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Recombinant PcrV Induced Poly-Isotypic Humoral Immune Responses Against *Pseudomonas aeruginosa* in BALB/c Mice

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Type III secretion system plays an important role in pathogenesis of *Pseudomonas (P.) aeruginosa*. PcrV protein is a structural component of this system. Following the expression and purification of recombinant PcrV (r-PcrV), three shots of immunization were performed. Specific total and isotype (IgG_1, IgG_2a, IgG_2b and total IgM) antibodies were measured after each shot. In addition, four weeks after the last immunization, immunized and nonimmunized groups of mice were burned and challenged with *P. aeruginosa* PAO1. To study the efficacy of the immunization, survival rate and bacterial quantity were evaluated in the skin, liver and spleen. All immunized mice were able to produce protective antibodies against *P.aeruginosa* PAO1. Anti-r-PcrV antibodies effectively enhanced (65%) survival rate of the immunized group in contrast to control group against challenge strain. Isotype antibody analysis revealed that the IgG_1 was produced more than IgG_2a in the immunized group. This phenomenon indicated, although both humoral and cellular immune systems were activated, of which the humoral immunity was the dominant immune response against *P.aeruginosa*.

Key words: Immunization, PcrV, Poly Isotypic Antibodies, *P.aeruginosa.* Vaccine.

Pseudomonas (P.) aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections[1,2]. Almost all of these associated with the onset of weakness, and defects in the host immune system, such as infections occur in patients with human immunodeficiency virus (HIV), cancers, severe burn wounds and cystic fibrosis[3-5].

Burns are one of the most common and deteriorating forms of trauma that predispose patients to be infected by variety of potential pathogens, such as *P.aeruginosa* because of damage of the protective skin barrier and suppression of the immune system[6-7]. On the other hand, ability of *P.aeruginosa* to survive under different environmental conditions, combined with its inherent resistance to different antibiotics, allows it to colonize and proliferate within the burned wound[8,9]. This localized proliferation may lead to systemic sepsis, which is often associated with a high rate of mortality in burn patients. Statistical studies indicate that approximately 10% of burn patients become infected with *P.aeruginosa* and of those, up to 80% die from *P.aeruginosa* septicemia[7-9]. Hereon these patients require immediate specialized care. As explained above, *P.aeruginosa* has provided extensive resistance against antibiotic therapy, therefore immune prophylaxis for *P.aeruginosa* infections by active and passive immunization in high risk people)welders, firefighter, etc), has been widely considered[10-12].

These features have motivated scientists to search for serum therapy instead of antibiotic

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therapy for prevention of post burn infections caused by *P.aeruginosa*[10-12]. Serum therapy has various advantages: lower cost, lower toxic effects and a potential of this process to overcome antibiotic resistance strains[13-15]. Hereupon, extensive research studies were done on the pathogenesis mechanisms of *P.aeruginosa* in burn infections, in order to discover efficient virulence associated factor that can be used as an immunogenic agent against Pseudomonas infections. One of these virulence factors is type III secretion system that injects effector proteins and toxins directly into adjacent eukaryotic cytosol across the eukaryotic plasma membrane[14-18]. Type III secretion system produced by three functional sets of genes encoding different structural and functional proteins (secretion, chaperone and effectors) were involved in the delivery or translocation of effectors and toxins into the cytoplasm of eukaryotic cells[18, 19]. PcrV protein, (also known as V antigen) plays a unique role in this phenomenon. However, the mutants of *P.aeruginosa* lacking the genes of PcrV were unable to intoxicate eukaryotic cells[18-21].

Previous studies have revealed that active and passive immunization against PcrV can increase survival in animal models with lung infection induced by *P.aeruginosa*[18-23]. However, there are few studies that focused on PcrV immunization efficiency in burn infections. The main purpose of the present study was an explana-tion on the possibility of using PcrV as a vaccine candidate against *P.aeruginosa,* and also to investigate how PcrV would promote the humoral immune responses. On the other word we going to reveal how PcrV immunization can protect burned mouse against *P.aeruginosa,* infections and fuscous on Immunoreactivity in this procedure. Our findings, for the first time showed how PcrV immunization is able to induce poly-isotypic humoral immune response.

MATERIALS AND METHODS

Bacterial Strains and Vectors

P.aeruginosa strain PAO1 was obtained from Pasteur Institute (Iran). *E.coli* strains *DH5α* (Invitrogen, California USA) and *BL21* (DE3) (Novagen Wisconsin, USA) were used for cloning and expression of recombinant PcrV protein, respectively. *pET24a* (Novagen, Wisconsin USA) was used as expression vector.

PCR of *PcrV* Gene Segment

Genomic DNA of *P.aeruginosa PAO1*, was extracted by using bacterial genomic DNA isolation kit (Me-tabion, South Korea), according to manufactures instructions. The extracted genomic DNA was used as template to amplify *PcrV* gene fragment.

The designed primers used for amplifying PcrV, had nucleotide sequence as follows: Forward primer, containing a restriction site for *BamHI* (5´-AT**GGATCC**GAAG TCAGAAACCTTAATGC-3´) and reverse primer with *HindIII* restriction site (5´-GGC**AAGCTT**G TAGATCGCGCTGAG-3´).

