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Ceasing down *Pseudomonas aeruginosa* Invasiveness in A Mouse Burn Wound Sepsis Model by Recombinant OprF

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ABSTRACT

***Background*:** Bacterial infections in burn and wound patients are common and difficult to control. The aim of the current study was to evaluate the ability of full length OprF to elicit the production of protective IgG in mice burn wound sepsis model against *P. aeruginosa* infection.
***Methods*:** OprF protein was expressed and purified by Ni-NTA. The purified protein as used to immunize BALB/c mice. The antibody raised against OprF was confirmed by ELISA and evaluated by immunoblot analysis. After burn and bacterial challenge, mortality rate was monitored in the control and immunized mice groups. Bacterial quantity in skin, blood, spleen and liver was evaluated to study spread or inhibition of the infection.
Results: Immunization of mice with OprF brought about a significant rise in anti-OprF sera titer. Protection was imparted in the immunized group resulting in 100% survival against 1000 fold LD50 challenge with P. aeruginosa. The antiserum against OprF was able to significantly inhibit the systemic spread of P. aeruginosa infection from the infection site to internal organs.
Conclusions: The results suggest that anti-*P. aeruginosa* OprF antibodies elicited in burn wound sepsis model by active immunization are protective against infection with *P. aeruginosa*, and provide a rational for further development of the vaccine for prevention against *P. aeruginosa* infection in burn patients.

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Introduction

Burns are very frequent and affect approximately 1% of the general population every year (1). In the last few years patients who survived to burn injury has increased, but despite advances in the management of burn patients, infections remain the most common cause of morbidity and mortality following burn injury (1, 2). The mortality rate in burn centers varies depending on different factors, like age of patients, being the extremes of life more vulnerable, and particularly the pediatric population aged less than four years. Furthermore, type and mechanisms of burns have also been associated with mortality. Full thickness burns, burns secondary to flame associated with inhalation injury have higher rate of infections and mortality (1, 3). Localization of burns in perineal area and face has increased risk of mortality too. In developing countries mortality due to infections is very high and an important risk factor to prevent. Bacterial infections in burn and wound patients are common and subsequent sepsis is often fatal (4). It requires more interest and attention than it currently receives as it is responsible for a great deal of morbidity and mortality among hospitalized patients in addition to unavailability of records, statistics or enough information about the problem as well as lack of universal program or approach to control it (5). The infection of burn wounds with multiple organisms, with superadded problem of drug resistance, illustrates the need for a drug policy by the hospitals for burn patients. The isolated bacteria exhibited multiple resistance to antibiotics (6). Burns provide a suitable site for bacterial multiplication and are more persistent richer sources of infection than surgical wounds, mainly because of the larger area involved and longer duration of patient stay in the hospital (7). *Pseudomonas aeruginosa* is a major agent of hospital-acquired infections, and a pathogen of immunocompromised, cystic fibrosis and burn patients. It uses a type III secretion system for the injection of toxins directly into host cells, through a translocon assembled in the host cell membrane (8). There is currently no approved vaccine against *P. aeruginosa*, the major cause of morbidity and mortality in cystic fibrosis (CF) patients and a major pathogen in ventilated and burn patients. In a previous study, it was demonstrated that the immunization of mice with OprF311–341–OprI–type A- and B-flagellin fusion proteins dramatically enhanced clearance of nonmucoid P. aeruginosa in young (4–6 months old) African green monkeys. Intramuscular immunization of African green monkeys with 1, 3, 10, or 30 μg of OprF311–341–OprI–flagellins generated robust antigen-specific IgG responses. In addition, immunization with OprF311–341–OprI–flagellins elicited high-affinity anti-flagellins, OprI, and OprF IgG that individually promoted extensive deposition of complement component C3 on *P. aeruginosa* and synergized to facilitate maximal C3 deposition (9). Risk factors for, and outcomes of *Pseudomonas aeruginosa* bacteraemia in adult burns patients have been identified (10). Rapid acquisition of multi-drug resistance by *P. aeruginosa* leads to high morbidity and mortality, especially in burn centers. Majority of imipenem and meropenem resistant isolates (85–100% and 76–100%) demonstrated cross-resistance to all the other antibiotics. Almost all of the antibiotic resistant isolates also showed cross-resistance to the majority of penicillins and cephalosporins with or without β-lactamase inhibitors, from which ticarcillin/clavulanate demonstrated this phenomenon at the highest level (11). Immunoglobulin derived from multiple donors has variable anti-pseudomonas antibody titers. An Ig-inactivated transgenic mice was reported to reconstitute with human immunoglobulin loci, to generate human Mab against a single serotype of *P. aeruginosa* lipopolysaccharide O-specific side chain (PS) (12). Since there is no vaccine available, passive antibody prophylaxis against protective epitopes is an alternative strategy to prevent *P. aeruginosa* infection. The aim of this study was to determine whether the antibodies raised in mice burn wound sepsis model by active immunization with a *P. aeruginosa* OprF protein have a protective effect against infection with *P. aeruginosa*.

