag protein in *E. coli* BL21. After running the SDS-PAGE, the protein transferred onto PVDF membrane and detected with an anti-His monoclonal antibody. (Lane M) PageRuler™ Prestained Protein Ladder; (lane 1) total cell lysate of non-induced bacteria; (lane 2 and 3) Total cell lysate of bacteria after 4 hr induction with IPTG; (lane 4) purified periplasmic PilQ$_{380\text{-}705}$-PilA-6His by Ni-NTA agarose

carry the unfolded fusion protein to the periplasmic space of the *E. coli* (18). The signal peptide is cleaved by an enzyme in the inner membrane named signal peptidase. Results from SDS-PAGE analysis of expression products revealed that the fusion protein over-expressed 4 hr after induction with IPTG. The expression product of fusion protein PilQ$_{380\text{-}705}$-PilA was approximately 62 kDa in molecular size (Figure 3), furthermore, almost all of the fusion protein was expressed as a soluble form in the cell disruption supernatant. Western blot analysis of total cell extracts (induced and non-induced) and the purified protein using anti-His-tag-specific antibodies showed that the fusion protein was substantially expressed by using the pET26b/*pilQ*$_{380\text{-}705}$-*pilA* expression vector when IPTG was added at the early-exponential phase of growth and collecting the cells 4 hr after induction (Figure 4). The checkerboard titration demonstrated that the optimal dilution of the anti r-fusion protein antibody to react with the r-fusion protein is 1:128. The yield of the purified fusion protein was about 3.84 mg per liter of culture media. No LPS was detected above the lower limit of the assay (< 0.08 endotoxin unit/ml).

Opsonophagocytic killing activity

To determine the bioactivity of anti r-fusion protein IgG *in vitro*, its ability to promote phagocytosis of bacteria was evaluated by incubating *P. aeruginosa*

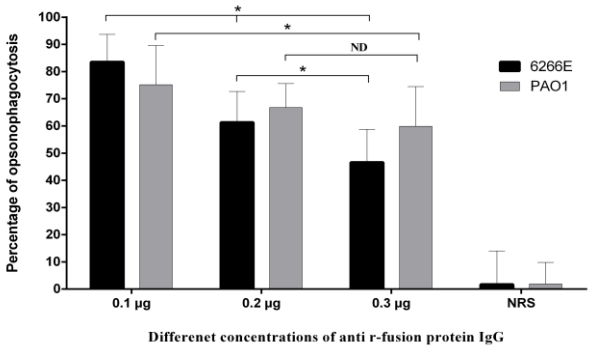

Figure 5. The opsonic killing activity of three different concentration of anti r-fusion IgG against *P. aeruginosa* strain PAO1and 6266E. To do this test, desired strains were incubated with different concentrations (0.1, 0.2 and 0.3 µg) of rabbit anti r-fusion IgG and mouse macrophage in the presence of rabbit complement. A significant opsonic killing activity was observed when specific r-fusion antiserum was treated with PAO1 and 6266E strains. No cross reaction was detected between normal rabbit serum and the strains. Bars represent means of triplicate determinations, and error bar indicate SD. Results were accepted to be significant at *P* less than 0.05. The asterisks represents the groups which were significantly different (*P*<0.05) and ND indicates not detectable differences

PAO1 and 6266E strains with different concentration of anti r-fusion antibody and mouse macrophages in the presence of rabbit complement. In the presence of normal rabbit serum (NRS), as the control group, only 1.8% phagocytosis-mediated killing was

monitored, which is probably an index of non-opsonic phagocytosis. This present study also showed that addition of anti r-fusion IgG promoted phagocytosis of *P. aeruginosa* 6266E strain and the number of viable bacterial cells decreased over 83.5% after 90 min as compared with the control group (Figure 5). When antibodies against fusion protein were treated with the PAO1 strain, 75.2% opsonic killing was detected. These data indicate that the anti r-fusion protein IgG act as a good opsonin for killing of the clinical piliated strain (6266E) and PAO1 strain of *P. aeruginosa*.

Twitching inhibition assay

Immunized and non-immunized mouse sera were tested in the twitching inhibition assay for their functional activity to inhibit the motility of *P. aeruginosa* strains PAO1 and 6266E. In this assay, NRS was used as control group. As shown in Table 2, the r-fusion protein IgG was able to inhibit the motility of the PAO1 strain. This antibody also inhibited the motility of the 6266E strain, so that the motility zone was significantly decreased compared to control group. In the presence of NRS, no immobilization was observed.