Pfu DNA polymerase (fermentase) was used as a polymerase enzyme in PCR reactions. Then, PCR products were purified by using PCR purification kit (Roche Germany), according to the manufacture's instructions. Then, the purified *PcrV* fragment was digested with restriction enzymes) *HindIII* and *BamH1* (and ligated into the digested *pET24a* vector. This process provides six histidine residues, (His-tag), on the C-terminal side of the expressed PcrV protein. Recombinant construct was transformed into the competent *E.coli DH5α* cells. The completeness of the recovered plasmid was confirmed by controversial methods such as colony PCR, restriction enzymes digestion method and sequencing[24, 25].

PcrV Expression

E.coli BL21 (DE3) was transformed with *pET24a*-PcrV plasmid to express recombinant protein. Transformed bacteria were cultured at 37 ºC in LB medium containing the kanamycin (50 *mg/ml*), until exponential phase (OD600 nm=0.5-0.6). Following this step, induction process was done by using 1 *mM* IPTG (fermentase). Samples were collected every 3 hours during 24 hours, and analyzed by SDS-PAGE, to determine the optimal time point of protein expression .SDS-PAGE and western blot analysis were carried out to confirm protein expression[24,25].

Purification of Recombinant Protein

Transformed *E.coli BL21* (DE3) was grown in large scale and the pellets of them harvested and resuspended in lysis buffer (0.1 M NaH_2PO_4, 8 M urea, and 0.01 M Tris, pH 8.0) containing protease inhibitors .Cell suspension was sonicated and centrifuged for 15 min at 10000 *rpm*,

recombinant protein (approximately 34*kDa*) was purified via its His-tag, by using Ni-nitrilotriacetic acid affinity chromatography (Ni-NTA Agarose; Qiagen), according to manufacturer's instructions.

PcrV protein solution was dialyzed against 0.1 M phosphate buffered saline (PBS, pH 7.4) for 72 hours to remove urea and stored at -70 ºC until use. It must be reminded that the absence of detectable levels of endotoxin in final solution was verified by a Limulus amoebocyte lysate assay after purification process[24, 25].

Western Blot Analysis

Separated proteins by SDS-PAGE were transferred to a nitrocellulose membrane (Schliecher & Schuell). Blocking process was done by using Tris-Buffered Saline (TBS) containing 5-10% Bovine Serum Albumin (BSA) overnight at 4 ºC. The membrane was then washed three times (each time 10 min) with TBS containing 0.05% Tween 20 (TBST). Subsequently the membrane was incubated for 2 hours at room temperature, with diluted (1:10000) mouse anti-His-tag antibody (Qiagen, USA) in TBST. After incubation period the membrane was washed with TBST and incubated with anti-mouse immunoglobulin G, (Heavy and light chain) Horseradish peroxidase (HRP) conjugated antibody (diluted 1:5000 in TBST) for 2 hours at room temperature. After three times of washing, membrane was treated with DAB solution (Sigma Saint Louis, MO, USA), and placed in the darkness, until the appearance of the protein band[24, 25].

Mice Immunization

The groups of mice (Six to eight weeks old female BALB/c mice) obtained from Pasteur Institute of Iran. The mice were kept under standard laboratory conditions for one week before applying the antigen. They were given free access to water and food and maintained in a light/dark cycle with lights (12 h / 12 h). In next step, the mice were assigned into the two groups (N=14) and immunized with 10 *µg* of r-PcrV or *PBS* (for control group).

Hereupon, the concentration of 0.2 *mg/ml*, of r-PcrV protein in PBS was prepared and mixed with equivalent ratio (1:1) of Complete Freund's adjuvant (Sigma, Saint Louis, MO, USA). Finally, 0.1 *ml* of this solution (containing 10 *µg of* r-PcrV) was used for each injection. On the day 0, the groups of mice were immunized subcutaneously (Behind the neck) with r-PcrV (PcrV- injected group or immunized group) and PBS (PBS injected group or Control group). Immunization procedure was performed according to the specific schedule that is shown in Fig. 1. Booster doses were prepared, the same as primary injection condition in PBS, and mixed with Equivalent ratio (1:1) of incomplete Freund's adjuvant. Two weeks after each immunization, mice were bled via the retro-orbital sinus and the sera were separated and stored at -20 ºC until future analyzed (Fig. 1)[9, 19, 26].

Specific Total IgG

Optimized indirect enzyme-linked immune sorbent assay (ELISA) method was used to assess the existence of anti-r-PcrV antibodies in sera of immunized mice.

A 96-well micro titer plate was coated with 100 *µL* of 10 *µg/ml* r-PcrV protein, diluted in PBS

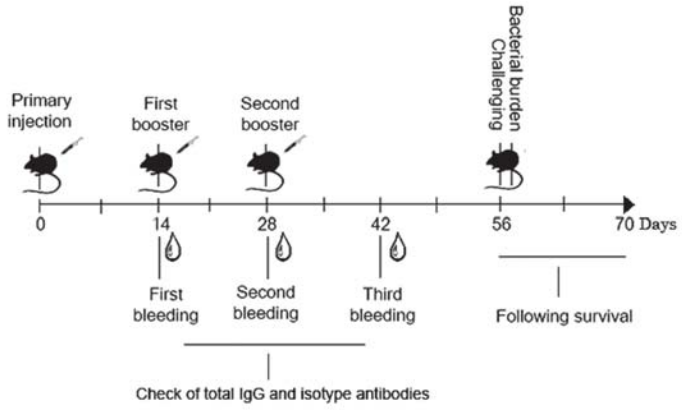

Fig. 1. Immunization: Schematic diagram of immunization process, and timing of future studies to evaluate the efficacy of this immunization courses