Material and method

Expression and purification of OprF

The PCR primers F: 5′- TTAA AAGCTT ATGAAACTGAAGAACACCTTAG -3′ and R: 5′- TATA CTCGAG TTACTTGGCTTCRGCTT CT -3 were used to amplify the full length *OprF* gene (13). The PCR product was digested with *Hind* III and *XhoI* and cloned into pET28a (+) vector. OprF-pET28a construct was transformed into Escherichia coli BL21 (DE3) and expression was induced with 1 mM IPTG. The bacterial cells were centrifuged and harvested. Sonication was employed to lyse the pellet suspended in buffer (100 mM NaH2PO4, 10 m MTris. Cl, 8 M urea). Centrifugation at 12000×g for 30 min resulted in separation of the supernatant which was then analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant OprF supernatant was purified by Ni-NTA chromatography according to the supplier's (Qiagen) directions.

Burnt mouse model

6–8-week-old male BALB/c mice weighing 20-25 g were procured from Pasture Institute, Karaj, Iran. All murine procedures were approved by Shahed University Animal Care and Use Committee. The burn procedure was as previously described (14). Briefly, the hair is clipped from the backs of 22–25 g, male BALB/c mice, and the mice were anesthetized with methoxyflurane. A flame-resistant card is pressed against the shaved area, the exposed back is covered with 0.5 ml ethanol, which is ignited and allowed to burn for 10 s. The mice are immediately given 0.5 ml saline for fluid resuscitation. This procedure produces a 15% total body surface area non-lethal third degree burn, which immunosuppresses the host, reducing an LD50 of *P. aeruginosa* from >106 cfu to an LD90–100 of 102–103 cfu.

The animals were divided into four different groups of 10 mice each. The mice were injected according to the regimen described below: Group 1: Negative control with burn trauma only; Group 2: Positive control with burn and infection; Group 3: Test group immunized with OprF followed by burn and infection; Group 4: Test group immunized with OprF followed by burn without infection. The BALB/c mice were immunized intraperitoneally (IP). On days 0, 15, 30 and 45, 10 µg of the recombinant protein was injected subcutaneously to two groups of 15 mice per group using Freund's complete adjuvant (Razi Vaccine and Serum Research Institute, Karaj, Iran) at the first dose and incomplete adjuvant in the subsequent doses. Blood samples were collected prior to immunisation and 2 weeks after each immunisation. Sera were collected from the retracted clot, and clarified by centrifugation (6500 ×g). Sera were aliquots (0.5 ml) and stored at -70 oC. Results were demonstrated as the percentage of the number of survivors/the total number of mice challenged in each group.

