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Induction of Specific Humoral Immune Response in Mice against a *Pseudomonas aeruginosa* Chimeric PilQ/PilA Protein

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Abstract

Background: *Pseudomonas aeruginosa*, an opportunistic pathogen, is a common cause of healthcare-associated infections in immunocompromised individuals. The rapid emergence of multidrug-resistant strains has made *P. aeruginosa* infections progressively difficult to treat. In this study we evaluated the effect of a chimeric protein containing a *P. aeruginosa* PilQ fragment and the PilA disulfide loop (PilA-DSL) on the humoral immune response in BALB/c mice.
Methods: A chimeric gene encoding an immunogenic region of PilQ and the PilA-DSL was synthesized. Following bacterial expression and purification, the protein was administered to mice and the humoral immune response analyzed. The resulting antibodies were analyzed using an opsonophagocytic killing assay.
Results: The anti-recombinant protein antibody titer was significantly greater in immunized mice than in controls. In addition, antibody titers were significantly increased after booster immunizations, and the immunizations induced opsonophagocytosis of *P. aeruginosa* PAO1.
Conclusions: These results suggest that an anti-adhesion-based vaccination may be effective in preventing *P. aeruginosa* infections. Further studies are needed to evaluate the abilities of such bivalent proteins to induce strong immune responses.

Keywords: Chimeric protein, PilQ, *Pseudomonas aeruginosa*, Type IV pili, Vaccine

Introduction

Pseudomonas aeruginosa (*P. aeruginosa* or PA), a common opportunistic pathogen, can cause serious complications in immunocompromised patients, particularly those with cystic fibrosis (CF), open wounds, and severe burns (1). PA is also notorious for its ability to acquire antibiotic resistance, often showing multi-drug resistance (2, 3). The rapid development of this antibiotic resistance has increasingly complicated treatment (3); thus, non-antimicrobial-based treatments are urgently needed (4). Currently, vaccination is a promising method for infectious disease prevention (5). The pathogenesis of PA is considered to be multifactorial, depending on bacterial flagella, pili, alginate, and extracellular proteases (6). The first step in the infection process is attachment of the bacterial ligands to appropriate host cell receptors; thus, anti-adhesion therapy could prevent bacterial infections (7, 8).

Type IV pili (Tfp), critical factors in the initial invasion steps of all gram-negative bacteria, are also involved in bacterial pathogenicity during acute infections (9). These pili are composed of thousands of pilin copies that can stimulate the host immune

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response, and therefore, be an effective vaccination target (9, 10). PA Tfp contains PilA subunits and mediates biofilm formation, bacterial attachment, and bacterial twitching motility on both biotic and abiotic surfaces (11). The Tfp binding domain, located in the PilA c-terminus, is termed the disulfide loop (DSL) (12). It has been shown that the 50% lethal dose (LD50) for piliated PA is tenfold greater than that of non-piliated mutants (13). Furthermore, immunological studies in animal models indicated that the specific humoral immune response against the DSL is protective (14, 15). In PA, PilQ is encoded in the terminal sequences of the polycistronic operon pilMNOPQ, affiliated with the secretin superfamily, and plays an essential role in extruding the pilus through the bacterial outer membrane (16).

The protective immune response following vaccination can be enhanced using bivalent antigens. To date, numerous studies have indicated the efficacy of recombinant fusion protein vaccines to prevent *Pseudomonas* infections (17, 18). In this study we analyzed the effect of a chimeric PilQ and PilA-DSL protein from PA on the humoral immune response in BALB/c mice.

Materials and methods

Ethics statement

All animal procedures were approved by the Animal Ethics Committee of Iran University of Medical Sciences. (NO= IR.IUMS.REC 1395.9221133204).

PilQ/DSL-PilA Construct design

The nucleotide sequences of the secretin pilQ and DSL region of pilA gene from *P. aeruginosa* PAO1 were obtained from the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov) in FASTA format. The sequences were combined using the amino acid linker (EAAAK)4. Finally, the fusion protein was analyzed *in silico* and by immunoinformatics as previously described (12). The QA-encoding construct was produced in the pET28a (+) vector by Biomatik, Inc., Canada.