buffer (1 µg/well) and incubated overnight at 4 ºC. Then, each immunoplate was washed three times with PBS containing 0.05% Tween 20 (washing buffer) and blocked with PBS containing 3-5% BSA (blocking buffer) for 2 hours at 37 ºC. Plates were washed and 100 μL of (1/100 to 1/204800), diluted sera in blocking buffer was added into each well and incubated for 2 hours, at 37 ºC. In next step, the wells were washed and incubated with diluted (1:7000) HRP-conjugated anti mouse-IgG antibodies, (Sigma, USA) (as secondary antibody), at 37 ºC for 2 hours. Then, immunoplates were washed five times and incubated for 30 min with 100 μL tetramethylbenzidine (TMB) as the substrate in darkness. The enzymatic reaction was stopped by adding 100 μl of H_2SO_4 (2N). Optical density was read at 450 nm (OD 450). This test was done, to compare and analyzed alteration of total specific anti-r-PcrV antibodies after each immunization process[9,25,27].

Isotypes Antibodies

After third immunization, the existence of anti-r-PcrV specific subclass IgG_1, IgG_2a, IgG_2b, and IgM antibodies were analyzed using indirect-ELISA as described by the manufacturer (R&D Systems, Minneapolis, MN, USA).

After antigen coating and blocking process, immunoplates were washed and then 100 μL of 1:100 diluted sera (in blocking buffer) was added into each well. Following the incubation process (one hour) , wells were washed and 100 μL of 1:1000 dilutions of anti-mouse IgG_1, IgG_2a, IgG_2b, and IgM subclasses, as secondary antibodies, were added. After washing three times, 100 μL of HRP-conjugated anti-mouse antibodies (Sigma) were added to each well. The plates were washed five times and incubated with 100 μL of TMB substrate (30 *min)* in the darkness. The Peroxidase-TMB reaction, was stopped by adding H_2SO_4 (2N), and the color density was measured at 450 nm[9, 25, 27].

Thermal Injury Model

Thermal injury process was done according to the method developed by Stieritz and Holder with slight modifications[9, 19]. Four weeks after the last immunization, female BALB/c mice, were anesthetized with mixture of ketamin hydrochloride, (100 *mg/ml*, alfasan Woerden-Holand), and xylazine (20 *mg/ml* Alfasan Woerden-Holand), in distilled water intraperitonellay. The back hairs of the mice, were shaved (2.5×2.5cm), and subjected to a 20s, ethanol flame burn on shaved area (12-15% of total body). After the thermal injury process, they received 0.5 ml of sterile saline solution intraperitoneally without interruption as fluid replacement therapy. It must remained that this burned model has a partial-thickness and not lethal[9, 19, 26].

Bacterial Challenge

To evaluate the protective efficacy of produced anti-sera against r-PcrV, the survival of a burned mouse model against a lethal dose of *P.aeruginosa* PAO1, infection was investigated. Previous studies revealed that thermal injury caused immune suppression and reduced the lethal dose of *P.aeruginosa* from 10^6 CFU to a 10^2 - 10^3 CFU in these models[9, 19].

For bacterial challenge, *P.aeruginosa* PAO1 was grown overnight on brain-heart infusion broth (BHI). 50-100 μl of 24h culture, were transferred into the 5ml BHI broth under agitation (200 rpm) at 37ºC and incubated for 3-4 hours to reach favorite optical density (OD of 0.2 at 620 nm). Bacterial cells were harvested by centrifugation and resuspended in 10 ml of sterile saline solution. The number of bacteria in injected inoculate, was counted by plating 100 μL of each dilution onto MacConckey agar plates. An inoculums of 5-6×10^2CFU (3 folds higher than LD_{50}) was used and administered in 100 μL volume subeschar immediately after thermal injury process in burned site .A new group was defined in this section that contained only burned models without the challenge as witness group. The animals were monitored for survival twice a day up to 14 days[9, 19, 26].

Bacterial Burden

The efficacy of anti-r-PcrV antibodies to inhibit the distribution of *P.aeruginosa* PAO1 from infected wound to internal organs was assessed. Twenty five hrs after the challenge (post burn/ infection), bacterial load was analyzed in skin, liver and spleen.

Four mice of each group were sacrificed and a biopsy of their burned skin (1.5×1.5 cm), spleen and liver was prepared in aseptic condition. Then, each biopsy sample was rinsed, weighed, excised, and finally homogenized in PBS (2ml) , by using a Sorvall Omni mixer (Ivan Sorvall, Inc, Norwalk, Conn.). For bacterial counting, serial

dilutions (10^{-1} -10^{-4}fold) of homogenates were prepared in PBS. In next step, 100 µl of each diluted suspension were cultured in triplicate on *Pseudomonas* selection agar plates (Merck, Germany), then plates were incubated at 37ºC and the number of CFUs was counted after 24-48 hours. Results were expressed as $\log_{10}$CFUg^{-1} of wet weight for infected organs[9,19,26].

Statistical analysis

For statistical analysis, the software statistical package for social sciences (SPSS) 20.0 was used. All data were reported as the mean ±S.D. One-way analysis of variation (ANOVA) and student's t-test (Statview) were used to analyzed obtained results. A *P*-value less than 0.05 was considered as statistically significant[9,25].

RESULTS

PcrV* amplification and construction of *pET24a-PcrV

Genomic DNA of *P.aeruginosa* PAO1 was extracted and used as template to isolate PcrV gene. The expected size of PCR product was 885*bp*, (Fig. 2).