Determination of antibody titers

An enzyme-linked immunosorbent assay (ELISA) was used for analysis of antibodies of the immunoglobulin G (IgG) class elicited in response to OprF. The sera were collected 15 days after each immunization. Unimmunized mice sera were used as control. 96-well microtiter plates coated with 5 mg purified recombinant OprF protein were incubated at 4°C overnight. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST). 100 µl/well of mouse serum serial dilutions from 1:250 to 1:10000, were then added to the wells and blocked with 5% skimmed milk. After washing three times with PBST, the plates were incubated at 37°C/1 hr with 100 µl per well of horseradish peroxidase conjugated (HRP-conjugated) secondary antibody already diluted to 1:1000 with PBST. The wells of the plates were then washed three times with PBST.

A 100 µl of tetramethylbenzidine (TMB) solution were added to each well and incubated for 20–30 min at 37°C in dark. The reaction was stopped by addition of 2 M H2SO4. In order to measure stability of the antibody titer, samples were collected after 2 weeks, 1-4 months after immunization or bacterial inoculation.

Western blot analysis

SDS-PAGE in 12% gel was used to separate the protein samples which were then transferred to nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris–base, 0.037% SDS, and 20% methanol). Ten ml blocking buffer (5% skim milk in PBST [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and 0.05% (v/v) Tween-20]) was used to incubate the membrane at room temperature under constant shaking for 1 hr. The membrane was washed 3 times with PBS-T and incubated again in a 1:1000 dilution of mice anti-His-tag -conjugated HRP in PBS-T for 1 hr at room temperature. The membrane washed with PBS-T. To visualize the membrane, 3, 3′-diaminobenzidine for 1 hr at room temperature was used.

Preparation of bacterial inocula

For bacterial challenge, the *P. aeruginosa* strain isolated from a hospitalized burn patient was grown overnight on brain–heart infusion (BHI) agar plates. The bacteria from these plates were incubated on BHI broth under agitation (200 rpm) at 37 oC for 3–4 h for desired optical density (OD620 of 0.2). The bacterial cells were then harvested by centrifugation and resuspended in sterile BHI broth. The number of infected bacteria was calculated by plating serial dilutions of the injected inocula onto MacConkey agar plates. An inoculum of 5×105 cfu (1000×LD50) was administered in a 10µl volume subeschar at the burn site. Animals survival was monitored for one week.

Determination of lethal dose (LD_{50}) in mice

The 50% lethal dose (LD50) was determined in the following manner: Bacteria at doses ranging from l04 to l010 CFU/0.5 ml mixed with equal volume of PBS were administered subcutaneously to four groups of ten BALB/c mice per group. LD50 was estimated by the number of survivals on day 5.

Bacterial load in skin and internal organs

To assess bacterial load at infection location and in internal organs, the mice were sacrificed 24 h after subeschar infection with *P. aeruginosa*. Aseptically, skin (burnt skin and the surrounding edges of normal skin, 15 mm × 15 mm), blood, spleen and liver were harvested. Each biopsy sample was weighed, diced, ground and finally homogenised in saline. For bacterial enumeration, serial 10-fold dilutions of the blood and homogenates were prepared using PBS. Then, 1ml portions of diluted suspensions were plated in triplicate. Plates were then incubated at 37 oC, and the colonies were counted after 24h. Results were calculated as log cfu per g of wet weight for infected organs.

Statistical analysis

The software Statistical Package for Social Sciences (SPSS) 16.0 was used for statistical analysis. All data were expressed as the mean ± S.D. Data were analysed using one-way analysis of variance (ANOVA) and Student's t-test (Statview). A P value less than 0.05 was considered significant.

Result

Isolation of the OprF gene and expression of OprF protein

The expected size of the PCR product for OprF was about 1053 bp. A 38 kDa recombinant OprF protein was successfully expressed using IPTG as inducer (Figure 1).

Western blotting

Western blot using anti-His-tag antibodies confirmed expression of the recombinant protein (Figure 2).