Expression of integrated antigen

Escherichia coli strain BL21 (DE3) competent cells were transformed with the construct. The transformed bacteria were cultured in 5 mL of Luria-Bertani (LB) broth (Merck Co, Darmstadt, Germany), containing 100 µg/mL of kanamycin, overnight at 37 °C. Transformation was verified by polymerase chain reaction (PCR) and enzymatic digestion. Then, one mM Isopropyl-D-thiogalactopyranoside (IPTG) (Sigma, USA) was added to induce expression and the cells were incubated at 37 °C for 5 h. The cells were centrifuged at 8000 g and pellets were resuspended in lysis buffer containing 20 mM sodium phosphate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1% [v/v] Triton X-114, pH 7.5, followed by three freeze- thaw and 5 sonication cycles on ice (Misonix Inc., Farmingdale, NY). The pellet was denatured and solubilized in 20 mM sodium phosphate buffer containing 500 mM NaCl and 6 M guanidine HCl, pH 7.4 for 1 h.

Purification of the recombinant protein

The expressed protein was purified by Ni-NTA affinity chromatography under denaturing conditions as previously described with slight modification and optimization (12). Briefly, the supernatant was shaken with 1.5 ml His60 Ni superflow resin (Takara, Dalian CO, China) for 35 min at room temperature (RT) and transferred to a chromatography column. The resin was washed with buffer containing 100 mM NaH2PO4, 10 mM Tris-HCl, and 8 M urea, pH 6.3. Urea was removed stepwise by washing the column with decreasing concentrations from 8 to 0 M urea. Finally, the recombinant protein was eluted with 20 mM sodium phosphate buffer containing 500 mM NaCl and 1M imidazole, pH 8.0. The eluted protein was dialyzed against 20 mM Tris–HCl, pH 7.4, to remove the imidazole. The purified protein was analyzed by 12% SDS-PAGE and the protein concentration was determined with a micro BCA protein assay kit (Thermo Fisher Scientific, Waltham, USA) (19).

Western blot analysis

The immunoreactivity of the recombinant QA fusion protein was evaluated by western blotting. The recombinant protein was electrophoresed by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked overnight with 2% bovine serum albumin. The membrane was then incubated with rabbit anti-6X His tag antibody (Abcam, USA) diluted 1:1000 for 1 hour at RT. After extensive washing with tris-buffered saline Tween-20 (TBST) membrane was incubated with horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG (Abcam, USA) diluted 1:10000 for 1 hour at RT. Following another washing, the antibody-bound protein was visualized using an electrochemiluminescent substrate (GE Healthcare, Uppsala, Sweden).

Toxicity assay

Lipopolysaccharide (LPS) contamination was quantified by Limulus amoebocyte lysate (LAL) assay (20). Lipopolysaccharide toxicity in mice was also considered to identify possible side effects of remaining LPS in the purified protein solution. Mice were injected subcutaneously with 10 to 100 µg of the recombinant protein in sterile PBS. Mice survival rates were recorded at 6-hour intervals for seven days (21).

Immunization of mice with the recombinant protein

Thirty BALB/c 6-8-week-old female mice weighing 30 to 35 g were purchased from the Pasteur Institute, Tehran, Iran. The mice were housed in cages in pathogen-free conditions. The protocols for all animal experiments were approved by the Animal Ethics Committee of IUMS, Tehran, Iran, before starting the experiments. The mice were divided into two groups of 14 mice each. The test group was injected with 10 µg of QA at the first day. On Days 14 and 28 post-initial immunizations, the same formula was administered for booster immunizations. Fourteen days after each vaccination the mice were bled via the retro-orbital sinus and the sera were collected and stored at -70 ºC. The control mice received PBS only on the same schedule.

Measurement of Specific Serum Immunoglobulin

The specific anti-QA antibody level was measured in serum samples by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, wells of 96-well microtiter plates (Greiner-Labortechnik, Frickenhausen, Germany) were coated with the purified recombinant protein at a concentration of 2.5 µg/mL in bicarbonate buffer, pH 9.6. After coating, the wells were blocked with 1% BSA for 2 h, washed with PBST, and incubated with 100 µL of 1:1000 diluted mice sera for 2 h at 37 °C. The plates were then washed three times and incubated with HRP-conjugated anti-mouse antibody (1:1000) (Abcam, USA) for 1 h at 37 °C. After another wash color was developed at RT with tetramethylbenzidine substrate (Sigma-Aldrich, München, Germany). The reaction was stopped with 2N H_2SO_4 and the optical density was determined at 450 nm on a Multiskan microplate reader (Labsystems, Montigny-le-Bretonneux, France).