The integrity of PcrV into the *pET24a* was confirmed by colony-PCR method that used specific designed primers and double digestion method with *HindIII* and *BamHI* as restriction enzymes (Fig. 3). Finally, sequencing process was done to reveal identity and orientation of PcrV gene with the references sequence. The results showed 100% homology between them.

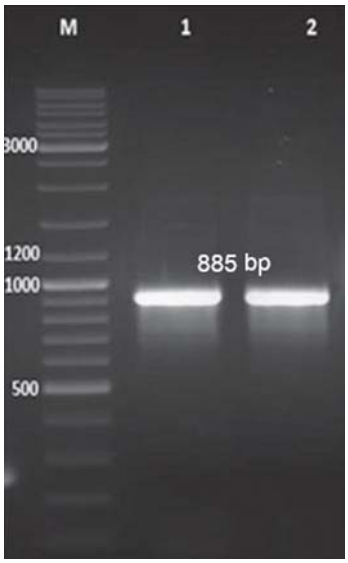

Fig. 2. *pcrV* amplification : Electrophoresis of PCR product on 1% agarose gel. 885 bp (lane 1 and 2) fragments were amplified by specific primers from *pcrV* gene in *P.aeruginosa* PAO1. lane M, DNA size marker

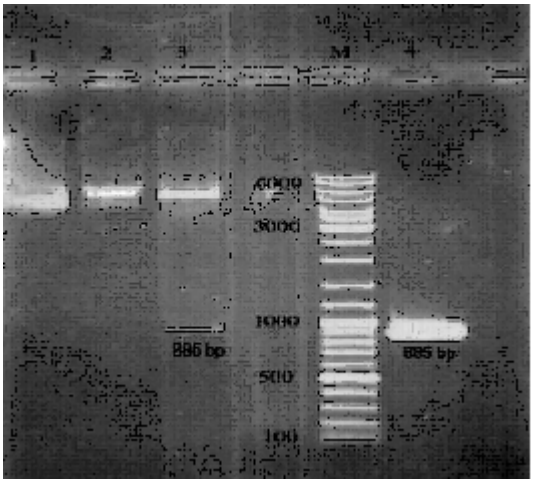

Fig. 3. Restriction enzyme digestion method was done to confirm existence of *pcrV* fragment in recombinant vector.The plasmids were extracted and digested with appropriate restriction enzymes. Lane 1 *pET24a* vector, Lane 2, undigested recombinant vector. Lane 3, Recombinant vector digested with *BamHI* and *HindIII*, Lane 4 PCR product of *pcrV* gene (885*bp*), Lane M 1kb DNA size marker on 1% agarose gel

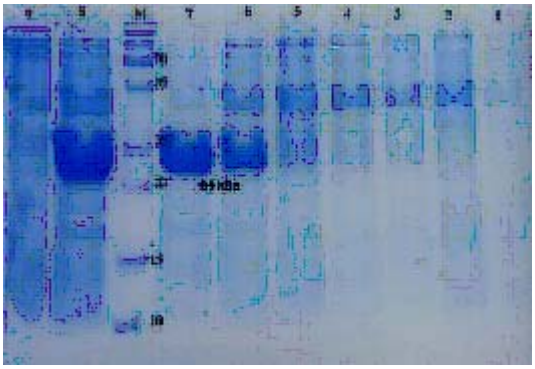

Fig. 4. SDS-PAGE: Confirm the expression and purification of recombinant PcrV protein by SDS-PAGE (12% W/V).Amount of protein loaded in each well was about 50 µg. Lane 1 to 5 flows through material after washing and solubilizing. Lane 6, and 7 purified r-PcrV from Ni-NTA Agarose column with elution buffers, Lane 8 induced bacteria with IPTG, and Lane 9 uninduced bacteria

SDS-PAGE Analysis

SDS-PAGE results revealed that one major band around the 34*kDa* position in lysate of samples that induced with IPTG which was expected position for PcrV fraction. In next step, recombinant protein was purified with Ni-NTA affinity chromatography (Fig. 4).

Western Blot Analysis

Western blot analysis was conducted to confirm the expression of desired protein. The major band observed in SDS-PAGE was analyzed with anti-r-PcrV antibodies (anti his-tag antibodies) to demonstrate r-PcrV protein (about 34*kDa*) (Fig. 5).

Antibody Responses

Titration results in immunized (*r*-PcrV injected) group, after the second booster, revealed that immunization with *r*-PcrV significantly increased the total IgG antibody responses at the dilution of 1/100 up to 1/12800, as compared with non-immunized (PBS injected) group ($P<0.05$) (Fig. 6).

The result of the total IgG antibody assay revealed that all animals immunized with recombinant immunogen (*r*-PcrV) were able to produce specific antibodies against *r*-PcrV and the highest antibody titer were detected after the second booster (Fig. 7).

To determine the specific isotype antibodies, the existence of anti-PcrV IgG subclasses (IgG_1/IgG_2a/IgG_2b), and total IgM were analyzed. Results indicated that the levels of specific anti-r-PcrV, subclass antibodies in the serum of infected mice differed significantly among the two groups in this experiment. The immunized (*r*-PcrV injected) group, showed higher level of r-PcrV-specific IgG_1 ($P<0.001$), IgG_2a ($P<0.001$), IgG_2b ($P<0.001$) and IgM ($P<0.001$), in contrast to control group (PBS injected) (Fig. 8).

