Fakoor MH, et al. Iran J Immunol. 2020; 17(2):121-136
https://doi.org/10.22034/iji.2020.85590.1718.

ORIGINAL ARTICLE

In-Silico Analysis and Protective Efficacy of the PcrV Recombinant Vaccine against *Pseudomonas Aeruginosa* in the Burned and PA-Infected BALB/c Mouse Model

Mohammad Hadi Fakoor[1], Parviz Owlia[2*], Seyed Latif Mousavi Gargari[3], Azar Sabokbar[1]

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, [2]Molecular Microbiology Research Center (MMRC), [3]Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran

ABSTRACT

Background: *Pseudomonas aeruginosa* is considered as the most severe cause of infections in burn patients and pneumonia infections. **Objective:** To study the protective effects of recombinant protein vaccine harboring the PcrV of *P. aeruginosa* in the mice model of burn and respiratory infections. **Methods:** Recombinant protein vaccine harboring the PcrV was expressed in the *E.coli* BL-21 strain. Mice were immunized with the purified recombinant protein and the antibody titer was measured in the sera obtained from the immunized mice. Immunized and control mice were challenged by active and passive immunization. The microbial counts in the lung, skin, liver, spleen, and kidney were compared with the control mice. **Results:** Bioinformatics analysis indicated that the PcrV protein was conserved in 1552 clinical and environmental isolates. Also, the isoelectric point (pI), molecular weight, and Grand Average of Hydropathy (GRAVY) score were analyzed. Mice were injected with recombinant protein and serum from immunized mice reacted strongly with recombinant antigen at a dilution of 1:64000. The survival rate of the mice infected with 5 x LD50 of the PA was significantly increased up to 75% in the standard strains (PAO1 and PAK), and the number of bacteria, especially in the internal organs (kidney, spleen, and liver) significantly reduced compared to the mice immunized with the placebo. **Conclusion:** Our results demonstrated that the PcrV protein could be an effective candidate vaccine for generation of immunity against the *P. aeruginosa* infection.

Received: 2020-03-02, Revised: 2020-06-15, Accepted: 2020-06-29.
Citation: Fakoor MH, Owlia P, Mousavi SL, Sabokbar A. In-Silico Analysis and Protective Efficacy of the PcrV Recombinant Vaccine against *Pseudomonas Aeruginosa* in the Burned and PA-Infected Balb/c Mouse Model. *Iran J Immunol.* 2020; 17(2): 121-136. doi: 10.22034/iji.2020.85590.1718

Keywords: Burn, *Pseudomonas Aeruginosa*, Recombinant Protein, Respiratory, Vaccine

---

*Corresponding author: Dr. Parviz Owlia, Molecular Microbiology Research Center, Shahed University, Tehran, Iran, e-mail: powlia@gmail.com

INTRODUCTION

In recent years, Multi-Drug-Resistant (MDR) *P. aeruginosa* strains have been developed. *P. aeruginosa* is commonly found in hospitals due to environmental adaptation and antibiotic resistance (1,2). The preventive approach is the optimal strategy against these infections (3). Vaccines are considered a successful strategy in preventing bacterial diseases. Antigens, including the Outer Membrane Proteins (OMPs), Lipopolysaccharides (LPS), toxins, fimbriae, and flagella, are targets for vaccine design. PcrV in the *P. aeruginosa* is identified as a needle-tip and surface-expressed protein from the Type III Secretion System (TTSS) (4). As a major virulence mechanism, TTSS is present in most of the gram-negative species through which bacteria can introduce their toxins into their targets (5). PcrV is present in most *P. aeruginosa* strains causing acute infections, particularly those associated with poor clinical outcomes. Additionally, the gene coding for PcrV is almost conserved among the strains and is not related to antibiotic resistance (6). The PcrV characteristics hinted above have made it a more effective vaccine candidate for *P. aeruginosa*. Currently, novel methods have been proposed to evaluate the antigen protection via bioinformatics analysis (7). In the present research, *in-silico* study is conducted to compare the similarities of the PcrV structures in different bacterial strains. PcrV protein is then produced after confirmation by the bioinformatics studies; finally, it is tested on the BALB/c mice. The effect of active and passive immunization on the burned and *P. aeruginosa*-infected mice is further investigated.

MATERIALS AND METHODS

Sequence Availability, Similarity Search, and Alignments. The sequences of the protein PcrV from *P. aeruginosa* PAO1 strain (Protein Accession No: NP-250397) were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein). For further analyses, the sequences were saved in the FASTA format. In order to check the sequences, UniProt (http://www.uniprot.org) and ExPASy (8) (http://www.expasy.org) portals were evaluated. The genes and proteins were further investigated on the Pseudomonas website (9) available at (http://beta.pseudomonas.com). To collect the homologous sequences, the NCBI BLAST (10) was used against the reference sequence database. The BLAST parameters were once restricted to *P. aeruginosa* (taxid: 287). The highest BLAST protein sequences were aligned for an accurate homology analysis. The amino acid sequences of the reference with sequence identity (Identity(I)> 97%) and query coverage (100%) were selected; then, the similarities were checked in the T-COFFEE and PRALINE servers (http://www.tcoffee.crg.cat/apps/tcoffee/do:regular; http://www.ibi.vu.nl/programs/praline) (11).

Microorganisms and Animals. In this study, the microorganisms, including *P. aeruginosa* strains of PAO1 (ATCC$_{15692}$) and PAK (ATCC$_{25102}$), were used. *P. aeruginosa* R$_5$, aggressive and antibiotic-resistant hospital strain, was collected from the Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran). Six to eight-weeks-old male BALB/c mice, weighing 22-25 grams, were purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). For active and passive immunization, eleven and eight mice were assigned to each group, respectively.

Signal Peptide and Physicochemical Parameters. LipoP 1.0 (12) and SignalP 4.1 (13) servers were used (http://www.cbs.dtu.dk/services/LipoP/; http://www.cbs.dtu.dk/services/SignalP) to predict the signal peptides. The ExPASy ProtParam tool was used to measure the isoelectric point (pI), molecular weight, extinction coefficient, physicochemical indices, and Grand Average of Hydropathy (GRAVY) (14).

Prediction of Secondary and Tertiary Structures of the PcrV Protein. The Self-Optimized Prediction Method with Alignment (SOPMA) (15) and Phyre^2 (16) servers were used to predict the consensus-driving secondary structure prediction. The protein tertiary structure was predicted by I-TASSER and Phyre^2 servers.

Selection of the Protective Antigens and Antigenicity Prediction. The Protegen server (http://www.violinet.org/protegen/) (17), described as an analysis system, besides a central database of protective antigens and the Vaxign system (http://www.violinet.org/vaxign/index.php), were checked (18) to select the protective antigens. In order to determine the antigenicity of the PcrV protein, the VaxiJen v.2.0 server (19) (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used.

