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Active Immunization with Recombinant PilA protein Protects Against *Pseudomonas aeruginosa* Infection in a Mouse Burn Wound Model

Fatemeh Korpi¹, Gholamreza Irajian²*, Mehdi Mahdavi³, Mohammad Motamedifar⁴, Mehdi Mousavi⁴, Parisa Laghaei⁵, Negin Raei⁶ and Bahador Behrouz⁶*

¹Department of Microbiology, Iran University of Medical Sciences, International Branch, Tehran, Iran
²Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran
³Department of Immunology, Pasteur Institute of Iran, Tehran, Iran
⁴Department of Bacteriology and Virology, Shiraz University of Medical Sciences, Shiraz, Iran
⁵Department of Biology, Mohaghegh Ardabili University, Ardabil, Iran
⁶Department of Microbiology, Tehran University of Medical Sciences, Tehran, Iran

* Corresponding author: Department of Microbiology, Iran University of Medical Sciences, Telephone and Fax: +982188058649, E-mail: dr.irajian@gmail.com.

* Corresponding author: Department of Microbiology, Tehran University of Medical Sciences, Telephone and Fax: +982188955810, E-mail: bahador.behroz3@gmail.com.

Running title: *P. aeruginosa* pilin as a vaccine candidate
Abstract

Pseudomonas aeruginosa as an opportunistic human pathogen that causes lethal infections in immunocompromised patients. Type IV pili are critical factors in virulence and colonization of P. aeruginosa in acute burn wound infection. The immunogenicity and efficacy of P. aeruginosa recombinant PilA (r-PilA) was evaluated in an experimental model of burn wound sepsis as a vaccine candidate. In this study, female C57BL/6 mice were divided into five groups. Mice in the experimental groups received either r-PilA vaccine alone or in combination with the alum adjuvant or complete Freund's adjuvant (CFA). Mice in the negative control group received phosphate-buffered saline (PBS). In order to characterize the response of Th1-Th2 to immunization, the cytokine profiles of spleen cells isolated from r-PilA immunized mice were investigated. Total IgG titers and isotopes were measured using ELISA method and finally, in order to study the systemic infection, bacterial titers in the liver, spleen and blood were also determined. Active immunization with r-PilA, which is followed by two booster shots, was sufficient to generate a robust immune response in mice. Cytokine analysis demonstrated the secretion of IL-4 and INF-γ from splenocytes in response to in vitro antigen stimulation. The IgG response to r-PilA was a Th2 type response consisting predominantly of the isotype IgG1 accompanied by lower levels of IgG2a. In conclusion, in this burned mouse model, vaccination with r-PilA can increase the humoral immunity, thereby leading to an effective protection against P. aeruginosa infection.

Keywords: Pseudomonas aeruginosa, recombinant PilA, immunization, burn
**Introduction**

*Pseudomonas aeruginosa* is a well-known colonizing microbial pathogen for cystic fibrosis (CF) patients and a common infectious agent in nosocomial infections, including patients with severe burns, cancer, transplantations, acquired immunodeficiency syndrome (AIDS), and other immunocompromising conditions [18]. *P. aeruginosa* burn wound infection, due to its rapid proliferation within the injured tissues, bacteremia, and septic shock, has been associated with high rates of mortality and morbidity [10, 40]. It has been estimated that 75% of all deaths caused by burns are related to infection [10]. It is therefore important to develop an effective new therapeutic vaccine that is important for the treatment of *P. aeruginosa* infection, because of the effective resistance mechanisms to commonly used antibiotics and high occurrence of multi-drug resistant (MDR) strains [10, 48]. The polar pili of *P. aeruginosa* strains belong to a class of type IV pili, also known as N-methylphenylalanine pili, which are responsible for twitching motility, adhesion, and invasion of tissue [20]. The pilus filament is composed of pilin protein, which is encoded by the *pilA* gene [25]. Most of the structural and functional features of pilin are determined by the N- and C-terminal regions, which are involved in Structural determination and receptor binding [12, 14, 22]. The C-terminal disulphide-bonded loop of PilA subunits mediate binding to asialo-GM1, asialo-GM2 and lactosylceramide [16, 21, 33, 34, 46]. Previous studies have demonstrated the importance of pili in the invasion of *P. aeruginosa* to damaged tracheal cells, indicating that pili are crucial virulence factor in the initial colonization of the immunocompromised host tissue [53]. In the animal model of *P. aeruginosa* acute pneumonia and corneal infections, non-piliated mutants showed a decrease in virulence with a reduced ability to colonize the host tissues [6, 11, 20, 25]. It has also been shown 90% of the adherence to host cells mediates via pili and antibody raised against it inhibited binding to epithelial cells [9, 14, 41, 50]. There is no report on the protective efficacy of full-length r-PilA of *P. aeruginosa* in
the burn wound sepsis model. The purpose of this study was to determine the immunogenic potential of the r-PilA vaccine candidate against *P. aeruginosa* burn wound infection. Important implications of this study are the development of humoral immune responses to r-PilA following active vaccination and to determine *in vitro* the protective activity of elicited antibody.