Discussion

P. aeruginosa is an opportunistic microorganism which commonly considered as an infectious agent in immunocompromised hosts. In these patients, infections are often severe and life-threatening. There is a great attention in developing vaccines to prevent *P. aeruginosa* infection, given that immunization is always considered the most economic and efficient means for such prevention, specially in developing countries. The selection of immunogenic targets is critical for vaccine design. It has been proposed that adhesions act as a key role in the initial stages of infection, where they allow the bacteria to anchor themselves to the epithelial layers, which can lead to successive colonization and potential invasion (22). A large number of published data showed that pili might be the most definitive antigen candidate (23, 24). T4P is essential virulence factor of *P. aeruginosa* that has been studied in the mice model (9, 25). The contribution of T4P to pathogenesis lies primarily in its ability to promote the initial attachment of the pathogen to various cell surface receptors before other adhesins are able to attach. As soon as attachment take places, the coordinated expression of other virulence factors facilitate invasion (26). Because of the its role in early pathogenesis of infection, T4P has been suggested as an attractive vaccine target (27). A number of studies based on monoclonal antibody-binding data have shown that DSL mediates attachment of bacteria to epithelial cell receptors (8, 13). In another study, it was demonstrated that antibodies elevated against RBD can obstruct pilus-mediated attachment and therefore, it was the basis for the vaccine development projects (28). Therefore, development of a new DSL-based vaccine against *P. aeruginosa* can be useful to combat infection. *P. aeruginosa* type IV pili is thought to be extruded and retracted by the secretin domain of PilQ. The secretin is a member of a family belonging to integral outer membrane proteins with conserved C-terminal regions involved in the type IV pilus biogenesis (29). Since the secretin domain of PilQ is exposed to the bacterial surface, it is likely to be important for induction of effective immune responses. As adhesion and colonization are the initial stages of infection and play important roles in the pathogenicity of *P. aeruginosa*, therefore we developed a novel T4P-based fusion protein to combat infection.

In our previous study, we investigated the sequence diversity of *pilA* gene among CF isolates obtaining from Tehran hospitals. After amplification of *pilA* gene by specific primers, the PCR products was sequenced. Alignment of the sequencing data showed that there are two consensus sequences above 89% homology at the C-terminal region of the PilA protein (data not shown). Therefore, the coding sequence of six copies of 19-amino acid residues and three copies of 17-amino acid residues (12) from DSL region was designed in the pET26b vector as a part of the $PilQ_{380-705}$-PilA fusion protein. The secretin domain of the PilQ ($PilQ_{380-705}$) was chosen as another part of the fusion protein.

Engineered fusion proteins containing two or more functional polypeptides linked by a peptide linker are important in the fields of biotechnological research. The distance between two functional units may affect epitope access and avidity binding; thus the availability of a wide variety of linkers with different degrees of flexibility or rigidity would be helpful for fusion protein design attempts (30). Hence, in the present study, we used a rigid linker (A(EAAAK)2ALEA(EAAAK)2A) between polypeptides to prohibit unwanted intramolecular interactions and Gly/Ser flexible linkers to separate pilA tandems and domains.

Recombinant protein production is a very useful and powerful tool for supplying proteins for various purposes within life sciences, from basic research to biopharmaceuticals and drug discovery. Here, we offer a rapid, stepwise strategy for isolation of soluble protein using *E. coli* as the expression host. As *E. coli* does not naturally produce high amounts of secretory protein, recovery of a recombinant gene product can be greatly simplified by a secretion strategy that minimizes contamination from host proteins (18). The pET system was chosen because it is a very potent system developed specially for the cloning, expression and purification of recombinant proteins in *E. coli.* A series of vectors with signal

sequence-directed secretion has been designed such as pET26b. The pET26b vector produces recombinant protein with N-terminal PelB as a signal peptide for periplasmic localization and a C-terminal His-tag for detection and purification. Since the PilA protein contains at least eighteen cysteine residues, it must be expressed as secretory strategy, hence we constructed the fusion gene into the pET26b vectors. If the protein expressed (for example, under pET28a vector) as inclusion bodies in *E. coli*, it will be contain intramolecular misfolding due to aggregation of the target protein. The promising results from the fusion protein purification, indicate beneficial effects on the solubility of proteins with high amount of cysteine residue using secretory system. The *E. coli* strain BL21(DE3) was already used for this kind of cytoplasmic expression. In this work, a reliable protocol was examined to rapidly express and purify the $PilQ_{380-705}$-PilA fusion protein in *E. coli* expression strain BL21 (DE3). We successfully purified the fusion protein with high yield and approximate homogeneity on SDS-PAGE by Nickel affinity chromatography. Due to the ease, time effective and low cost of this purification process, we recommend this vector for similar purposes as well as more directed studies regarding one or few hard-to-get protein targets.

The current study expands our understanding regarding the functional activities of the $PilQ_{380-705}$-PilA fusion protein from *P. aeruginosa.* Following investigation of opsonic killing activity of anti r-fusion protein antibody to *P. aeruginosa*, we concluded that antibodies raised against r-fusion protein have excellent opsonic killing activity following treatment with 6266E strain. The antisera induced phagocytosis of PAO1 strain, so that the number of viable bacterial cells decreased over 75.2% as compared to the control group. These results indicate that the anti r-fusion protein antibody can act as a suitable opsonin for eradication of the organism. We also showed that the antisera raised against r-fusion protein can increasingly inhibit the twitching motility of PAK and 6266E strains; the result was in agreement with the results of opsonophagocytosis test.

Conclusion

To the best of our knowledge, for the first time, we have designed and developed a novel fusion protein vaccine including $PilQ_{380-705}$ and multivalent DSL-based PilA for prevention and treatment of *P. aeruginosa*. Taken all together, due to efficient immobilization of the strains and excellent killing activity mediated by opsonic antibodies, it seems that the $PilQ_{380-705}$-PilA fusion protein could be an appropriate candidate vaccine against *P. aeruginosa* infections. Although, there's still a tough and long-way on examination of the vaccine until being applicable to the clinical therapeutics.

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Conflict of interest

The authors declare no conflict of interest associated with the present manuscript.

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