Prediction of the Antibody Epitopes and B Cells. In the tertiary protein structure, the BepiPred-2.0 server (20) (http://www.cbs.dtu.dk/services/Bepipred/) was used to predict linear B cell epitopes. Further, the LBtope (21) (http://crdd.osdd.net/raghava/lbtope/) server was searched to find more reliable linear B cell epitopes. The IEDB database (http://tools.iedb.org/bcell/) was another server used to analyze the data (granted by the National Institute of Allergy and Infectious Diseases (NIAID)) (20).

Expression, Purification, and Verification of the PcrV Protein. For protein production, the *pcrV* genes of *P. aeruginosa* PAO1 (GenBank Accession No: NC_002516.2) reading framework were isolated and amplified by the specific primers (*pcrV*-Forward: 5′-*CACGGAATTCATGGAAGTCAGAAACCTTA*-3′ and *pcrV*-Reverse: 5′-*ACCTAAGCTTCTAGATCGCGCTGAGAATG*-3′). The Pfu DNA polymerase (Fermentas Company, Lithonia) was used to perform amplification. The gene was inserted into the pET28a expression vector with restriction sites of the *EcoRI* and *HindIII* (Fermentas Company, Lithonia) at the 5′ and 3′ of *pcrV* gene, respectively; then, it was transferred into the *E. coli* BL21-DE3 strain (Novagen Co. Germany). The recombinant PcrV protein was induced by 1 mM Isopropyl β-D-1-Thiogalactoside (IPTG) and purified by the Ni-NTA column (Qiagen Company, USA) using the imidazole gradient in a natural condition. The purified recombinant protein was analyzed on SDS-PAGE and approved by the Western blotting (22).

Immunization of the Mice and Determination of the Antibody Titer. Different mice groups were immunized with 10 µg of the recombinant protein subcutaneously on days 0, 14, 28, and 42 for active immunization. The first injection was performed with complete Freund's adjuvant and three boosters with incomplete Freund's adjuvant. Blood samples were collected from the mice's eyes after the second, third, and fourth injections, and the antibody titer of the sera was measured as described earlier (22). The blood was collected pre-immunization, and the antibody titers against the PcrV protein of the sera were measured by ELISA. For the PcrV, the ELISA test was negative.

Induction of the Respiratory and Burn Infection. All animals were anesthetized as described before (22). For induction of an acute pneumonia model, after anesthesia, bacteria were injected into the right nasal cavity of the animals (23). The burned immunized mice were challenged with the subcutaneous injection of the bacteria at the center of burn. Bacterial counting was done by estimating the OD_{600} using a two-beam spectrophotometer (UV-Vis T80 Plus-PG, PG Instruments Ltd., UK), and the graph was plotted using the Excel 2016 software. In order to obtain the LD_{50}, mice were burned and then, were challenged with different concentrations of bacteria (10, 10^2, 5×10^2, 10^3, 2×10^3, 10^4 CFU). Intranasal injection of mice were also carried out with different concentrations (2×10^7, 3.6×10^7, 9×10^7, 3.6×10^7, and 3.6×10^8 CFU) of *P. aeruginosa* strains (PAO1, PAK, and R_5). The results were analyzed by applying the Probit test in the SPSS software version 20, and LD_{50}, $2xLD_{50}$, $5xLD_{50}$, 10xLD50, and $20xLD_{50}$ were determined. The test was performed three times individually. Finally, 14 days after injection of the third booster, the immunized mice were challenged with the burned and intranasal inoculations of $2xLD_{50}$, $5xLD_{50}$, and 10xLD50 of the bacteria and were maintained for one month (23).

Organ Collection for Bacterial Burden. Twenty-four hours after challenging the burned and PA-infected mice with $5xLD_{50}$ bacteria, three mice from each group were sacrificed. Skin, lung, liver, spleen, and kidney samples were obtained Sample preparation and counting the colony-Forming Units (CFU), in grams of tissue (cfu/g) was performed according to methods described in our previous work (22).

Passive Immunization and Respiratory Challenge. Mice groups each with six to eight week old female BALB/c mice were selected and the sera obtained from the mice immunized with recombinant proteins were isolated. 200 µl of the sera was mixed with $2xLD_{50}$ and $5xLD_{50}$ of the bacteria (PAO1, PAK, and R_5) in a Luria-Bertani broth and was incubated at 37 °C for 30 min in a shaking condition. The bacterial suspension was washed three times using the PBS and was collected by centrifugation. Finally, 30 µL of the suspension was injected into the right nasal cavity of the mice. Burn mice were subcutaneously administered with 100 µL of the suspension at the center of the burned skin.

Statistical Analysis. Data were analyzed by the Analysis of Variance (ANOVA) and t-test using the SPSS software version 20. The mean, standard deviation, and significance level were calculated, and the *p*-value of < 0.05 was considered as statistically significant. The Excel 2016 and GraphPad Prism software, version 6 were used to draw the charts, graphs, and data contents.

RESULTS

Sequence Availability, Similarity Search, and Alignments.

The PcrV sequences of the NCBI, UniProt, and ExPASy databanks were equal to 294 amino acids. The PcrV protein conserved in 1552 clinical and environmental isolates was also studied.

Physicochemical Parameters and Signal Peptide.

Physicochemical parameters such as the isoelectric point (pI), molecular weight, formula, total number of atoms, and GRAVY score were obtained as 5.

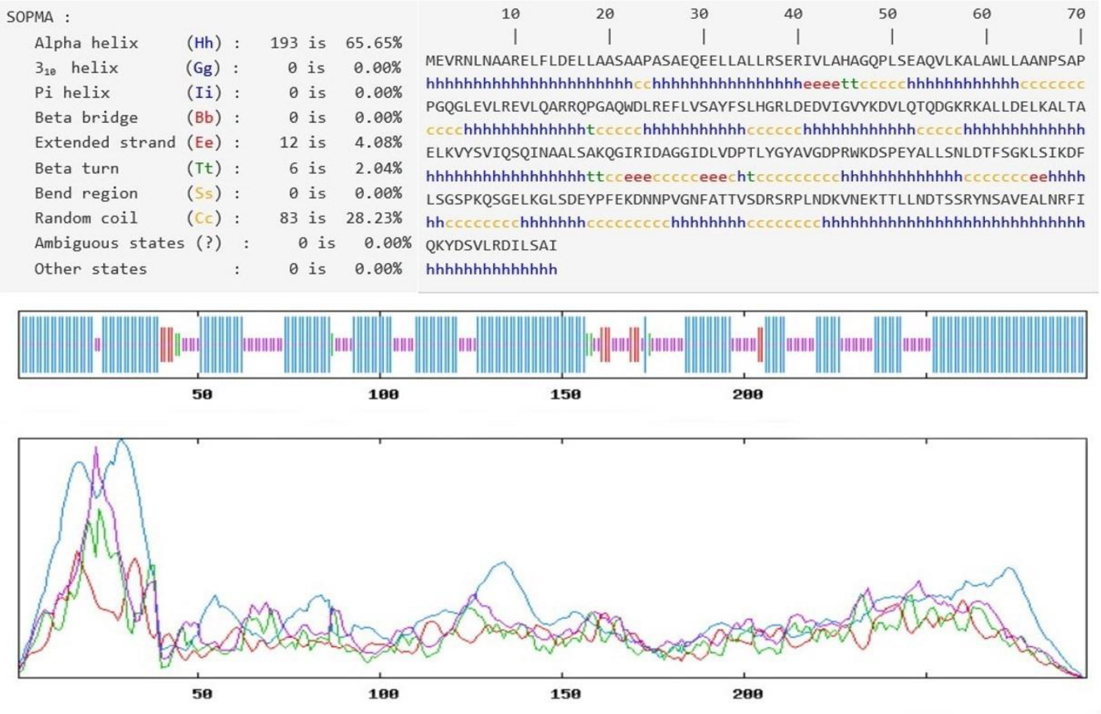
03, ~33 kDa, $C_{1436}H_{2301}N_{395}O_{447}S_1$, 4580, and -0.257, respectively for the PcrV. The PcrV protein had not any signal peptide.