In vitro Opsonophagocytosis killing assay (OPA)

An *in vitro* opsonophagocytosis killing assay (OPA) was performed using immunized mice sera to determine the efficacy of the recombinant protein (22). We used pooled sera from five non-immunized mice as the negative control. In brief, to test for opsonophagocytic killing, 100 uL of each of the components including 1) mice peritoneal macrophages at a density of 1×10^6 cells; 2) overnight culture of PAO1 strain propagated on LB broth to reach mid-log phase of approximately 1×10^7 cells/ml; and 3) Anti-sera containing opsonizing antibodies diluted 1:2, 1:4, 1:8, 1:16 and 1:32. The opsonophagocytic activity of immune sera was compared with its pre-immune sample. The bactericidal activity was calculated using the formula: opsonophagocytosis (%) = [1- (CFU of immune serum/CFU of pre-immune serum)] × 100.

Statistical Analysis

Statistics were analyzed using Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL, USA version 16) and GraphPad Prism 5 (GraphPad Software, Inc., USA). All data were expressed as means ± standard deviations. P values <0.05 were considered statistically significant.

Results

Characterization of the fusion gene

Using PCR amplification, restriction enzyme digestion, and sequencing methods (not shown), the appropriate size and identity of the transformed construct was confirmed. Figure 1 shows a Coomassie blue-stained SDS-PAGE gel of the *E. coli* lysate after and before induction of recombinant protein expression (lanes 2 and 3, respectively). The target protein was expressed as insoluble form at high yield and migrated with an apparent molecular mass of 40 kDa. Purification of the His-tagged fusion protein by Ni-NTA resin chromatography resulted in a single band of 40 kDa on SDS-PAGE (Fig. 1, lane 4).

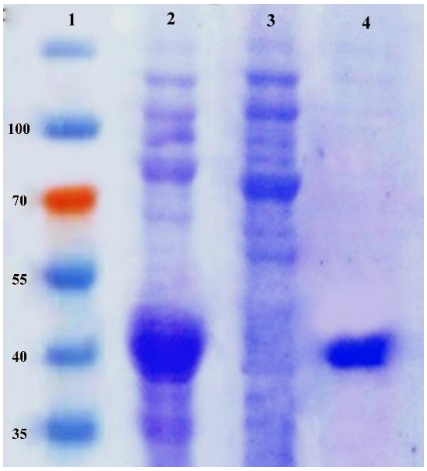

Fig. 1. SDS-PAGE of the expression and purification steps of chimeric pilQ and PilA DSL region fusion protein (QA). Lane 1: Protein marker. Lane 2: Total protein of the IPTG-induced bacteria. Lane 3: Total protein of uninduced bacteria. Lane 4: Purified protein.

Western-blotting

Expression of the recombinant protein was analyzed by Western blotting. The purified recombinant protein was bound by the anti-His tag antibody (Fig 2).

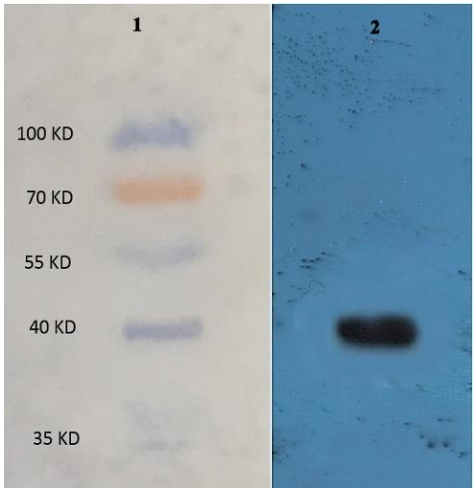

Fig. 2. Western blot of QA with anti-His tag antibody. The Ni-NTA-purified protein was electrophoresed by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with rabbit anti-6X His tag antibody. Lane 1: pre-stained protein marker. Lane 2: Western blot of the purified *Pseudomonas aeruginosa* QA.