Serological responses

The antibody titers of sera from immunized mice showed a significant ($P < 0.001$) rise after the third booster as compared to the control group injected with adjuvant (Figure 3). Determination of LD50 and animal challenge with *P. aeruginosa*. LD50 was determined for unimmunized, burnt and immunized mice as 3×105, 5×102 and 5×108 CFU respectively by IP. The immunization with the recombinant protein induced protection against high doses (1000 fold LD50) of *P. aeruginosa*.

Determination of LD50 and animal challenge with P. aeruginosa

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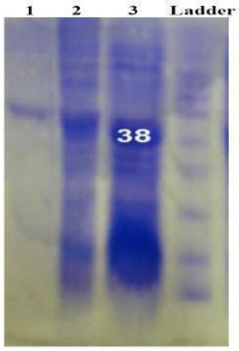

Figure 1: The 38 kDa recombinant OprF protein expressed using IPTG as inducer. Lane 1: Recombinant OprF purified by Ni-NTA; Lanes 2 and 3: Recombinant OprF protein prior to purification

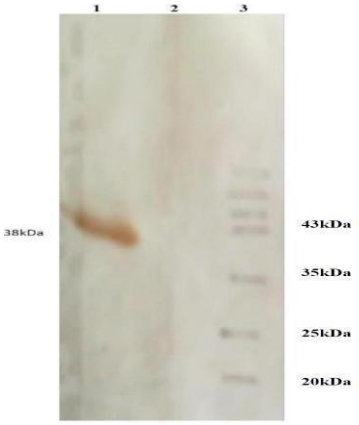

Figure 2: Western blot analysis of recombinant OprF using anti His tag. Lane 1 Purified recombinant OprF; Lane 2: Uninduced bacterial cell lysate; Lane 3: Protein weight markers

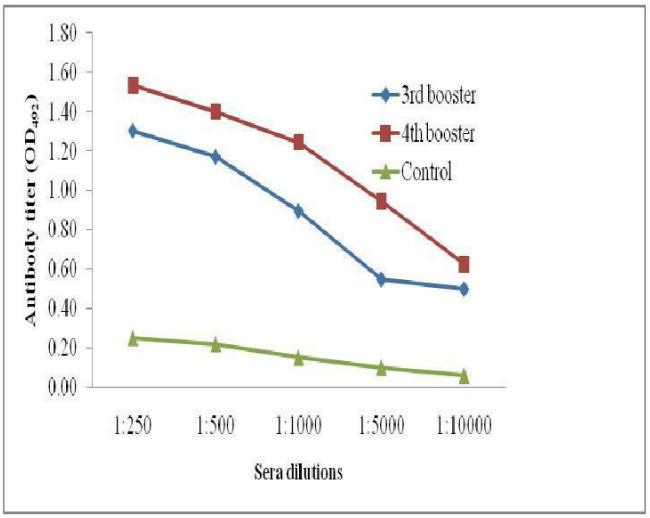

Figure 3: Indirect ELISA of serum from mice immunized with OprF protein and control group showing significant difference (P-value <0.001)

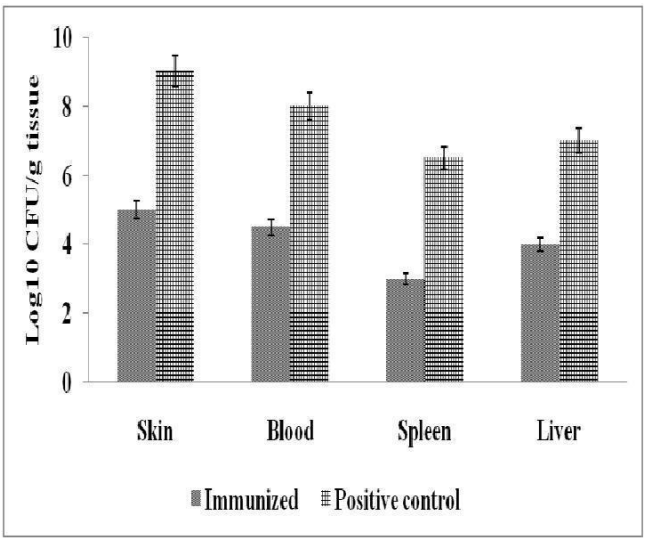

Figure 4: Local and systemic spread of *P. aeruginosa* in immunized and control burnt mice sepsis models.