Protective Antigens and Antigenicity Prediction.

The PcrV was recorded as protective antigen for *P. aeruginosa* in the Protegen server. The adhesion probability for the PcrV was reported to be 0.214. The score for the antigenic properties of the PcrV was obtained as 0.4760 in the VaxiJen server.

Analysis of the Secondary and Tertiary Structure Prediction for the PcrV.

The secondary structure of the PcrV was predicted by SOPMA the online software and is exhibited in Figure 1. The 3D model structure of the PcrV protein was generated by the I-TASSER software and Phyre^2 server. The confidence score (C-score) indicating the quality of the predicted models by the I-TASSER software was equal to -0.26. The expected TM-score for PcrV was obtained as 0.68 ± 0.12.



```
SOPMA :
   Alpha helix      (Hh) :   193 is  65.65%
   310  helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    12 is   4.08%
   Beta turn        (Tt) :     6 is   2.04%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    83 is  28.23%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

```
         10        20        30        40        50        60        70
          |         |         |         |         |         |         |
MEVRNLNAARELFLDELLAASAAPASAEQEELLALLRSERIVLAHAGQPLSEAQVLKALAWLLAANPSAP
hhhhhhhhhhhhhhhhhhhhhhcchhhhhhhhhhhhhhhheeeettccccchhhhhhhhhhhhccccccc
PGQGLEVLREVLQARRQPGAQWDLREFLVSAYFSLHGRLDEDVIGVYKDVLQTQDGKRKALLDELKALTA
cccchhhhhhhhhhhhhtccccchhhhhhhhhhhcccccchhhhhhhhhhhhccccchhhhhhhhhhhhh
ELKVYSVIQSQINAALSAKQGIRIDAGGIDLVDPTLYGYAVGDPRWKDSPEYALLSNLDTFSGKLSIKDF
hhhhhhhhhhhhhhhhhttcceeecccccceeechtccccccccchhhhhhhhhhhhhccccccceehhhh
LSGSPKQSGELKGLSDEYPFEKDNNPVGNFATTVSDRSRPLNDKVNEKTTLLNDTSSRYNSAVEALNRFI
hhcccccccchhhhhhhccccccccchhhhhhhhcccccccchhhhhhhhhhhhhhhhhhhhhhhhhhhh
QKYDSVLRDILSAI
hhhhhhhhhhhhhh
```

Figure 1. Secondary Structure Prediction. The results of secondary structure prediction for the recombinant protein using the SOPMA software.

As shown in Figure 2(a), the N-terminal region of the protein begins with several helixes and sheets where the C-terminal of the protein is consisted of several helixes. The Phyre^2 server results showed an overall good quality for the predicted 3D model (Figure 2b). Totally, 253 residues (86% of the sequence) were modelled with 100.0% confidence by the single highest scoring template. Figure 2b shows the final model of PcrV protein.

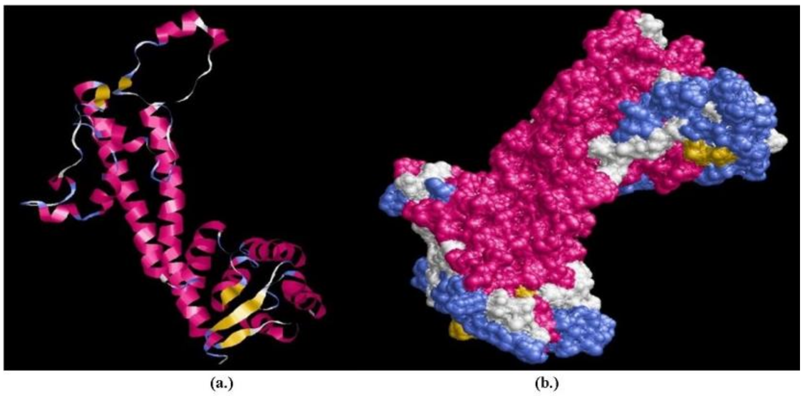


Figure 2. Tertiary Structure Prediction. The results of tertiary structure prediction for the PcrV protein by the Phyre2 server and I-TASSER software. The results were checked by the RasMol-MODEL3.PDB software. **(a)** View of the ribbons, **(b)** View of the molecular surface.

The PcrV Protein Expression, Purification, and Verification.

The colonies are analyzed with clony-PCR (Figure 3a) and further confirmed by enzyme digestion (Figure 3b). A ~33-kDa protein band appeared on the SDS-PAGE. The recombinant protein was purified by the Ni-NTA affinity column (Figure 3c) and confirmed with western blotting (Figure 3d). The concentration of recombinant PcrV was 85 mg/L.

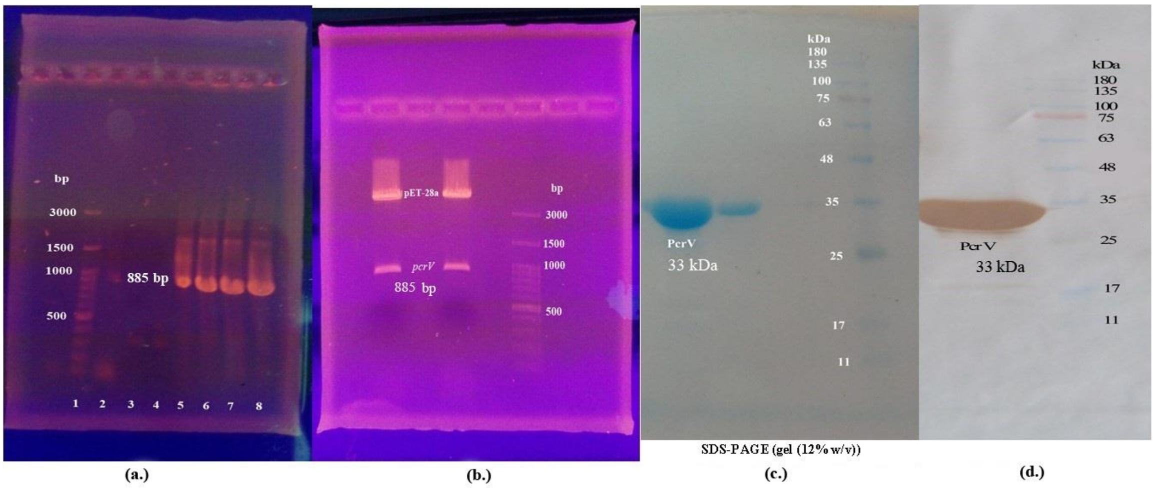


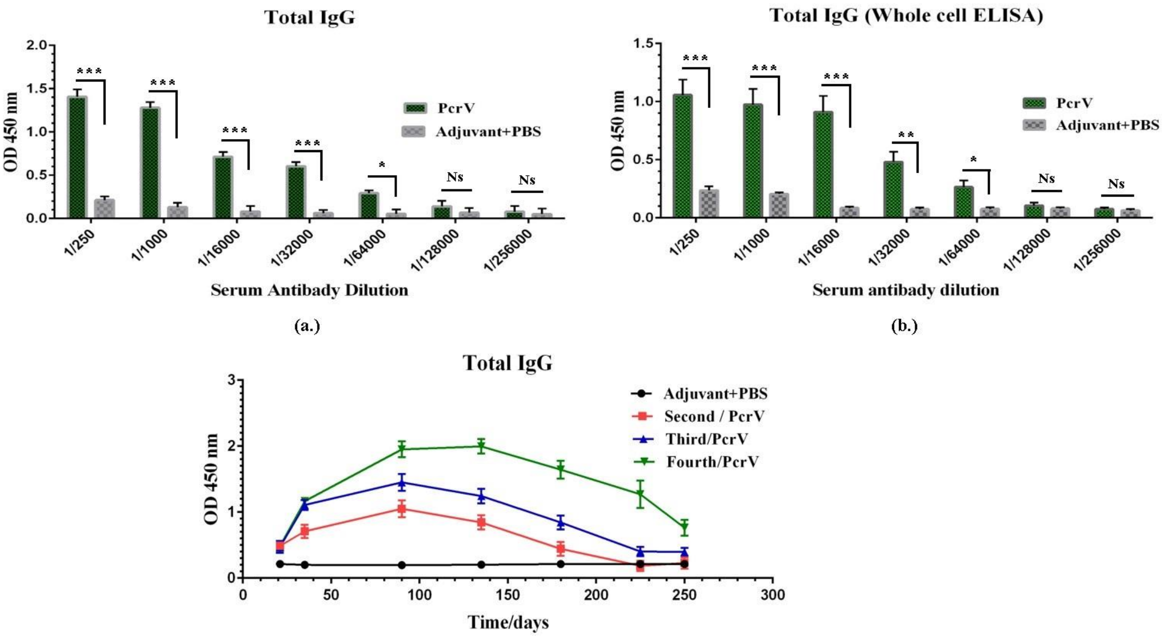
Figure 3. Colony PCR Analysis on Agarose Gel. (a.) PCR products obtained from colony PCR was analyzed on 1% agarose gel electrophoresis. Lane 1: molecular weight marker; Lanes 2-8: colony PCR from 1, 2, and 3 clones, respectively; Lanes 2-4: Negative control (Lane 2: without enzyme; Lane 3: *E.coli* without pET-28a-*pcrV*; Lane 3: without template); Lane 8: Positive control (*Pseudomonas aeruginosa* PAO1). (b.) The results of 1% agarose gel electrophoresis on the recombinant pET-28a. Lanes 1 and 2: Double digests of pET28a-*pcrV* with *EcoRI* and *HindIII* from 2 and 3 clones, respectively; b) Lane 3: molecular weight marker. (c.) the PcrV protein purified on SDS-PAGE (gel (12% w/v) was stained with Coomassie blue R-250). The column was rinsed by 250 mM imidazole. (d.) Confirmation of the PcrV protein through the Western blotting.