Antibody responses

Two weeks after each injection, mice sera were collected and the antibody titer against the recombinant antigen was measured by ELISA. Following each injection, the specific IgG titer increased, confirming appropriate immunization and boosting of the humoral immune response (Fig. 3).

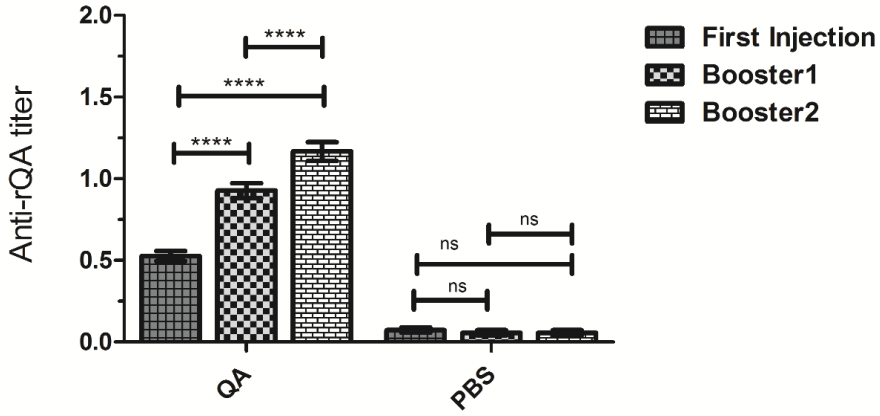

Fig. 3. Specific antibodies titer against PilQ/DSL-pilA (QA) in the sera of mice immunized with recombinant protein or PBS as negative control, following immunization steps. Experimental mice (QA group) were injected with 10 µg of recombinant protein and boosted with another 10 µg of the same protein 14 and 28 days later. Control mice only received PBS with the same vaccination schedule. Sera were collected 14 days after each vaccination. Antibody titers were determined by ELISA. Values are presented as means ± SDs of experimental groups. ****p<0.0001. QA: Integrated PilQ/ disulphide loop with pilA from *P. aeruginosa.*

In vitro OPA assay

The antisera were tested for their capacity to enhance complement-mediated killing of PAO1 strain *in vitro*. At all serum dilutions, the opsonic activity of the experimental sera showed significantly greater bactericidal activity than that from the control group. The bactericidal activities of the sera were 62 ± 2.5%, 25 ± 2.5%, 12 ± 2%, 7 ± 1%, and 5 ± 1% at 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions, respectively. These data showed that anti-QA antibodies may be a suitable opsonin to kill the PAO1 strain. No phagocytic killing occurred with sera from the control group.

Discussion

Therapeutic management of multidrug-resistant infections remains a concern worldwide. The expensive treatments of these infections and limited drug choices have recently led microbiologists to focus on novel therapeutic methods (23). Numerous *Pseudomonas* antigens including alginate, LPS, outer membrane proteins, flagella, Type III secretion system, and pili, have been studied as potential vaccine candidates and some are currently being tested in clinical trials (5, 24). Similar to *Neisseria meningitidis*, another gram-negative bacterium, PA expresses a cell-surface pilin, which has been well studied (11, 25). Also, alignments of multiple pilQ sequences illustrated that pilQ is conserved among various PA strains including PAO1, PA14, B136, LESB58, M18, PA7, and NCGM2. Additionally, the previous *in silico* and immunoinformatics analyses showed induction of both humoral and cellular immune responses after administration of the integrated antigen, which is a key platform for the design of novel vaccines (12). Therefore, we evaluated the immunogenic properties of a chimeric protein in a murine model. Serum IgG titer was significantly greater in mice immunized with QA than in the control mice. This result demonstrates the immunogenicity of this integrated antigen in mice. Hsieh et al. reported that intranasal immunization with a chimeric protein composed of a nontoxic form of PA exotoxin A and the C-terminal disulfide-bonded loop amino acid sequence of the PAK strain pilin protein prompted both mucosal and systemic immune responses capable of blocking pilin-mediated binding of PA to airway epithelial cells in early infection stages (26). Horzempa et al. showed that immunization with pure pilin from various PA strains provided O-antigen-specific protection against either the mouse respiratory model and/or the thermal injury model (27). Korpi et al. demonstrated that active immunization with recombinant pilin of PA admixed with alum increased the humoral immunity, improving opsonophagocytosis and inhibition of bacterial adherence (28). Previous findings showed that immunization of mice with type b flagellin combined with PilA induces cellular and humoral immune responses and provides protection in burned PA-infected mice (29). In this study, the sera from the experimental group had high opsonic killing activity against the PAO1 strain while sera from control mice showed no opsonic killing activity. These findings indicate that the antibodies induced by immunization with QA chimeric protein could opsonize bacterial cells and mediate phagocytic killing of PAO1 strain. Consistent with the initial bioinformatics predictions, the results of *in vivo* experiments showed that the recombinant fusion protein can be considered as a potential vaccine candidate, based on its ability to induce high anti-pilin antibody titers and opsonic killing. Additional *in vivo* and *in vitro* studies, optimization of the dose and finding a safe and effective adjuvant are required to evaluate the immunological and biological properties of the fusion protein.