Protective effects of OprF protein against P. aeruginosa infection

To study the protective efficacy of OprF protein in the survival of infected mice, we used a burnt mouse model against a lethal murine mode of *P. aeruginosa* infection. In this model, burn injury itself did not cause mortality in negative control group (Group 1). The infectious dose for the in vivo studies was 5×10^2 cfu per. There was no protection in the positive control group (Group 2) and all the mice died. The antibody against OprF brought about 100% survival following challenge with *P. aeruginosa* strain in test group (Group 3). Group 4 i.e. test group immunized with OprF followed by burn without infection survived.

Quantitative estimation of bacteria in skin and internal organs

In order to investigate the efficacy of the antibody in the inhibition of *P. aeruginosa* from surface wound infection to internal organs, the bacterial loads in skin, blood, spleen and liver were enumerated 24 h after the inoculation. The immunized mice showed significant decrease in load in the skin, blood, liver and spleen Bacterial compared to the positive control group ($p<0.001$) (Figure 4).

Discussion

Increasing antibiotic resistance in *P.aeruginosa* is a major therapeutic concern. That is why the majority of research work is focused on developing an effective vaccine. It is well known that burn patients suffer from an impaired immune system, which may lead to systemic inflammatory response syndrome or to immunosuppression. LPS-based vaccines or vaccines produced by

extraction of antigens from whole bacterial organisms may induce the liberation of inflammatory cytokines which may cause local or systemic side effects (15). The present study analyzes the anti-*P. aeruginosa* immunity elicited by an outer membrane protein F (OprF) of *P. aeruginosa*. The results indicated that immunization of mice with OprF induced significant elicitation of anti-OprF IgG. A recombinant OprF–OprI vaccine against *P. aeruginosa* based on recombinant outer membranes has been developed. After intramuscularly injecting into patients with severe burns, antibodies against *P. aeruginosa* were induced. Vaccination was well tolerated. Intranasal application of the vaccine into volunteers induced specific s-IgA antibodies. It has been concluded that the newly developed vaccine may be suitable for protection of the main risk groups of *P. aeruginosa* infections. In particular, for the protection of burn patients and patients with cystic fibrosis (16). Recently a report was published on the protection against an extracellular bacterial pathogen using a DNA vaccine encoding the outer membrane protein F gene (oprF) of *P. aeruginosa*. This vaccine afforded protection upon subsequent challenge with the bacterium in a mouse model of chronic pulmonary infection. The oprF vaccine provided significant protection not only against the development of severe lung lesions (present in 43% of vaccinated mice versus in 76% of control mice; P = 0.002) but also by promoting the clearance of the inoculated challenge organisms (sterile lungs in 50% of vaccinated mice versus in 29% of control mice; P = 0.035). From that study it was apparent that the route of immunization (i.d. by gene-gun versus i.m. injection) dramatically affected the efficacy of the DNA vaccine (17). In the present study we observed no fatal effect of 1000 fold LD50 dose of P. aeuginosa in the immunized group.