Prediction of the B cell and Antibody Epitope. Table 1 presents the B cell epitopes predicted by the "BepiPred" LBtope, and IEDB servers for the PcrV.

Table 1. The linear B-cell epitopes predicted by BepiPred LBtope, and IEDB for the PcrV.

Row	Server	Peptide/BepiPred	Start position	End position	Length
1	LBtope	NLNAAREL	5	12	8
	IEDB	ASAAPASAEQE	20	30	11
2	LBtope	SAAPASAEQEEL	21	32	12
	IEDB	GQPLSEA	47	53	7
3	LBtope	HAGQPLSEAQ	45	54	10
	IEDB	ANPSAPPGQGLE	64	76	12
4	LBtope	NPSAPPGQGL	66	75	10
	IEDB	QARRQPGAQWD	83	93	11
5	LBtope	VLQARRQPGAQWD	81	93	13
	IEDB	RLDE	108	111	4
6	LBtope	R	108	108	1
	IEDB	I	114	114	1
7	LBtope	D	110	110	1
	IEDB	VLQTQDGKRKA	120	130	11
8	LBtope	QTQDGKRKALLD	122	133	12
	IEDB	N	153	153	1
9	LBtope	AALSAKQGIRIDAGGIDLVDPTLY GYAVGDPRWKDSPEYALLSNLDT	154	268	114
	IEDB	FSGKLSIKDFLSGSPKQSGELKGL SDEYPFEKDNNPVGNFATTVSDR SRPLNDKVNEKTTLLNDTSSR ALS	155	157	3
10	LBtope	S	285	285	1
	IEDB	K	159	159	1
11	LBtope	-	-	-	-
	IEDB	GIRIDAGGIDLV	161	172	12
12	LBtope	-	-	-	-
	IEDB	P	174	174	1
13	LBtope	-	-	-	-
	IEDB	LYGYAVGDPRWKDSPEYA	176	193	18
14	LBtope	-	-	-	-
	IEDB	TF	200	201	2
15	LBtope IEDB	FLSGSPKQSGELKGLSDEYPFEKD NNPVGNFATTVSDRSRPLNDKVN EKTTLLNDTSSRY NSAV	210	273	64

Antibody Titer. The ELISA was used to determine the antibody titers raised against the PcrV proteins (Figure 4). As indicated in Figure 4c, the antibody titers toward the PcrV protein remained high for 135 days. The results of ELISA showed a significant increase ($P<0.001$) in the antibody titer (total IgG) of the test mice compared to the control after each injection until 1:32,000 dilution of the mouse serum (Figure 4a). Results regarding the whole-cell neutralization showed that it was performed well at a concentration of 1:64,000 dilution (*P*<0.05) as clearly shown in Figure 4b.



(a.)

(b.)

(c.)

Figure 4. ELISA Analysis of Antibody Titers. (a.) The total IgG bar chart after injection of the third booster in serial dilution antibody for PcrV recombinant protein. (b.) The binding power of specific antibodies to the *P. aeruginosa* PAO1 whole -cell in the PcrV recombinant protein. (c.) Total IgG level in the mice immunized with the PcrV recombinant protein at different times. Ns: Not Significant, **P*<0.05, ***P*<0.01 and ****P*<0.001

LD$_{50}$, Active and Passive Immunization, Challenges in the Animals.

Table 2 presents the results of different doses of LD50 (LD50, 2xLD50, 5xLD50, 10xLD50, and 20xLD50) for each bacteria. The immunized burned and PA-infected mice were challenged by injection and inoculations of *P. aeruginosa* strains including PAO1, PAK, and R5 at 2xLD50, 5xLD50, and 10xLD50 doses to determine the protective potency of our recombinant proteins respectively. A significant increase (*P*<0.05) was observed in the survival rate of the mice vaccinated with the PcrV protein compared to the control burned and PA-infected group that were challenged by injection or inoculations of bacteria at 5xLD50 and 10xLD50 doses for active immunization (Figure 5). It is noteworthy that the survival (%) in a high percentage of the mice immunized with the PcrV protein was high after injection of the bacteria at 5xLD$_{50}$ in both burned and PA-infected mice (Figure 5a and 5b). Also, 50% of the mice immunized with the PcrV protein successfully survived following the injection of 5xLD$_{50}$ of R$_5$-resistant strain. Although, the survival rate (%) was higher in the mice challenged with injection of PAO1 and PAK standard strains (Figure 5a and 5b). For passive immunization, the survival rate