**Materials and Methods**

**Bacterial strains, vector, and media**

*P. aeruginosa* strain PAO1 was obtained from Accidents and Burns Hospital, Tehran, Iran. This strain was used for the challenge and *in vitro* experiments. *Escherichia coli* TOP10F’ and *E. coli* BL21 (DE3) were used as preservation and expression bacterial hosts. pET22b(+) (Novagen, USA) was used as an expression plasmid. Luria Bertani (LB) medium (Merck, Germany), tryptic soy broth (TSA) (Merck, Germany) and tryptic soy agar (TSA) (Merck) were used for routine cultivation of all bacterial strains.

**Cloning and expression of pilA gene**

Specific primers were designed for pilA sequences of *P. aeruginosa* strain PAO1 from NCBI (GenBank Accession No: NC_002516.2), including a forward 5' - CTCGGATCCACTGATGATCGTGGTTGCGA-3' and reverse 5' - ACGAAGCTTCCGATCGATCGTGGTTGCGA-3'. *BamHI* and *HindIII* restriction sites were incorporated at 5' terminus of forward and reverse primers, respectively. Amplifications were carried out using *Pfu* DNA polymerase (Fermentas, Lithonia) with regular polymerase chain reaction (PCR) ingredients [19]. Briefly, predenaturation was carried out at 94 °C for 1 min,
followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension
at 72 °C for 10 min. The purified pilA fragment was digested and ligated into the BamHI and
HindIII sites of the pET22b vector, which provided six His residues at the N-terminus of the
expressed protein. The ligation product was transformed into competent E. coli TOP10F’ and the
transformants were selected on LB agar plates containing 100 µg ampicillin/ml. Screening of the
transformants was performed using PCR and restriction analysis. Positive pilA clones were
sequenced for analysis of the sequence integrity.

Expression and purification of r-PilA

E. coli BL21 (DE3) carrying pET22b-pilA was grown in 1 L of LB broth, supplemented with 100
µg/ml ampicillin. After the culture had reached an OD 600 nm of 0.4–0.8, the expression of r-
PilA was induced by adding to the cultures 1 mM of isopropyl β-D-1-thiogalactopyranoside
(IPTG; Sigma, USA). After 4 h of induction, the bacterial cultures were pelleted and dissolved in
lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF; pH 8.0).
The suspension was subsequently centrifuged and supernatant was dissolved in buffer B (100
mM NaH2PO4, 10 mM Tris–HCl, and 8 M urea; pH 8) and the debris was omitted by
centrifugation (18,000×g, 30 min, 25 °C). Ni–NTA agarose (QIAGEN) was added to the lysate
and incubated on a rotary shaker at room temperature for 1 h. The mixture was applied to a Ni–
NTA column and followed by washing with buffer C (100 mM NaH2PO4, 10 mM Tris–HCl,
and 8 M urea; pH 6.3). Urea was removed stepwise using washing buffers with decreasing urea
concentrations (8, 6, 4, 2, 1, and 0 M urea). Finally, the r-PilA was eluted with buffer E (50 mM
NaH2PO4, 300 mM Tris–HCl, and 250 mM imidazole; pH 4.5). In order to remove imidazole,
the protein solution was dialyzed against phosphate buffered saline (PBS, pH 7.4) overnight. The
total amount of purified solubilized protein from 1 L of bacterial culture was quantitatively measured using a NanoDrop 2000 spectrophotometer system (Thermo Scientific, USA). The r-PilA protein was tested for lipopolysaccharide (LPS) contamination using a commercially Pierce™ LAL Chromogenic Endotoxin Quantitation Kit.

**Immunobloting**

Western blot was performed as previously described [1]. Purified protein sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% polyacrylamide mini-gels. This protein was electrotransfered to polyvinylidene fluoride (PVDF; Invitrogen) membranes in a semi-dry transfer cell at 15 volts/10 min (Trans-Blot®SD, Bio-Rad). Ponceau S staining was used to check protein transfer. Lanes on membranes were cut to 3-mm-wide strips which were de-stained by distilled water and washed with tris-buffered saline (TBS-T) that consist of 20 mM Tris–HCl (pH 7.4), 0.5 M NaCl, and 0.05% Tween 20. Then, membranes were blocked for 1 h in TBS buffer containing 10% (w/v) bovine serum albumin (BSA, Sigma). Monoclonal anti-His tag antibody (Sigma, USA) was tested to evaluate its ability to recognize this r-PilA, with an horseradish peroxidase (HRP)-conjugated-anti-His (Sigma) diluted 1:10,000 as a secondary antibody. After adequate washes with TBS-T, strips were developed by enhanced chemiluminescence (ECL) substrate.