The increased protective ability of the oprF/I vaccine compared to the oprF vaccine may be due in part to the presence of antibodies directed against the second immunogen (OprI) (17). Vaccination against *P. aeruginosa* is a desirable, yet challenging strategy for prevention of airway infection in patients with cystic fibrosis. Formation of antibodies in lower airways induced by systemic and mucosal vaccination strategies were compared by immunising 48 volunteers in six vaccination groups with either a systemic, a nasal, or four newly constructed oral live vaccines based on attenuated live *Salmonella* (strains CVD908 and Ty21a), followed by a systemic booster vaccination. All vaccines were based on a recombinant fusion protein of the highly conserved *P. aeruginosa* outer membrane proteins OprF and OprI as antigen. While systemic and mucosal vaccines induced a comparable rise of serum antibody titers, a significant rise of IgA and IgG antibodies in the lower airways was noted only after nasal and oral vaccinations. Nasal and oral OprF-OprI vaccines are promising candidates for development of antipseudomonal immunisation through inducing a specific antibody response in the lung (18). For efficacious vaccine development against *P. aeruginosa*, the immunogenicity of multivalent DNA vaccine has been evaluated. Three different plasmids each targeting a fusion of outer membrane proteins (*OprF*/*OprI*), a protein regulating type III secretion system (PcrV), or an appendage (PilA) were prepared and mice were immunized with single (monovalent) or a combination of these plasmids (multivalent) *via* intramuscular electroporation (imEPT) or gene gun. Immunization with multivalent DNA vaccine *via* imEPT induced the most potent protection against lethal pneumonia.

P. aeruginosa genome. SigX is involved in the transcription of *oprF*, encoding the major outer membrane protein. OprF is a pleiotropic porin that contributes to the maintaining of the wall structure, and is essential to *P. aeruginosa* virulence. Proteomic investigations have revealed lower production of 8 porins among which 4 gated channels involved in iron or hem uptake, OprF, and the three substrate-specific proteins OprD, OprQ and OprE (20). Bacterial load was determined in skin, blood, spleen and liver at 24 h after the inoculation and treatment. A significant ($p<0.001$) decrease in bacterial load was observed in the skin, blood and internal organs of the immunized mice infected with *P. aeruginosa* as compared to positive control group. The data suggest that anti OprF IgG plays an important role in inhibiting the systemic spread of *P. aeruginosa* from infection site to internal organs. Based on our results, OprF protein is effective in mice and nonhuman primates and thus merit additional development as a potential vaccine for use in humans. Immunization with OprF protein can develop protection against *P.aeruginosa* infections. OprF may be exploited in protective measures in *P.aeruginosa* infections. This observation and the fact that the majority of patients in other risk groups such as transplant patients or patients undergoing major surgery can be easily vaccinated before the onset of treatment make vaccination against *P. aeruginosa*, as well as against other nosocomial pathogens, a promising tool to reduce the high incidence of infections and decrease the high costs of intensive care treatment (21).

Conclusion

The results suggest that anti-*P. aeruginosa* OprF antibodies elicited in burn wound sepsis model by active immunization are protective against infection with *P. aeruginosa*, and provide a rational for further development of the vaccine for prevention against *P. aeruginosa* infection in burn patients.

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Conflict of interest

None declared conflicts of interest.

References

1. Macedo JLSd, Santos JB. Predictive factors of mortality in burn patients. Revista do Instituto de Medicina Tropical de Sao Paulo 2007; **49**: 365-70.
2. Alp E, Coruh A, Gunay GK, *et al*. Risk factors for nosocomial infection and mortality in burn patients: 10 years of experience at a university hospital. *J Burn Care & Res* 2012; **33**: 379-85.
3. Rodgers GL, Mortensen Joel, Fisher MC, *et al*. Predictors of infectious complications after burn injuries in children. *Ped Infect Dis J* 2000; **19**: 990-5.
4. Armour AD, Shankowsky HA, Swanson T, *et al*. The Impact of Nosocomially-Acquired Resistant *Pseudomonas aeruginosa* infection in a burn unit. *J Trauma Inj Infect Crit Care* 2007; **63**: 164-71.
5. Abdel RA, Hafez SF, Abdelhakam SM, *et al*. Antimicrobial resistant bacteria among health care workers in intensive care units at Ain Shams University Hospitals. *J Egypt Soc Parasitol* 2010; **40**: 71-83.
6. Roberts JA, Kruger P, Paterson DL, et al. Antibiotic resistance-Whats dosing got to do with it. *Crit care Med* 2008; **36:** 2433-40.