(%) was significant (*P*<0.01) in the burned and PA-infected mice challenged with injection of the bacteria at 2xLD50.

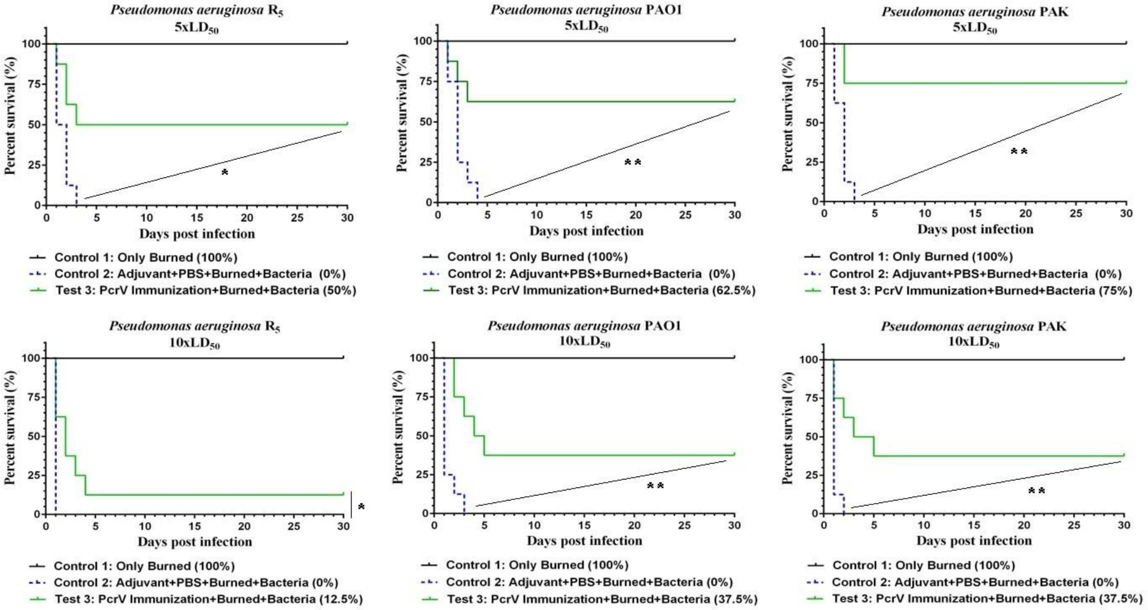


Fig. 5(a.)

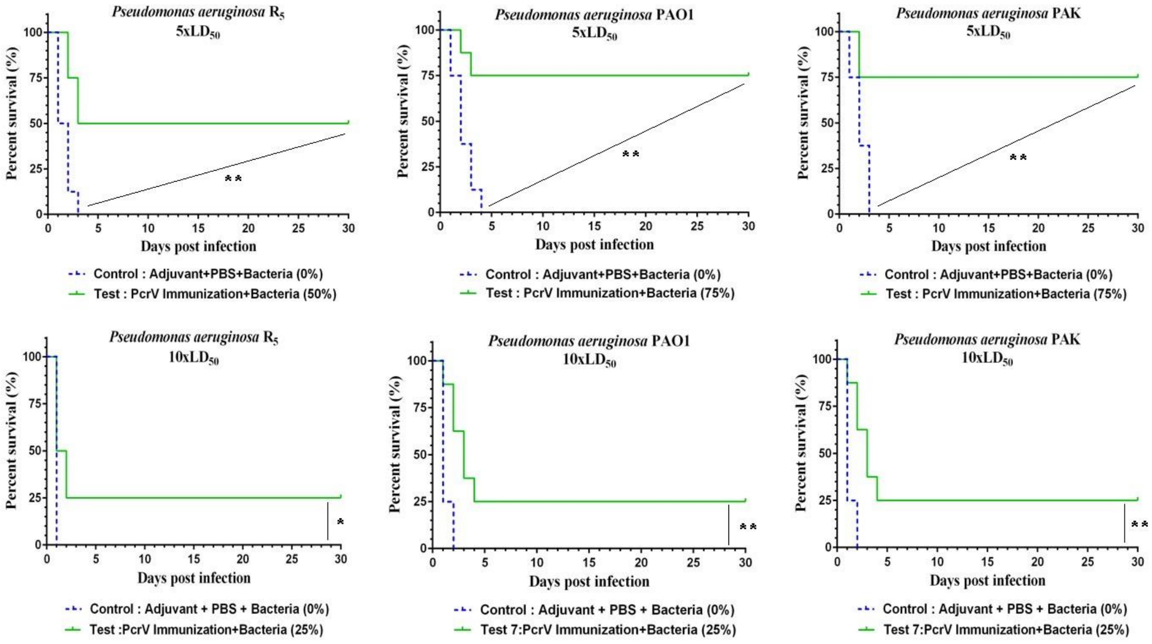


Fig. 5(b.)

Figure 5. Survival Rate Estimation Analysis of Active Immunization. Active immunization by comparing the survival rates of the burned (a) and PA-infected (b) mice treated with the placebo (adjuvant with PBS) and PcrV vaccine candidate and challenged with the inoculations of *P. aeruginosa* strains including PAO1, PAK, and R$_5$. Numbers in parentheses indicate percent (%) survival. **P*<0.05, and ***P*<0.01

Table 2. Lethal doses of *Pseudomonas aeruginosa* strains, including PAO1, PAK, and R_5, in the burned and PA-infected mice, challenged with the inoculations of bacteria.

Bacteria	Dose	CFU/Intranasal	CFU/Burned
***P. aeruginosa* PAO1**	LD_{50}	1.02×10^8	1.4×10^3
	$2xLD_{50}$	2.04×10^8	2.8×10^3
	$5xLD_{50}$	5.1×10^8	7×10^3
	$10xLD_{50}$	1.02×10^9	1.4×10^4
	$20xLD_{50}$	2.04×10^9	2.8×10^4
***P. aeruginosa* PAK**	LD_{50}	1.0×10^8	1.7×10^3
	$2xLD_{50}$	2.0×10^8	3.4×10^3
	$5xLD_{50}$	5.0×10^8	8.5×10^3
	$10xLD_{50}$	1.0×10^9	1.7×10^4
	$20xLD_{50}$	2.0×10^9	3.4×10^4
***P. aeruginosa* R_5**	LD_{50}	5.5×10^7	6×10^2
	$2xLD_{50}$	1.1×10^8	1.2×10^3
	$5xLD_{50}$	2.7×10^8	3×10^3
	$10xLD_{50}$	5.5×10^8	6×10^3
	$20xLD_{50}$	1.1×10^9	1.2×10^4

However, the PcrV protein was more successful in producing the passive immunity than the placebo. So, the results were not acceptable. Figure 6a and 6b present the results of passive immunization.

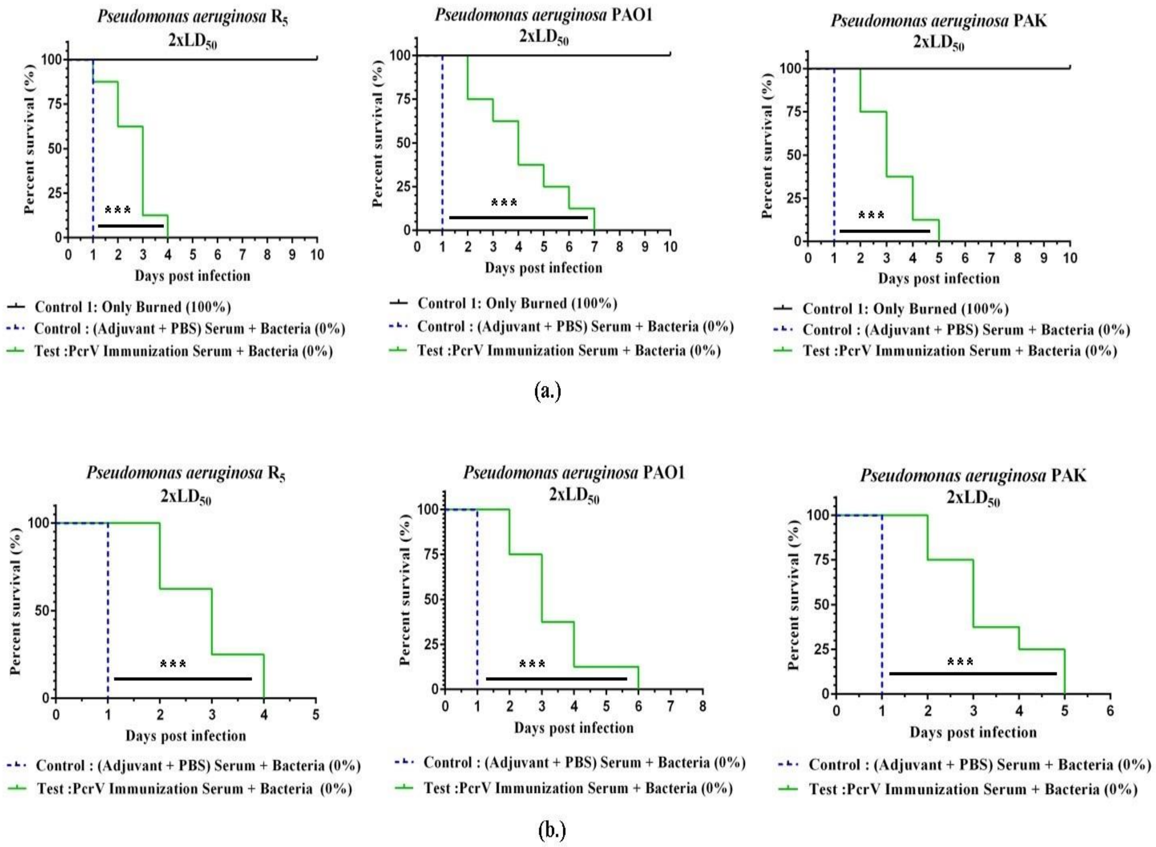


Figure 6. Survival Rate Estimation Analysis of Passive Immunization. Passive immunization by comparing the survival rates of the burned (a) and PA-infected (b) mice treated with the placebo (adjuvant with PBS) and PcrV vaccine candidate and challenged with the inoculations of *P. aeruginosa* strains including PAO1, PAK, and R_5. Numbers in parentheses indicate percent (%) survival. ****P*<0.001

Bacterial Burden. The mice immunized with the PcrV protein showed significantly lower bacterial loads (*P*<0.01) in the organs including skin, lung, liver, spleen, and kidney, in comparison with the control group 24 h after injection of the bacteria at 5xLD50 in the PA-infected (Figure 7b) and burned (Figure 7a) mice. These results indicate that immunization with the PcrV protein could reduce the bacterial local colonization and systematic spreading simultaneously.

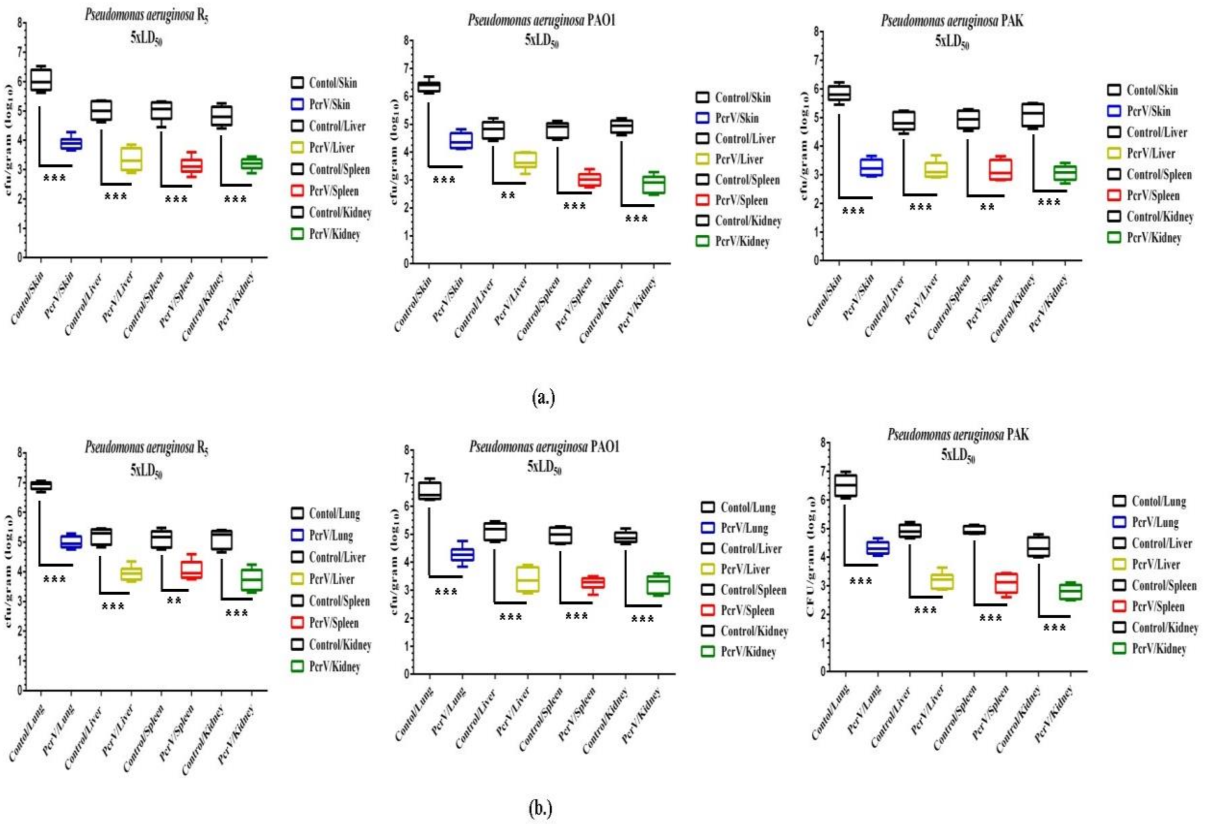


Figure 7. Bacterial Load of Different Organs. Bacterial loads in the skin, lung, liver, spleen, and kidney of the immunized burned (a) and PA-infected (b) mice challenged with the inoculations of PAO1, PAK, and R_5 strains at $5xLD_{50}$ 24 h after infection. $^{**}P<0.01$ and $^{***}P<0.001$

DISCUSSION

PcrV is a potentially protective antigen of *P. aeruginosa*, as immunogenic molecules especially in epitopes of B cells (Table 1) (24,25). The sum of hydrophobicity scales of all the amino acids is described as the GRAVY score for a protein or peptide. Our results indicated the negative GRAVY score (-0.257) for all the proteins. The hydrophobicity of the bacterial surface acts as opsonin, creating the phagocytic capacity and contributing to the extent of the complement system and IgG opsonization (26). Antigen probability for PcrV estimated by the VaxiJen server showed that PcrV protein was highly antigenic. Therefore, with high binding strength of the recombinant protein to the antibody (Figure 4a, 4b), B-cell can be stimulated at high levels and humoral immunity occurs. Sequence similarity was determined for finding a conserved region in PcrV sequences; this region should be conserved in the majority or all the pathogenic strains. Our results revealed that the protein sequences of PcrV were highly conserved in 1552 clinical and environmental isolates, respectively. Worgall *et al.