**Immunizations**

Female C57BL/6 mice (6-8 weeks) were purchased from Pasteur Institute (Tehran, Iran). All animal experiments were performed in compliance with the Animal Ethics Committee guidelines of Iran University of Medical Sciences. Female mice were divided into five groups. Group I was
immunized subcutaneously (s.c) at the multiple sites with 10 µg of adjuvanted r-PilA in alum.

Group II received r-PilA in CFA (1:1, Sigma) administered subcutaneously and boosted twice
with 10 µg of the purified r-PilA in incomplete Freund’s adjuvant (1:1). Group III was injected
with 10 µg of r-PilA alone with the same conditions. In the control groups (Group IV), mice
were injected with sterile PBS and alum adjuvant only. Non-immunized, that is, non-infected
burn mice were identified as burn control group (witness group). Mice were injected on days 0,
14 and 28 and blood samples were collected via retro-orbital bleeding 2 weeks after the last
immunization. The optimal dose for immunization was determined by preliminary titration (data
not shown). At day 48, mice were burned and challenged as previously described by Stieritz and
Holder [37]. In this model, back hairs of anesthetized mice were clipped and burn wound (12-15%
of the body surface area) induced with ethanol flame for 15 s. Immediately after the burn, 0.5 ml
of sterile saline solution was injected intraperitoneally to the mice as fluid replacement therapy.
The acetaminophen (0.25 mg/ml) was used as a post burn analgesic. After that, burned-mice
were infected by subcutaneous injection of PAO1 strain (3-5 ×10² colony forming unit) at the
burn site. The survival rate of experimental mice was monitored for one week. In addition, 24 h
after subeschar infection, five mice from each group were sacrificed. To determine local spread,
the skin sections (approximately 15 by 15 mm) were removed from the burned skin of infected
mice at the site of injection (inoculation site). To determine systemic spread, liver, spleen, and
blood of each animal were removed. Individual skin sections and internal organs were weighed
and homogenized in PBS. For bacterial enumeration, portions of the homogenates were plated in
triplicate on TSA and were incubated at 37°C for 24 h. The number of CFUs from each sample
was calculated per gram of tissue (CFUs/g).
**Cytokine assays**

Two weeks after the last immunization, the spleens of immunized mice were removed under sterile conditions and suspended in PBS containing 2% fetal bovine serum (FBS, Sigma). Red blood cells (RBCs) were lysed with RBC cell lysis buffer (eBioscience, San Diego, CA) and single-cell suspension of splenocytes (4×10⁶ cells/ml) and plated on RPMI 1640 (Gibco, Germany) supplemented with 5% FBS, 50 µM 2-mercaptoethanol (2ME, Sigma), 0.1 mM non-essential amino acid, 4 mM L-glutamine, 1 mM sodium pyruvate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), and 100 U of penicillin-streptomycin (Sigma). The cell suspensions were stimulated with 10 µg of r-PilA and cultured in 24-well plates (Nunc; Naperville, IL) for 72 h at 37°C in the presence of CO₂ (5%). After incubation, supernatants were harvested and kept at -70°C until ELISA for interleukin-4 (IL-4) and interferon-γ (IFN-γ) levels (eBioscience) was performed following manufacturers' instructions. The concentration of each secreted cytokine was calculated as pg/ml according to the standard curve.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was performed as previously described [13]. Briefly, microtiter plates (Nunc) were coated with 1µg the r-PilA (PBS, pH 7.4) and incubated at 4 °C overnight. Then, the plates were washed three times with 0.05% Tween-PBS (T-PBS). Blocking was carried out with PBS containing 3% (w/v) bovine serum albumin (BSA, Sigma) at 37 °C for 2 h. Mouse sera to be analyzed were incubated on plates overnight at 4°C. The plates were washed again three times with T-PBS, and then 100 µl of 1:7000 diluted goat anti-mouse conjugated to HRP (Sigma) was added. After 1 h incubation at room temperature, the plates were washed five times with T-PBS.
Then, 100 µl of the substrate, 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system (Sigma) was added into each well. After 30 min of color development at room temperature (RT), the reaction was stopped with 2 N H₂SO₄ and the optical density was read at 405 nm. The IgG isotypes in mice sera were determined using a procedure similar to the one described earlier. Isotype-specific secondary anti-mouse IgG1 and IgG2a conjugated to HRP (Sigma) were added at a 1:8000 dilution (100 µl/well) in place of the standard HRP conjugated anti-mouse IgG. The ELISA data were presented by use the SOFTmax PRO software (Molecular Devices Corp., Sunnyvale, CA).