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Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Sousa D, Justo I, Dominguez A, Manzur A, Izquierdo C, Ruiz L, et al. Community-acquired pneumonia in immunocompromised older patients: incidence, causative organisms and outcome. Clin Microbiol Infect. 2013;19(2):187-92.
2. Bejestani FB, Hakemi-Vala M, Momtaheni R, Bejestani OB, Gholami M. The frequency of imp and vim genes Among *Pseudomonas aeruginosa* isolates from Children's medical center of tehran. Arch Clin Infect Dis. 2015;10(1).
3. Gholipourmalekabadi M, Bandehpour M, Mozafari M, Hashemi A, Ghanbarian H, Sameni M, et al. Decellularized human amniotic membrane: more is needed for an efficient dressing for protection of burns against antibiotic-resistant bacteria isolated from burn patients. Burns. 2015;41(7):1488-97.
4. Sedlak-Weinstein E, Cripps AW, Kyd JM, Foxwell AR. *Pseudomonas aeruginosa*: the potential to immunise against infection. Expert Opin Biol Ther. 2005;5(7):967-82.
5. Sharma A, Krause A, Worgall S. Recent developments for *Pseudomonas* vaccines. Hum Vaccin. 2011;7(10):999-1011.
6. Streeter K, Katouli M. *Pseudomonas aeruginosa*: A review of their Pathogenesis and Prevalence in Clinical Settings and the Environment. Infect Epidemiol Med. 2016;2(1):25-32.
7. Kline KA, Fälker S, Dahlberg S, Normark S, Henriques-Normark B. Bacterial adhesins in host-microbe interactions. Cell host & microbe. 2009;5(6):580-92.
8. Krachler AM, Orth K. Targeting the bacteria–host interface: strategies in anti-adhesion therapy. Virulence. 2013;4(4):284-94.
9. Craig L, Pique ME, Tainer JA. Type IV pilus structure and bacterial pathogenicity. Nature Rev Microbiol. 2004;2(5):363-78.
10. Ofek I, Hasty DL, Sharon N. Anti-adhesion therapy of bacterial diseases: prospects and problems. FEMS Immunol Med Microbiol. 2003;38(3):181-91.
11. Martin PR, Hobbs M, Free PD, Jeske Y, Mattick JS. Characterization of pilQ, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol Microbiol. 1993;9(4):857-68.
12. Chirani AS, Majidzadeh R, Dabiri H, Rezaei J, Esmaili A, Kord YA, et al. Immunological study on integrated PilQ and disulfide loop region of PilA against acute *Pseudomonas aeruginosa* infection: In silico analysis and *in vitro* production. J Acute Dis. 2016;5(2):131-42.
13. Sato H, Okinaga K, Saito H. Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. Microbiol Immunol. 1988;32(2):131-9.
14. Castric PA, Deal CD. Differentiation of *Pseudomonas aeruginosa* pili based on sequence and B-cell epitope analyses. Infect immun. 1994;62(2):371-6.
15. Spangenberg C, Fislage R, Sierralta W, Tümmler B, Römling U. Comparison of type IV-pilin genes of *Pseudomonas aeruginosa* of various habitats has uncovered a novel unusual sequence. FEMS Microbiol Lett. 1995;125(2-3):265-73.
16. Tammam S, Sampaleanu LM, Koo J, Manoharan K, Daubaras M, Burrows LL, et al. PilMNOPQ from the *Pseudomonas aeruginosa* type IV pilus system form a transenvelope protein interaction network that interacts with PilA. J Bacteriol. 2013;195(10):2126-35.
17. Hassan R, El-Naggar W, El-Aziz AMA, Shaaban M, Kenawy HI, Ali YM. Immunization with outer membrane proteins (OprF and OprI) and flagellin B protects mice from pulmonary infection with mucoid and nonmucoid *Pseudomonas aeruginosa*. J Microbiol Immunol Infec. 2017.
18. Peluso L, De Luca C, Bozza S, Leonardi A, Giovannini G, Lavorgna A, et al. Protection against *Pseudomonas aeruginosa* lung infection in mice by