*, (27) showed that an OprF epitope (14 amino acids) from *P. aeruginosa* (Epi-8) boosted the cellular anti-Epi8 and humoral anti-OprF responses. In a previous study, full-length PcrV$_{1\text{-}294}$ significantly enhanced the active immunization in the mice challenged with *P. aeruginosa* (25). As can be seen in Table 1, the epileptic segments of PcrV stimulating the immune system are further located in the C-terminal portion. It seems to be useful to combine the C-terminal PcrV protein or Epi-9 and Epi-15 (Table 1) with another epitopes evaluated in other proteins such as OprF, OprI. A strong immunogenicity was found in the mice, which could protect mice toward *P. aeruginosa* challenge (Figure 4 and 5). Evidence shows that the PcrV is able to induce the humoral and cellular immunity (28). In a study, higher antibody titers were induced against the OprI, OprF, and flagellin B through subcutaneous immunization with OprI and OprF (with or without flagellin B). The antibodies could effectively opsonize the nonmucoid or mucoid strains, thereby activating the complement terminal pathway, which is known to eliminate the nonmucoid strains through complement-mediated lysis. In addition, a significantly longer survival reduced the lung damage, and low-grade of bacteremia was induced in the immunized mice in comparison with the non-immunized control mice (24). In our research, an increase in the antibody titers was also quite evident to 1:64000 dilution (Figure 4a). Numerous surveys have formerly suggested that the protection mediated by anti-PcrV antibodies is Fc -independent, as these antibodies are not able to activate the complement-mediated killing (29). Thanabalasuriar *et al.*, (30) by applying the multi-laser spinning-disk intravital microscopy found that the protection against anti-PcrV antibodies is highly dependent on the inhibition of cytotoxic activity of the T3SS and the acidification of endosomal compartments. Chung Wan *et al.*, (31), however utilized the PcrV-derived chimeric antibodies and displayed that the uptake of neutrophils by anti-PcrV antibody is a prominent feature in the response to *P. aeruginosa* infection killing the bacteria in a number of highly effective molecules. They also observed vigorous Th_2 immune response after immunization with the $PcrV_{NH}$ (the Nter and H12 domains of PcrV). Anti-$PcrV_{NH}$ antibodies were expected to contribute to the engulfment of *P. aeruginosa* by the neutrophils, because Chung Wan *et al.*, (31) ,in an *in -vivo* study (2019) pointed out a sharp increase in the *P. aeruginosa* internalization when anti-PcrV antibodies were given. Anti-PcrV antibodies were also capable of boosting the localization of ingested *P. aeruginosa* into acidified vacuoles (30), giving rise to an effective killing within the acidified phagosomes in the neutrophils (31). Their findings (31) implied that the $PcrV_{NH}$ protein could raise the level of antibodies (IgG_1, IgG_{2a}, and IgG_{2b}) and the titer of IL-4, IL-17, and IL-1β suggesting the humoral immunity. In addition, this protein is comprised of the N-terminal (1-127 amino acid) and the helix 12 in C-terminal (251-294 amino acids). In line with our finding, Dacheux *et al.