**Opsonophagocytic assay**

The opsonophagocytic assay was carried out according to as described previously [2]. Briefly, *P. aeruginosa* strain PAO1 was cultured in 3 ml TSB at 37°C until they reached an optical density of 0.2 at 650 nm. Isolated mouse peritoneal macrophages were counted and re-suspended at a concentration of 2×10⁹ in RPMI-1640 with 10% FBS and fresh infant rabbit serum was used as a complement source. Different dilutions of heat-inactivated mouse sera (1:2 to 1:8) were used as opsonic antibody. These components were mixed in sterile microfuge tubes. The opsonophagocytic activity of immune sera was compared with pre-immune serum. Negative control tubes, from which antibody, complement or macrophages were omitted, and 100 ml RPMI medium/fetal calf serum was substituted, were run with each assay. Following 90 min of incubation, 50 µl of the mixture was removed, diluted in 225-µl saline containing 0.05% Tween 20, and finally plated for bacterial enumeration. Normal mouse serum (NMS) (1:2 dilution) was used as pre-immune serum (control IgG). Each experiment was performed in triplicate. The opsonic activity of the serum was calculated as follows:
Opsonophagocytic activity = \[1 - (\text{CFU of immune serum at 90 min} / \text{CFU of pre-immune serum at 90 min})\] \times 100.

**Statistical analysis**

For statistical analysis, the Statistical Package for Social Science (SPSS 21.0; SPSS Inc, Chicago, Illinois, USA) and GraphPad Prism 6 softwares were used. Titers for the ELISA data were determined using linear regression of the reciprocal dilution for the average of two replicates. The endpoint titer is the x intercept of this line. Differences in bacterial loads and ELISA result were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test. Analysis of the mean opsonic activity triplicate samples of serum were compared using Student’s t-test (Statview). Results were displayed as mean ± standard deviation (SD). For survival studies, data were analyzed using Kaplan-Meier curves and the log-rank test. A \( p \)-value of < 0.05 was considered statistically significant.

**Results**

**Cloning and expression of r-PiLA**

Electrophoresis of the PCR product resulted in a single band with an approximate size of 500 bp, which confirms amplification of the \( pilA \) gene. Existence of the insert (\( pilA \)) in recombinant vector (pET-22b/\( pilA \)) was also proved by digestion using \( BamHI \) and \( HindIII \) restriction enzymes. Finally, DNA sequencing confirmed the identity and orientation of the \( pilA \) gene in the recombinant construct (data not shown). \( E. \) \( coli \) BL21 (DE3) was transformed with recombinant plasmid pET22b/\( pilA \) containing a T7 promoter as the expression host. Protein production was
induced with IPTG (1 mM). SDS-PAGE gel detected approximately 18 kDa proteins after staining with coomassie blue G-250 (Fig. 1). The product was purified by Ni–NTA affinity chromatography under native conditions (Fig. 1). Significantly high amounts of the recombinant protein were obtained via denaturing procedures. This purification approach yielded 28 mg of highly purified r-PilA from 1 L of the induced culture. The concentration of LPS found in this protein was lower than 0.1 EU/ml. Recombinant protein and recombinant plasmid pET22b/pilA were detected in purified form in the crude cell lysate of induced cultures using anti-His monoclonal antibody (Fig. 2).

Cytokine response to r-PilA

In order to evaluate r-PilA in the induction of specific humoral response, IL-4 and IFN-γ cytokines responses were investigated. As shown in Fig. 3A-B, mice immunized with r-PilA with adjuvants contained high levels of IL-4 and IFN-γ cytokines \( (p < 0.006) \) compared with the other groups; however, no significant difference in IL-4 and IFN-γ cytokine productions was observed between r-PilA/alum and r-PilA/CFA immunized groups \( (p > 0.05) \). Mice immunized with the r-PilA alone, produced significantly more IL-4 and IFN-γ cytokines when compared with the control group (PBS plus alum) \( (p < 0.003) \) (Fig. 3A-B).