Survival Rate

To evaluate the protective efficacy of produced anti-sera against r-PcrV, the survival rate of burned mouse model was investigated against lethal dose of *P.aeruginosa* PAO1 infection.

The results indicated that the antibodies against r-PcrV caused 65% survival in immunized group, *within* 14 days after the burn wound infection with *P.aeruginosa PAO1*. However, there was no protection effect observed in the control group, and all mice died only 4 days after the challenge process (Fig. 9).

Bacterial Burden

To reveal whether anti-r-PcrV antibodies have high efficacy to inhibit the distribution of infection from infected wound (eschar) to internal organs, bacterial burden in skin and internal organs (liver and spleen), was examined 25 hour after the burn wound infection, with *P.aeruginosa* PAO1.

The results indicated that immunized (*r*-PcrV injected) mice had a lower number of colony-forming units (CFU) of *P.aeruginosa* PAO1 in liver and spleen, in comparison with non-immunization (PBS injected) mice, ($P<0.001$) (Fig. 10). The

Fig. 5. Western blot: Reactivity of anti r-PcrV antibodies (anti-His 1:10000) to r-PcrV of *P.aeruginosa*in immunoblot analysis. Lane 1: purified *P. aeruginosa* produced r-PcrV (Molecular weight of the protein was about 34*kDa)*, Lane 2: induced samples and Lane 3 none induce samples. Lane M: Protein size marker (*kDa*)

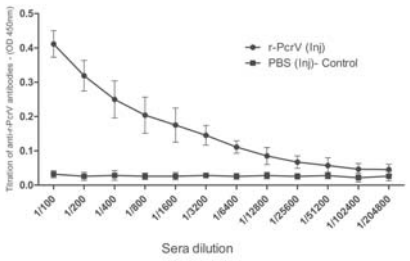

Fig. 6. Titration of anti-r-PcrV antibodies: Sera of immunized (PcrV injected) and nonimmunized (PBS injected) group was diluted and ELISA was done to reveal total IgG antibody at the dilution of 1/100 to 1/204800. Values presented as mean ±S.D of experimental group (n=14)

bacterial loads in skin (eschar) of immunized (*r*-PcrV injected) mice decreased but was not statistically significant (P>0.05) in comparison with the control (PBS injected) mice (Fig. 10).

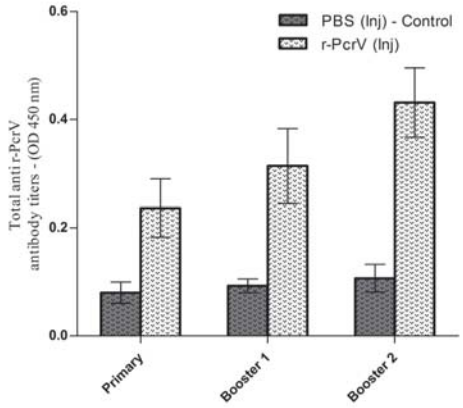

Fig. 7. Total IgG antibody response: Variation of total IgG anti-r-PcrV antibodies in sera of immunized group (PcrV injected group) in contrast to nonimmunized group (PBS injected group) after each shot of immunization. Data presented as mean ±S.D of experimental group. (n=14)

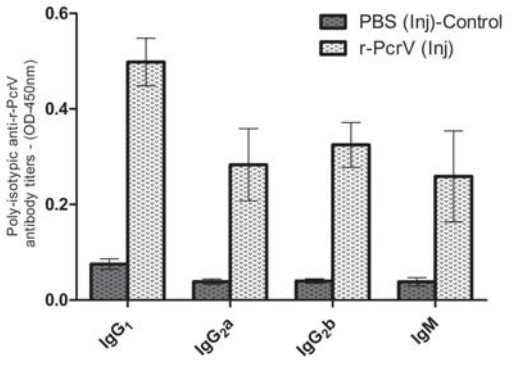

Fig. 8. Poly-isotypic anti-r-PcrV antibody titers : Immunization with candidate vaccine (r-PcrV) significantly increased specific IgG_1, IgG_2a, IgG_2b and IgM antibodies compared with control group (P<0.001). Experiments carried out triplicate and values are shown as the mean ± S.D

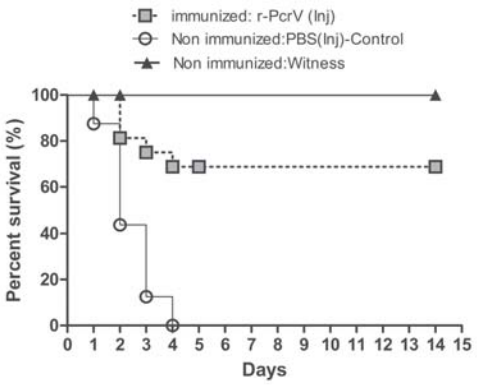

Fig. 9. Survival rate: Antibodies against r-PcrV effectively enhanced survival rate of immunized (r-PcrV injected) group in contrast to non-immunized (control or PBS injected) group after challenge with *P.aeruginosa* PAO1. Also it must be noted that all mice in witness group (only burned without challenge) survived