* (32) found that the antibodies could act against the PcrV, OprI, and OprF virulence factors of *P. aeruginosa*, as well as interfering with the early stages of its pathogenesis and later stages such as systemic dissemination to the organs. Our results showed that resistance to the pathogenic bacteria was started from the lung (nasal) or skin (burned), and more bacteria were killed in these areas. *P. aeruginosa* (PAO1, PAK, and R_5) was not observed in the internal organs at LD_{50}, while the bacteria were able to cross the lung and skin in small amounts at $5xLD_{50}$ (Figure 7a and 7b). Passive immunotherapy with anti-r-PilA IgG protected the burned mice infected with *P. aeruginosa* strains of PAO1 and a clinical Isolate (CI) and enhanced the opsonophagocytosis of these strains. Anti-r-PilA IgG successfully reduced the bacterial burden in the infected mice (33). IgY antibodies raised against the PcrV, decreased the invasiveness of *P. aeruginosa*, and increased killing of *P. aeruginosa* and survival of the infected mice (34). Although our findings demonstrated that the PcrV protein could be protect against the *P. aeruginosa* infection, it is recommended to use chimeric proteins where combination of several effective proteins in a vaccine acts against the *P. aeruginosa* infection. It is believed that the whole PcrV or Epi-9 and Epi-15 of PcrV (Table 1) in the chimeric recombinant antigen may effectively stimulate the immune system and prevent the *P. aeruginosa* infections.

ACKNOWLEDGEMENTS

The data were extracted from a PhD thesis by Mohammad Hadi Fakoor at Islamic Azad University (Karaj Branch) in collaboration with Shahed University (Grant No. 1398.057). The authors extend their gratitude to Molecular Microbiology Research Center (MMRC) for providing facilities to conduct this study.

REFERENCES

1. Salimi H, Yakhchali B, Owlia P, Lari AR. Molecular epidemiology and drug susceptibility of pseudomonas aeruginosa strains isolated from burn patients. Lab Med. 2010; 41:540-4.

2. Chen J, Chen Y, Hu P, Zhou T, Xu X, Pei X. Risk assessment of infected children with Pseudomonas aeruginosa pneumonia by combining host and pathogen predictors. Infect Genet Evol. 2018; 57:82-7.

3. Warrener P, Varkey R, Bonnell JC, DiGiandomenico A, Camara M, Cook K, et al. A Novel Anti-PcrV Antibody Providing Enhanced Protection against Pseudomonas aeruginosa in Multiple Animal Infection Models. Antimicrob Agents Chemother. 2014; 58:4384-91.

4. Sato H, Frank DW. Multi-functional characteristics of the Pseudomonas aeruginosa type III needle-tip protein, PcrV; comparison to orthologs in other Gram-negative bacteria. Front Microbiol. 2011; 2:142.

5. Portaliou AG, Tsolis KC, Loos MS, Zorzini V, Economou A. Type III secretion: building and operating a remarkable nanomachine. Trends Biochem Sci. 2016; 41:175-89.

6. Lynch SV, Flanagan JL, Sawa T, Fang A, Baek MS, Rubio-Mills A, et al. Polymorphisms in the Pseudomonas aeruginosa type III secretion protein, PcrV - implications for anti-PcrV immunotherapy. Microb Pathog. 2010; 48:197-204.

7. Rappuoli R. Bridging the knowledge gaps in vaccine design. Nat Biotechnol. 2007; 25:1361-66.

8. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 2003; 31:3784-88.

9. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, et al. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. Nucleic Acids Res. 2010; 39(Database issue):D596-D600.

10. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008; 36(Web Server issue):W5-W9.

11. Di Tommaso P, Moretti S, Xenarios I, Orobitg M, Montanyola A, Chang J-M, et al. T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. Nucleic Acids Res. 2011; 39(Web Server issue):W13-W17.

12. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. 2003; 12:1652-62.

13. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011; 8:785-86.

14. Rahbar MR, Rasooli I, Gargari SLM, Sandstrom G, Amani J, Fattahian Y, et al. A potential in silico antibody–antigen based diagnostic test for precise identification of Acinetobacter baumannii. J Theor Biol. 2012; 294:29-39.