Antibody Response to r-PilA

In order to determine the specific antibodies induced by r-PilA with or without adjuvants in vaccinated mice, mice were bled retro-orbitally two weeks after the last injection and the sera were analyzed by indirect ELISA. As shown in Fig. 4A, sera from r-PilA injected mice with
alum induced higher levels of anti-PilA IgG compared with other groups \((p < 0.04)\). The IgG response in mice serum that received r-PilA and CFA or r-PilA alone were higher than those observed in the mice that was injected PBS with alum \((p < 0.009)\); however, no significant difference in IgG titers was observed between r-PilA/CFA and r-PilA immunized groups \((p > 0.05)\). As shown in Fig. 4B, the sera of mice immunized with r-PilA and alum adjuvant contained high levels of IgG1 subtype compared with the other groups \((p < 0.001)\). The group of mice that received the r-PilA with CFA or r-PilA alone showed high level of IgG1 response compared with the control group \((p < 0.0001)\) (Fig. 4B); however, no significant difference in IgG1 levels was observed between r-PilA/CFA and r-PilA immunized groups \((p > 0.05)\). Mice vaccinated with r-PilA with CFA had significantly more IgG2a response compared with the other groups \((p < 0.004)\) (Fig. 4C). Mice immunized with r-PilA with alum or r-PilA alone contained high level IgG2a subtype compared with the control group \((p < 0.0001)\) (Fig. 4C); however, no significant difference in IgG2a levels was observed between r-PilA/alum and r-PilA immunized groups \((p > 0.05)\).

### Opsonophagocytic killing activity

The opsonophagocytic assay was performed to determine opsonic potential activity of mouse sera against \(P. \text{aeruginosa}\), by incubating bacterium with mouse macrophages in the presence of rabbit complement and different dilution of immunized mice serum. The addition of serum obtained from immunized mice with r-PilA with alum adjuvant at a dilution of 1:2 promoted phagocytosis of \(P. \text{aeruginosa}\) and the number of viable bacterial cells decreased over 46.61% after 90 min as compared with the control group \((p < 0.001)\) (Fig. 5). When serum (at a dilution
of 1:2) obtained from immunized mice with r-PilA with CFA or r-PilA alone, was treated with 
the strain PAO1, 24.50% and 24.81 opsonic killing were detected, respectively ($p < 0.0006$, 
versus results for control group) (Fig. 5); however, the differences between the r-PilA/CFA and 
r-PilA immunized groups were not statistically significant ($p > 0.05$). In the presence of pre-
immune sera (as negative control) the number of viable bacterial cells was slightly reduced 
(approximately 3 %) (Fig. 5).

**Bacterial load in the skin and internal organs**

The protective efficacy of r-PilA with or without adjuvants was determined by measuring the 
bacterial cell count in the liver, spleen, blood, and skin of mice, 24 h after infection. The 
bacterial loads in the liver and spleen of the r-PilA with adjuvants immunized mice infected with 
PAO1, were significantly fewer than in the organs of the other groups ($p < 0.04$) (Fig. 6A-B);
however, no significant difference was observed between r-PilA/alum and r-PilA/CFA 
immunized groups ($p > 0.05$). Bacterial titers in the liver and spleen of r-PilA-vaccinated was 
significantly lower than those of the control group ($p < 0.001$) (Fig. 6A-B). Also, immunization 
of mice r-PilA with adjuvants resulted in a decrease of bacterial burden in the blood as compared 
with the other groups ($p < 0.005$); however, no significant difference was observed between r-
PilA/alum and r-PilA/CFA immunized groups ($p > 0.05$) (Fig. 6C). Immunization with r-PilA 
significantly decreased bacterial titer in the blood of the immunized mice infected with PAO1 
compared with the control group ($p < 0.006$) (Fig. 6C). The bacterial titers in the skin of the 
immunized mice (groups 1 to 3) also diminished, but these were not statistically significant ($p > 
0.05$) compared to the PBS-alum control group (Fig. 6D).
Survival analysis

As determined by ELISA, r-PilA was highly immunogenic via subcutaneous administration route and induces a strong antibody response. The antisera raised to r-PilA with alum resulted 71.42% survival (Fig. 7), compared to the PBS-alum control group ($p < 0.009$). Active immunization with r-PilA and CFA or r-PilA alone caused 57.14% survival (Fig. 7), compared with the mice in the control group ($p < 0.02$). A significant difference was observed between groups, based on statistical assessment of survival rates ($p < 0.002$).

Discussion

The skin barrier is compromised after severe burns and it opens up an opportunity for *P. aeruginosa* to infect the moist tissue underlying burn wounds [35]. Broad spectrum antimicrobial therapy that was used in burn units, resulted in the emergence of multi-drug resistant (MDR) *P. aeruginosa* strains, and the shrinking number of effective anti-pseudomonal antibiotics needed for new effective therapeutic vaccine increases since treatment of infections are facing serious challenges [29].