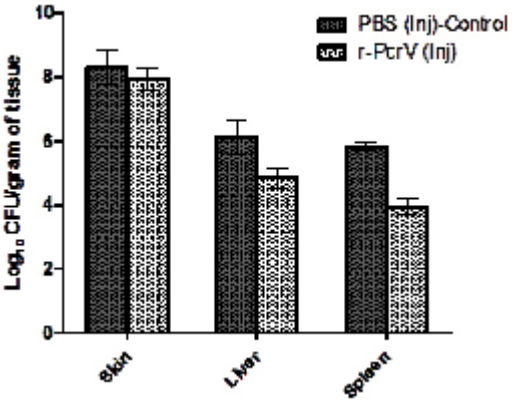

Fig. 10. Bacterial load in skin, liver and spleen: The effect of immunization process with r-PcrV on local and systemic spread of *P.aeruginosa PAO1* after the burn wound infection. (Mean±SD)

DISCUSSION

P.aeruginosa is an opportunistic pathogen that frequently causes severe infection and septicemia in immunocompromised hosts especially in patients with major burn injuries[28,30]. Burns are one of the most common and destructive forms of trauma that predispose patients to infect by *P.aeruginosa* because of the damage to protective skin barrier and suppression of the immune system. The burn process provides a susceptible environment for local colonization

and increases shedding of bacterium into the bloodstream, leading to systemic infection. Statistical studies indicate that this infection is associated with a high rate of mortality in burn patients[9, 19, 29, 31-33]. Hereon, burn patients require immediate specialized care.

The main purpose of this study was to evaluate the feasibility of using *r*-PcrV as a candidate vaccine against *P.aeruginosa* infections in burn injuries. Previous studies revealed the vital role of PcrV in *P.aeruginosa* burns infections[19]. This study demonstrates the protective efficacy of the anti-PcrV antibodies in thermal injury mouse models infected by *P.aeruginosa* PAO1. The results showed that PcrV can be a target immunogen in vaccine production against *P.aeruginosa*.

The ELISA results revealed that immunization process with *r*-PcrV can induce production of specific antibodies against PcrV protein in the immunized groups. On the other hand, to investigate the protective effect of these antibodies, survival rate and bacterial burden, (systemic and local spread), was evaluated in burned mice sepsis model that infected with *P.aeruginosa PAO1*. Antibodies could be a major factor in removal of extracellular pathogens, such as *P.aeruginosa*. In the presence of this pathogen, produced antibodies could bind to the target antigen of this bacterium and activate complement system. This phenomenon increased phagocytosis of this pathogen through opsonization process. The result of a similar study by Sawa showed that PcrV immunization provides protection in the lung infection mouse models[21]. In addition, we demonstrated that immunization with PcrV significantly increased survival of mice against infections caused by *P.aeroginosa PAO1,* in the burned wound model .The data described here, in confirms the results of a similar study, by Ian Alan Holder et al. While in their studies *pET16b* (belonging to pET family), vector was used to express PcrV protein, but we used *pET24a*[14,19]. In addition, there was a difference in immunization schedules .They used one booster dose after immunization without adjuvant, but in ours, two booster doses with incomplete Freund's adjuvant were used . Frank et al. showed polyclonal antibodies that bind to PcrV of *P.aeruginosa* inhibit the delivery of type III toxins, and enhance the clearance of high cytotoxic strain of *P.aeruginosa*, during acute lung infections. We obtained similar results in burn infections[22] .To date, four type III secretory effectors (ExoS, ExoT, ExoU, and ExoY) were identified in *P.aeruginosa*. A relation was found between bacterial counts in wound biopsy, the appearance of bacteremia and positive blood cultures and production of these toxins[33-35].

It seems that these toxins play an important role in the occurrence of *P.aeruginosa* bacteremia, and its lethality in burned patients. They contribute to dissemination of *P.aeruginosa* within the body of burned patients, and its horizontal spread within the burned skin. It is also responsible for overall virulence of *P.aeruginosa*. While the mentioned toxins, other proteins and enzymes secreted by type III secretion system, are important in burned wound infection. Thus, protective antibodies against PcrV could inhibit the proper function of type III secretion system and enhance the clearance process of *P.aeruginosa* in burned wound models. The death of non-immunized mice during 24-96 hrs post-burn/ infection with *P.aeruginosa* PAO1 indicate that this pathogen can proliferate in the burned area. This means the pathogen can overcome innate immunity, disseminate to internal organs , cause bacteremia, septicemia, septic shock and finally death of infected mice. These results support the findings of Holder[14,19].

Our findings provide in vivo evidence that specific antibodies against r-PcrV are have important in bacterial clearance and systemic spread of *P.aeroginosa* PAO1 infection, following burn injury. In general, we confirmed that systemic spread of the challenge strain in immunized (r-PcrV injected) group, is remarkably lower ($p<0.001$) than the control (PBS injected) group. Our results suggested that anti-r-PcrV antibodies play an important role in inhibiting the systemic spread of *P.aeruginosa* PAO1 from the site of infection to internal organs, whereby, antibodies against *r*-PcrV significantly reduced mortality and morbidity (65% in immunized group) of mice in the sepsis model of *P.aeroginosa* PAO1 infections.

However, we revealed that in the thermally injured immunized mice, *P.aeroginosa*, was not efficiently inhibited from colonizing at the primary infection site (burn wound), but could not spread systemically. It is possible that this inconsistency is due to the devascularized state of the burn

wound that allows an uncontrolled proliferation of *P.aeroginosa*. There was not a significant difference between the immunized and non-immunized group. The same observation was reported by Ian Alan Holder[14,19].