15. Geourjon C, Deleage G. SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput Appl Biosci. 1995; 11:681-84.

16. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015; 10:845-58.

17. He Y, Xiang Z. Bioinformatics analysis of bacterial protective antigens in manually curated Protegen database. Procedia Vaccinol. 2012; 6:3-9.

18. Xiang Z, He Y. Genome-wide prediction of vaccine targets for human herpes simplex viruses using Vaxign reverse vaccinology. BMC Bioinf. 2013; 14(Suppl 4):S2.

19. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinf. 2007; 8:4.

20. Larsen JEP, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immunome Res. 2006; 2:2.

21. Singh H, Ansari HR, Raghava GP. Improved method for linear B-cell epitope prediction using antigen's primary sequence. PloS one. 2013; 8:e62216.

22. Fakoor MH, Gargari SLM, Owlia P, Sabokbar A. Protective Efficacy of the OprF/OprI/PcrV Recombinant Chimeric Protein Against Pseudomonas aeruginosa in the Burned BALB/c Mouse Model. Infect Drug Resist. 2020; 13:1651-61.

23. Behrouz B, Mahdavi M, Amirmozafari N, Fatemi MJ, Irajian G, Bahroudi M, et al. Immunogenicity of Pseudomonas aeruginosa recombinant b-type fagellin as a vaccine candidate: protective efficacy in a murine burn wound sepsis model. Burns. 2016; S0305-4179:30028-6.

24. Hassan R, El-Naggar W, Abd El-Aziz AM, Shaaban M, Kenawy HI, Ali YM. Immunization with outer membrane proteins (OprF and OprI) and flagellin B protects mice from pulmonary infection with mucoid and nonmucoid Pseudomonas aeruginosa. J Microbiol Immunol Infect. 2018; 51:312-20.

25. Tabor DE, Oganesyan V, Keller AE, Yu L, McLaughlin RE, Song E, et al. Pseudomonas aeruginosa PcrV and Psl, the molecular targets of bispecific antibody MEDI3902, are conserved among diverse global clinical isolates. J Infect Dis. 2018; 218:1983-94.

26. Thomsen K, Christophersen L, Bjarnsholt T, Jensen PØ, Moser C, Høiby N. Anti-Pseudomonas aeruginosa IgY antibodies induce specific bacterial aggregation and internalization in human polymorphonuclear neutrophils. Infect Immun. 2015; 83:2686-93.

27. Worgall S, Krause A, Rivara M, Hee K-K, Vintayen EV, Hackett NR, et al. Protection against P. aeruginosa with an adenovirus vector containing an OprF epitope in the capsid. J Clin Invest. 2005; 115:1281-9.

28. Aguilera-Herce J, García-Quintanilla M, Romero-Flores R, McConnell MJ, Ramos-Morales F. A Live Salmonella Vaccine Delivering PcrV through the Type III Secretion System Protects against Pseudomonas aeruginosa. mSphere. 2019; 4:e00116-19.

29. Shime N, Sawa T, Fujimoto J, Faure K, Allmond LR, Karaca T, et al. Therapeutic administration of anti-PcrV F (ab′) 2 in sepsis associated with Pseudomonas aeruginosa. J Immunol. 2001; 167:5880-6.

30. Thanabalasuriar A, Surewaard BG, Willson ME, Neupane AS, Stover CK, Warrener P, et al. Bispecific antibody targets multiple Pseudomonas aeruginosa evasion mechanisms in the lung vasculature. J Clin Invest. 2017; 127:2249-61.

31. Chuang Wan JZ, Zhao L, Cheng X, Gao C, Wang Y, Xu W, et al. Rational design of a chimeric derivative of PcrV as a subunit vaccine against Pseudomonas aeruginosa. Front Immunol. 2019; 10:781.

32. Dacheux D, Goure J, Chabert J, Usson Y, Attree I. Pore-forming activity of type III system-secreted proteins leads to oncosis of Pseudomonas aeruginosa-infected macrophages. Mol Microbiol. 2001; 40:76-85.

33. Mousavi M, Behrouz B, Irajian G, Mahdavi M, Korpi F, Motamedifar M. Passive immunization against Pseudomonas aeruginosa recombinant PilA in a murine burn wound model. Microb Pathog. 2016; 101:83-8.

34. Ranjbar M, Behrouz B, Norouzi F, Mousavi Gargari SL. Anti-PcrV IgY antibodies protect against Pseudomonas aeruginosa infection in both acute pneumonia and burn wound models. Mol Immunol. 2019; 116:98-105.