Previous studies have revealed that pili mediated enhancement of virulence of *P. aeruginosa* were attributed to enhanced colonization of bacterium at the burned skin surfaces [44]. Due its important virulence properties; consequently, we used first time r-PilA as a vaccine candidate against *P. aeruginosa* infection in the burned-mouse model.
In this study, the r-PilA was successfully cloned, expressed and characterized. The PilA gene was isolated from *P. aeruginosa* PAO1 genome and then cloned in pET-22b expression vector. When recombinant vector was induced, SDS-polyacrylamide gels showed an apparent molecular mass of 18 kDa. PCR and sequencing analysis revealed that our specific designed primers amplified an approximately 500 bp length fragment that is in accordance with PilA gene. In another study using oligonucleotides probes which corresponded to a preserved region of *P. aeruginosa* pilin showed that products of cloned *P. aeruginosa* gene that were expressed in *E. coli* were localized to different parts than in *P. aeruginosa* [49]. It has been demonstrated that *P. aeruginosa* pilin has been synthesized in *E. coli* cytoplasm and inserted into the membrane, supposedly using the normal *E. coli* secretion apparatus while, no organelle assembly has occurred from this pool of membrane-associated subunits [17]. The expression of r-PilA in a heterogeneous host is associated with misfolding and aggregation to inclusion bodies. In the on-column re-solubilization method, urea solubilized inclusion bodies are physically immobilized to Ni–NTA resin, after which the denaturing agent is gradually replaced by a non-denaturing buffer. This protocol greatly helps the internal localization of hydrophobic domains and efficient superficial positioning of hydrophilic parts in the configured protein. The results of this study also indicated that using refolding of inclusion body protein on Ni-NTA column led to an enhance yield of soluble proteins.

In this study, it was demonstrated that active immunization with r-PilA/alum adjuvant significantly improved the survival rate of burned mice against *P. aeruginosa* infection. The protection in r-PilA with alum immunized mice was associated with reduction in bacterial burden in the peritoneal organs especially liver and spleen from infected mice and was assessed using CFU assays, signifying the role of the vaccine candidate r-PilA with alum in protection
against different *P. aeruginosa* strains and further highlighting the potential for passive immunotherapy. Aluminum-based mineral salts (alum) remains the only world-wide-approved adjuvant by the Food and Drug Administration (FDA) for human use. Alum alone is a potent inducer of antibody-mediated immunity response [32], which is required to combat *P. aeruginosa* infection. The mechanism of protection of water in oil (Freund's adjuvant) and alum adjuvants contains neutrophils early response to the site of infections [52].

The assessment of IgG subclasses of mice immunized with r-PilA/alum adjuvant exhibited an increased pattern of IgG1/IgG2a as well as IL-4/IFN-γ responses; therefore, it seems that antibody-mediated immunity is associated with protective immune responses against *P. aeruginosa* infection in the burned-mouse model [26]. In order to characterize the type of immune response to r-PilA immunization, the cytokine profiles of spleen cells isolated from immunized mice were investigated. Cytokine analysis demonstrated the secretion of IL-4 and INF-γ from the splenocytes. In the lung tissue, production of IFN-γ cytokine was associated with the increase of bacterial clearance, but its overproduction appears to contribute to the pathogenesis of *P. aeruginosa* infection in the eye [23, 43]. It was noted that IFN-γ treatment of rats with chronic *P. aeruginosa* lung infection decreases the inflammatory responses in the lung [30]. In addition, the protection in killed *P. aeruginosa* immunized animals was associated with the cell-mediated immune response [7]. Several previous studies demonstrated that CD4+ T cells capable of producing IL-4 and opsonic antibody are critical components for protective immune responses against acute *P. aeruginosa* infection [24, 31, 51].

In a study that mice were intratracheally immunized with pili protein of *P. aeruginosa*, it has been shown that the survival rate significantly increased, while no remarkable survival was detected in mice subcutaneously or intratracheally immunized with pili protein [39]. A study
established that immunization with strain 1244 pilin, a glycol protein in which the carbohydrate portion is an O subunit, provides O-antigen-specific protection against the bacterial challenge in the burned-mouse model [28]. Active immunization with flagellin led to significant increase in survival of mice infected with *P. aeruginosa* strains associated with fewer bacteria isolated from the liver [27]. Anti-flagellin antibody prevents the systemic dissemination of *P. aeruginosa* from the site of inoculation to liver [15]. This notion was concluded based on observation of fewer bacteria in the liver of immunized mice, while both immunized and control groups had the same number of bacteria in their skins. Also, Intraperitoneal administration of antibodies raised against N'-terminal region of flagellin of *P. aeruginosa* during septicemia led to significant increase in survival of lethal peritonitis (90% versus 12% in the control group) and burn infection (83% versus 8-17% in the control group) [38]. Others have shown passive immunization with anti-flagellin monoclonal antibody leading to high protection rate (96% versus 4% in the control group) in mice challenged with a clinical isolate of *P. aeruginosa* [5]. The inhibition or delay of dissemination of bacteria to the liver via the blood is the main reason for protection in the murine-infected burned model [36].