Our results display functional responses of the humoral immune system in immunized mice, in contrast to control group. Overall, it is seems that immunization of mice with r-PcrV immunogen induces poly-isotypic humoral immune re-sponse. Considering this fact that each isotype of antibodies has a unique function in humoral immune responses. The production of poly-isotypic antibodies shows that *r*-PcrV, could stimulate humoral immune response with excellent bioactivity[35-39]. Our results supported the resulrs of Neely who showed immunization with PcrV, enhanced survival of mice challenged with different serogroups of *P.aeruginosa*[14]. The pattern of expressed antibodies revealed that both cellular and humoral immune responses were involved in clearance of *P.aeruginosa* infections. Isotype antibody analysis, showed that the IgG_1 was produced more than IgG_2a in immunized (r-PcrV injected) group. This phenomenon (IgG_1/IgG_2a response) indicated although both humoral and cellular immune system are activated, humoral immunity is dominant immune response against *P.aeruginosa* in burn wound sepsis model. These data display functional responses of the humoral immune system in immunized groups, in contrast to control group. Humoral immune response eventually led to efficient immobilization of pathogen and moderate killing activity, mediated by opsonic antibody. Our results showed that systemic spread of this pathogen within the liver and spleen of burned/infected mice were significantly lower than those observed in control groups. These data suggested that anti PcrV antibodies play an important role in inhibiting the systemic spread of *P.aeruginosa* PAO1 from the site of infection to internal organs by prevention of type III secretion system function. Finally, according to the previous studies on the efficient dose of r-PcrV immunogen, we used the dose of 10 (*µg/mouse)* from r-PcrV for immunization of mice[19]. Different immunogen (r-PcrV) concentrations could be evaluated in future studies. We used the Freund's adjuvant, using other adjuvants can be used. In addition, we followed the survival rate of mice two weeks after challenge , and recommend that more time is needed to be spent on it in future studies.

CONCLUSION

We demonstrated that r-PcrV can be expressed in *E. coli BL21* (DE3) at a high level and stimulated humoral immune responses in burn wound infection murine model. Isotype antibody analysis showed that the IgG_1 was produced more than IgG2a in r-PcrV-injected group. This phenomenon indicates that humoral immunity is dominant immune response against *P.aeruginosa* in burned wound infection models. This process limits the systemic spread of infection and survival of infected models against lethal dose of *P. aeruginosa* PAO1. These positive effects reveal that PcrV protein can be used as a potential vaccine against *P.aeruginosa* strains. This however warrants future studies.

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REFERENCES

1. Richards MJ., Edwards JR., Culver DH., Gaynes RP. Nosocomial infections in medical intensive care units in the United States. *Critical care medicine*., 1999;**27**(5):887-92.
2. Barnea Y., Carmeli Y., Neville LF., Kahel-Reifer H., Eren R., Dagan S ., *et al*. Therapy with anti-flagellin A monoclonal antibody limits *P.aeruginosa* invasiveness in a mouse burn wound sepsis model. *Burns*., 2009; **35**(3):390-6.
3. Holder IA., *P.aeruginosa* burn infections: pathogenesis and treatment. *Pseudomonas aeruginosa* as an Opportunistic Pathogen: *Springer*.,1993; p. 275-95.
4. Döring G., Pier GB. Vaccines and immunotherapy against *P.aeruginosa* , *Vaccine*. . 2008; **26**(8): 1011-24.
5. Pier G. Application of vaccine technology to prevention of *P.aeruginosa* infections. *Expert Rev Vaccines*. 2005; **4**(5): 645-56.