recombinant OprF-pulsed dendritic cell immunization. BMC microbiology. 2010;10(1):9.
19. Tanom A, Farajnia S, Peerayeh SN, Majidi J. Cloning, expression and characterization of recombinant exotoxin A-flagellin fusion protein as a new vaccine candidate against *Pseudomonas aeruginosa* infections. Iran Biomed J. 2013;17(1):1.
20. Yin ET, Galanos C, Kinsky S, Bradshaw RA, Wessler S, Lüderitz O, et al. Picogram-sensitive assay for endotoxin: Gelation of Limulus polyphemus blood cell lysate induced lipopolysaccharides and lipid A from gram-negative bacteria. Biochim Biophys Acta. 1972;261(1):284-9.
21. Farajnia S, Peerayeh SN, Tanomand A, Majidi J, Goudarzi G, Naghili B, et al. Protective efficacy of recombinant exotoxin A—flagellin fusion protein against *Pseudomonas aeruginosa* infection. Can J Microbiol. 2014;61(1):60-4.
22. Ames P, DesJardins D, Pier GB. Opsonophagocytic killing activity of rabbit antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide. Infect immun. 1985;49(2):281-5.
23. Sedighi M, Moghoofei M, Kouhsari E, Poumajaf A, Emadi B, Tohidfar M, et al. In silico analysis and molecular modeling of RNA polymerase, sigma S (RpoS) protein in *Pseudomonas aeruginosa* PAO1. Rep Biochem Mol Biol. 2015;4(1):32.
24. Döring G, Pier GB. Vaccines and immunotherapy against *Pseudomonas aeruginosa*. Vaccine. 2008;26(8):1011-24.
25. Gholami M, Chirani AS, Moshiri M, Sedighi M, Pournajaf A, Tohidfar M, et al. In silico analysis and modeling of ACP-MIP–PilQ chimeric antigen from *Neisseria meningitidis* serogroup B. Rep Biochem Mol Biol. 2015;4(1):50.
26. Hsieh JC, Tham DM, Feng W, Huang F, Embaie S, Liu K, et al. Intranasal immunization strategy to impede pilin-mediated binding of *Pseudomonas aeruginosa* to airway epithelial cells. Infect immuni. 2005;73(11):7705-17.
27. Horzempa J, Held TK, Cross AS, Furst D, Qutyan M, Neely AN, et al. Immunization with a *Pseudomonas aeruginosa* 1244 pilin provides O-antigen-specific protection. Clin Vaccine Immunol. 2008;15(4):590-7.
28. Korpi F, Irajian G, Mahadavi M, Motamedifar M, Mousavi M, Laghaei P, et al. Active Immunization with Recombinant PilA protein Protects Against *Pseudomonas aeruginosa* Infection in a Mouse Burn Wound Model. J Microbiol Biotechnol. 2015.
29. Korpi F, Hashemi FB, Irajian G, Fatemi MJ, Laghaei P, Behrouz B. Flagellin and pilin immunization against multi-drug resistant *Pseudomonas aeruginosa* protects mice in the burn wound sepsis model. Immunol lette. 2016;176:8-17.