The opsonophagocytic killing assay was used to measure the functional capacities of antibodies [8]. Here, we have shown that, the antibodies elicited by active immunization with r-PilA/alum adjuvant were capable of opsonizing bacterial cells and mediate phagocytic killing of *P. aeruginosa*. In our previous study, it has shown that antibodies raised to recombinant flagellin can enhance killing of *P. aeruginosa* [47]. Anderson and Montie demonstrated that passive transfer of anti-flagellar antibodies would provide protection in burned mice, and these antibodies have the ability to inhibit the motility of *P. aeruginosa* and to improve opsonophagocytosis [3, 4]. Based on several studies, protective antibody should involve different
properties that includes an enhanced opsonophagocytosis, as well as an inhibition of bacterial adherence, and invasiveness [5, 8, 38, 45]. The results described in this study confirm protective actions of anti r-PiIA antibody.

In conclusion, this study provides additional evidence that the r-PiIA with alum adjuvant can protect burned mice infected with \textit{P. aeruginosa}. This protection appears to be due to enhanced opsonophagocytosis and inhibition of bacterial adherence. Thus, reduced systemic dissemination of the pathogen is associated with an increased survival of burned mice infected with \textit{P. aeruginosa}. The r-PiIA carried antigenic epitopes just like the native pili and the antibody raised against it had functional activity. Based on the initial result, our future goals are to evaluate the protective efficacy of r-PiIA in combination with other virulence factors for use as an alternative to antibiotic treatment in a murine-infected burned model.

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Conflicts of interest

There are no conflicts of interest.

References


**Figure legends:**

**Fig. 1.** SDS PAGE for detection of expressed and purified r-PilA. Lane M, low molecular weight protein size markers; lane 1, pellet of un-induced bacteria; lane 2, 3 and 4, ~18 kDa induced PilA protein from bacterial cultures after 2, 3 and 4 hours of induction; lane 5, purified r-PilA.

**Fig. 2.** Immunobloting results. Lane 1, crude cell lysate of 4 h-induced bacteria detected by monoclonal anti-His tag antibody; lane 2, purified r-PilA detected by monoclonal anti-His tag antibody.

**Fig. 3.** Cytokine profiles of supernatants from antigen-stimulated spleenocytes. IL-4 (A) and IFN-γ (B) cytokines in culture supernatants of spleenocytes collected from immunized and non-immunized mice. The splenocytes were taken from mice (n=5) and stimulated with r-PilA. The supernatants from the wells containing splenocytes were collected after 72 hours and used for sandwich ELISA method. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test (ND, undetectable differences). Bars are presented as mean ± SD of five mice in each group.

**Fig. 4.** Anti-r-PilA serum response 2 weeks after the last immunization of experimental groups. (A) Anti-r-PilA specific total IgG reactivity from serum. IgG1 (A) and IgG2a (B) subtypes of vaccinated r-PilA with and without adjuvants and PBS-alum treated mice. Statistical differences
were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test (ND, undetectable differences). Bars are presented as mean ± SD of five mice in each group.

**Fig. 5.** Opsonic potential activity of r-PilA immunized mice against *P. aeruginosa*. POA1 strain was incubated with immunized mouse sera and macrophages in the presence of rabbit complement. Differences in opsonic killing activity between groups of mice were determined using Student’s t test (ND, undetectable differences). Bars are presented as mean ± SD.

**Fig. 6.** The effect of active immunization (n = 5 mice/group) with r-PilA with or without adjuvants on local and systemic spread of *P. aeruginosa*. One day after the infection the liver (A), spleen (B), blood (C), and skin (D) were homogenized and plated on TSA. One day after culturing at 37 °C, CFUs were determined. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test (ND, undetectable differences). Bars are presented as mean ± SD.