6. Bodey GP., Bolivar R., Fainstein V., Jadeja L.Infections caused by *P.aeruginosa* . . 1983; **5**(2): 279-313.
7. Church D., Elsayed S., Reid O., Winston B., Lindsay R. Burn wound infections. *Clinical microbiology reviews*. 2006;**19**(2):403-34.
8. Wysocki AB.Evaluating and managing open skin wounds: colonization versus infection. *AACN Advanced Critical Care*. 2002;**13**(3):382-97.
9. Faezi S., Sattari M., Mahdavi M., Roudkenar MH.Passive immunisation against *P.aeruginosa* recombinant flagellin in an experimental model of burn wound sepsis. *burns*.2011;**37**(5):865-72.
10. Ekrami A., Hemadi A., Kalantar E., Latifi M., Kayedani A.Epidemiology of hospitalized burn patients during 5 years in Khuzestan province, Iran. *Archives of Clinical Infectious Diseases*. 2010; **5**(1):40-4.
11. Erol S., Altoparlak U., Akcay MN., Celebi F., Parlak M.Changes of microbial flora and wound colonization in burned patients. *Burns*. 2004;**30**(4):357-61.
12. Casadevall A., Scharff MD.Serum therapy revisited: animal models of infection and development of passive antibody therapy. *Antimicrobial agents and chemotherapy*. 1994; **38** (8):1695.
13. Campodónico VL., Llosa NJ., Grout M., Döring G., Maira-Litrán T., Pier GB.Evaluation of flagella and flagellin of *P.aeruginosa* as vaccines. *Infection and immunity*;.2010; **78**(2):746-55.
14. Neely AN., Holder IA, Wiener-Kronish JP, Sawa T.Passive anti-PcrV treatment protects burned mice against *P.aeruginosa* challenge. *Burns*. 2005; **31**(2): 153-8.
15. Casadevall A.Antibody-based therapies for emerging infectious diseases. *Emerging infectious diseases*.1996; **2**(3):200.
16. Galán JE., Collmer A.Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*; .1999; **284**(5418): 1322-8.
17. Dacheux D., Epaulard O., de Groot A., Guery B., Leberre R., Attree I., *et al*.Activation of the *P.aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase aceAB operon. *Infection and immunity*. .2002; **70**(7): 3973-7.
18. Imamura Y., Yanagihara K., Fukuda Y., Kaneko Y., Seki M., Izumikawa K., *et al*.Effect of anti-PcrV antibody in a murine chronic airway *P.aeruginosa* infection model. *European Respiratory Journal*.2007; **29**(5): 965-8.
19. Holder IA., Neely AN., Frank DW.PcrV immunization enhances survival of burned *P.aeruginosa* -infected mice. *Infection and immunity*. .2001; **69**(9): 5908-10.
20. Alan I.*P.aeruginosa* Virulence-associated factors and their rule in burn wound infections. *P.aeruginosa the Opportunist*.1992; 235.
21. Sawa T., Yahr TL., Ohara M., Kurahashi K., Gropper MA., Wiener-Kronish JP., *et al*.Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. *Nature medicine*. 1999; **5**(4):392-8.
22. Frank DW., Vallis A., Wiener-Kronish JP., Roy-Burman A., Spack EG., Mullaney BP., *et al*.Generation and characterization of a protective monoclonal antibody to *P.aeruginosa* PcrV. *Journal of Infectious Diseases*.2002;**186**(1):64-73.
23. Yahr TL., Goranson J., Frank DW.Exoenzyme S of *P.aeruginosa* is secreted by a type III pathway. *Molecular microbiology*. 1996; **22**(5):991-1003.
24. Goudarzi G., Sattari M., Roudkenar MH., Montajabi-Niyat M., Zavaran-Hosseini A., Mosavi-Hosseini K.Cloning, expression, purification, and characterization of recombinant flagellin isolated from *P.aeruginosa* . *Biotechnology letters*. .2009;**31**(9):1353-60.
25. Haghighat S., Siadat SD., Sorkhabadi SMR., Sepahi AA., Mahdavi M.Cloning, Expression and Purification of Penicillin Binding Protein2a (PBP2a) from Methicillin Resistant *Staphylococcus aureus*: A Study on Immunoreactivity in Balb/C Mouse. *Avicenna journal of medical biotechnology*. 2013; **5**(4): 204.
26. Rumbaugh KP., Colmer JA., Griswold JA., Hamood ANThe effects of infection of thermal injury by *P.aeruginosa* PAO1 on the murine cytokine responses. *Cytokine*. 2001; **16**(4):160-8.
27. Senna JP., Roth DM., Oliveira JS., Machado DC., Santos DS.Protective immune response against methicillin resistant *Staphylococcus aureus* in a murine model using a DNA vaccine approach. *Vaccine*. 2003; **21**(19): 2661-6.
28. Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R.Protein secretion systems in *P.aeruginosa* : A wealth of pathogenic weapons. *International Journal of Medical Microbiology*. 2010; **300**(8): 534-43.
29. Fothergill JL., Winstanley C., James CE.Novel therapeutic strategies to counter *P.aeruginosa* infections. *Expert Rev Anti Infect Theraphy* 2012; **10**(2): 219-35.
30. Estahbanati HK., Kashani PP., Ghanaatpisheh F.Frequency of *P.aeruginosa* serotypes in burn

wound infections and their resistance to antibiotics. Burns; 2002; **28**(4): 340-8.

31. Richard P. *P.aeruginosa* outbreak in a burn unit: role of antimicrobials in the emergence of multiply resistant strains, *J. Infect. Dis*. 1994; **170**: 377–383.
32. Faure K., Fujimoto J., Shimabukuro DW., Ajayi T., Shime N., Moriyama K., *et al*.Effects of monoclonal anti-PcrV antibody on *P.aeruginosa* -induced acute lung injury in a rat model. *Journal of immune based therapies and vaccines*; 2003; **1**(1):2.
33. Engel J., Balachandran P.Role of *P.aeruginosa* type III effectors in disease. *Current opinion in microbiology*.2009; **12**(1): 61-6.
34. Lee VT., Smith RS., Tümmler B., Lory S.Activities of *P.aeruginosa* effectors secreted by the type III secretion system in vitro and during infection. *Infection and immunity*. 2005; **73**(3): 1695-705.
35. Milla CE., Chmiel JF., Accurso FJ., VanDevanter DR, Konstan MW., Yarranton G., *et al*.Anti PcrV antibody in cystic fibrosis: A novel approach targeting *Pseudomonas aeruginosa* airway infection. *Pediatric pulmonology*. 2013; **49**(7): 650-8
36. Moriyama K., Wiener Kronish JP., Sawa T.Protective effects of affinity purified antibody and truncated vaccines against *Pseudomonas aeruginosa* V antigen in neutropenic mice. *Microbiology and immunology*; 2009. **53**(11): 587-94.
37. Sharma A., Krause A., Worgall S.Recent developments for *Pseudomonas* vaccines. *Human vaccines*. 2011; **7**(10): 999.
38. Shajiei A., Malal AR., Shahabi G., Farhoudi R., Faezi S., Tebianian M., *et al*.*Pseudomonas aeruginosa* Recombinant Flagellin Induced Poly-Isotypic Humoral Immune Responses in the Balb/C Mice. *Jundishapur Journal of Microbiology* 2013; **6**(7).
39. Song Y., Baer M., Srinivasan R., Lima J., Yarranton G., Bebbington C., *et al*.PcrV antibody–antibiotic combination improves survival in *Pseudomonas aeruginosa*-infected mice. *European journal of clinical microbiology & infectious diseases*; 2012; **31**(8):1837-45.