7. Agnihotri N, Gupta V, Joshi RM. Aerobic bacterial isolates from burn wound infections and their antibiograms GC; a five-year study. *Burns* 2004; **30**: 241-3.
8. Wager B, Faudry E, Wills T, *et al*. Current Fluctuation Analysis of the PopB and PopD Translocon Components of the Pseudomonas aeruginosa Type III Secretion System. *Biophys J* 2013; **104**: 1445-55.
9. Weimer ET, Ervin SE, Wozniak DJ, *et al*. Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-flagellin fusion proteins promotes the production of protective antibodies against nonmucoid *Pseudomonas aeruginosa*. *Vaccine* 2009; **27**: 6762-9.
10. Mahar P, Padiglione AA, Cleland H, *et al*. Pseudomonas aeruginosa bacteraemia in burns patients: Risk factors and outcomes. *Burns* 2010; **36**: 1228-33.
11. Japoni A, Alborzi A, Kalani M, *et al*. Susceptibility patterns and cross-resistance of antibiotics against Pseudomonas aeruginosa isolated from burn patients in the South of Iran. *Burns* 2006; **32**: 343-7.
12. Lai Z, Kimmel R, Petersen S, *et al*. Multi-valent human monoclonal antibody preparation against Pseudomonas aeruginosa derived from transgenic mice containing human immunoglobulin loci is protective against fatal pseudomonas sepsis caused by multiple serotypes. *Vaccine* 2005; **23**: 3264-71.
13. Kaloshin AA, Gatypova EV, Mikhailova NA. Obtaining recombinant forms of the outer membrane protein F of Pseudomonas aeruginosa and assessment of their immunogenic properties . *Appl Biochem Microbiol* 2011; **47**: 780-8.
14. Neely AN, Holder IA. A murine model with aspects of clinical relevance for the study of antibiotic-induced endotoxin release in septic hosts. *J Endotoxin Res* 1996; **3**: 229-35.
15. Lee NG, Jung SB, Ahn BY, *et al*. Immunization of burn-patients with a Pseudomonas aeruginosa outer membrane protein vaccine elicits antibodies with protective efficacy. *Vaccine* 2000; **18**: 1952-61.
16. Baumann U, Mansouri E, von Specht BU. Recombinant OprF-OprI as a vaccine against Pseudomonas aeruginosa infections. *Vaccine* 2004; **22**: 840-7.
17. Price BM, Galloway DR, Baker NR, *et al*. Protection against Pseudomonas aeruginosa Chronic Lung Infection in Mice by Genetic Immunization against Outer Membrane Protein F (OprF) of P. aeruginosa. *Infect Immun* 2001; **69**: 3510-5.
18. Bumann D, Behre C, Behre K, *et al*. Systemic, nasal and oral live vaccines against Pseudomonas aeruginosa: A clinical trial of immunogenicity in lower airways of human volunteers. *Vaccine* 2010; **28**: 707-13.
19. Saha S, Takeshita F, Sasaki S, *et al*. Multivalent DNA vaccine protects mice against pulmonary infection caused by Pseudomonas aeruginosa. *Vaccine* 2006; **24:** 6240-9.
20. Duchesne R, Bouffartigues E, Oxaran V, *et al*. Proteomic approaches of SigX functions in Pseudomonas aeruginosa outer membrane composition. *J Proteomics* 2013; **94:** 451-9.
21. Mansouri E, Blome-Eberwein S, Gabelsberger J, *et al*. Clinical study to assess the immunogenicity and safety of a recombinant Pseudomonas aeruginosa OprF-OprI vaccine in burn patients. *FEMS Immunol Med Microbiol* 2003; **37**:161-6.