**Fig. 7.** The survival rates of immunized mice (n = 7 mice/group) challenged with *P. aeruginosa* strain PAO1. Five group of mice were actively immunized with r-PilA (with or without adjuvants), PBS/alum. At day 48, the immunized mice were burned and infected with POA1 strain. Mice immunized with r-PilA plus alum and challenged with PAO1 (3×10^2 CFU/mouse; log-rank test; r-PilA plus alum versus PBS-alum control group, p < 0.009; r-PilA plus alum versus burn control group, p = 0.5780), Mice immunized with r-PilA plus CFA and challenged with PAO1 (3×10^2 CFU/mouse; log-rank test; r-PilA plus CFA versus PBS-alum control group, p < 0.02; r-PilA plus CFA burn control group, p = 0.4632), Mice immunized with r-PilA alone and challenged with PAO1 (3×10^2 CFU/mouse; log-rank test; r-PilA versus PBS-alum control group, p < 0.01; r-PilA versus burn control group, p = 0.4754) (C).
Table legends:

**Table 1.** Effects of active r-PilA protein immunization on mortality in burned mice infected with the *P. aeruginosa*. Fisher exact test reveals that there was a significant difference in the proportion of mortality between groups (*p* < 0.001).
<table>
<thead>
<tr>
<th>Group</th>
<th>Immunogen</th>
<th>% Survival (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Total= 7 per group]</td>
</tr>
<tr>
<td>1</td>
<td>r-PiLA + Alum</td>
<td>71.42 (5)</td>
</tr>
<tr>
<td>2</td>
<td>r-PiLA + CFA</td>
<td>57.14 (4)</td>
</tr>
<tr>
<td>3</td>
<td>r-PiLA</td>
<td>57.14 (4)</td>
</tr>
<tr>
<td>4</td>
<td>Positive control(^1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>5</td>
<td>Negative Control(^2)</td>
<td>100 (7)</td>
</tr>
</tbody>
</table>

\(^1\) PBS plus alum

\(^2\) Witness group (burn control group).
Fig. 1 SDS PAGE for detection of expressed and purified r-PilA. Lane M, low molecular weight protein size markers; lane 1, pellet of un-induced bacteria; lane 2, 3 and 4, ~18 kDa induced PilA protein from bacterial cultures after 2, 3 and 4 hours of induction; lane 5, purified r-PilA.
Fig. 2 Immunobloting results. Lane 1, crude cell lysate of 4 h-induced bacteria detected by monoclonal anti-His tag antibody; lane 2, purified r-PilA detected by monoclonal anti-His tag antibody.
Fig. 3A–B. Cytokine profiles of supernatants from antigen-stimulated spleenocytes. IL-4 (A) and IFN-γ (B) cytokines in culture supernatants of spleenocytes collected from immunized and non-immunized mice. The splenocytes were taken from mice (n=5) and stimulated with r-PilA. The supernatants from the wells containing splenocytes were collected after 72 hours and used for sandwich ELISA method. Statistical differences were analyzed by analyzed one-way ANOVA followed by Tukey’s multiple comparison test. The ND indicates not detectable differences between groups. Values represent the mean ± SD of five mice in each group.
Fig. Fig. 4A–C Anti-r–PilA serum response 2 weeks after the last immunization of experimental groups. (A) Anti-r–PilA specific total IgG reactivity from serum. IgG1 (B) and IgG2a (C) subtypes of vaccinated r–PilA with and without adjuvants and PBS–alum treated mice. Values for p were determined by one–way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. The ND indicates not detectable differences between groups. Values represent the mean ± SD of mice in each group.
Fig. 5. The opsonic killing activity of three different dilutions of r-PilA immunized mice serum against P. aeruginosa. To do this test, all sera of each group were pooled together. POA1 strain was incubated with different dilutions of immunized mice sera and mouse macrophage in the presence of rabbit complement. Differences in opsonic killing activity between groups of mice were determined using Student’s t test. The ND indicates not detectable differences between groups. Values represent the mean ± SD.
Fig. 6A–D. The effect of active immunization (n = 5 mice/group) with r-PiA with or without adjuvants on local and systemic spread of P. aeruginosa into the burned skin. Bacterial load was determined in the liver (A), spleen (B), blood (C), and skin (D) at 25 h after the inoculation. One day after culturing at 37 °C, CFUs were determined. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. The ND indicates not detectable differences between groups. Values represent the mean ± SD.
Fig. 7. The survival rates of immunized mice (n = 7 mice/group) challenged with P. aeruginosa strain PAO1. Five group of mice were actively immunized with r-PilA (with or without adjuvants), PBS/alum. At day 48, the immunized mice were burned and infected with PAO1 strain. Mice immunized with r-PilA with alum and subsequently challenged with PAO1 3×10^2 CFUs/burn; log-rank test: r-PilA/alum versus PBS/alum, p < 0.009; r-PilA/alum versus witness group, p = 0.5780), Mice immunized with r-PilA with CFA and challenged with PAO1 3×10^2 CFUs/burn; log-rank test: r-PilA/CFA versus PBS/alum, p < 0.02; r-PilA/CFA versus witness group, p = 0.4632), Mice immunized with r-PilA alone and challenged with PAO1 3×10^2 CFUs/burn; log-rank test: r-PilA versus PBS/alum, p < 0.01; r-PilA versus witness group, p = 0.4754) (C).