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Evaluation of systemic and mucosal immune responses of a multi-epitope vaccine candidate composed of virulence factors PcrV and OmpE against *Pseudomonas aeruginosa* in a BALB/c mouse model

Hamidreza Kalantari[1], Mehri Habibi[2]*, Atosa Ferdousi[1]*, Mohammad Reza Asadi Karam[3], Taher Mohammadian[1]

[1]Department of Microbiology, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran. [2]Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran. [3]Department of Molecular Biology, Pasteur Institute of Iran, Pasteur Ave., Tehran 1316943551, Iran.

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***Corresponding Authors:**

Mehri Habibi;
Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran, 1316943551,
Tel/Fax: (+98)2164112345; (+98)21 66492619.
Email: m_habibi1362@yahoo.com.

Atosa Ferdousi:
Department of Microbiology, ShQ.C., Islamic Azad University, Shahr-e Qods, Iran.
Email: dr.at.ferdousi@gmail.com

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is a major opportunistic pathogen causing urinary tract infections (UTIs), particularly in hospitalized or immunocompromised patients. Rising antibiotic resistance highlights the urgent need for effective vaccines. PcrV and OmpE as key virulence factors of *P. aeruginosa* are promising targets for vaccine development. In a previous study, a multi-epitope vaccine construct consisting B- and T-cell epitopes of PcrV and OmpE from *P. aeruginosa* was designed using immunoinformatics tools. In the present study, the immunogenicity of the recombinant protein was assessed in a murine model.
Methods: After purification of the multi-epitope protein PcrV.OmpE using Nickel resin, different vaccine formulations including multi-epitope protein alone and admixed with alum were injected into mice through the subcutaneous route. Thereafter, the levels of systemic humoral and mucosal immune responses were investigated using ELISA method.
Results: The multi-epitope vaccine construct alone elicited strong systemic IgG and IgA responses, as well as mucosal antibody in urine as compared to the control mice ($p<0.05$). The addition of alum significantly boosted systemic IgG and IgA, as well as mucosal IgG antibody response as compared to the multi-epitope alone ($p<0.05$).
Conclusion: These results support the potential of a multi-epitope composed of PcrV and OmpE antigens for the prevention of *P. aeruginosa*-associated UTIs, warranting further studies on cellular immunity and protective efficacy.

INTRODUCTION

Pseudomonas aeruginosa is a major opportunistic pathogen responsible for a wide range of healthcare-associated infections, including urinary tract infections (UTIs), pneumonia, burn wound infections, and septicemia [1]. UTIs caused by *P. aeruginosa* are particularly problematic in hospitalized patients, individuals with urinary catheters, and immunocompromised population [2]. The intrinsic resistance of this pathogen to different antibiotics, combined with its remarkable ability to acquire multidrug resistance, has significantly limited available therapeutic options and increased treatment failure rates [3, 4].
Vaccination represents a promising alternative strategy for preventing *P. aeruginosa* infections [5]. However, despite extensive researches, no licensed vaccine is currently available [6, 7]. One of the main challenges in developing an effective vaccine against *P. aeruginosa* is its antigenic diversity and complex virulence mechanisms [8].

Among the numerous virulence-associated proteins, PcrV as a key component of the type III secretion system, and OmpE as an outer membrane protein involved in bacterial survival and host interaction, have been identified as highly conserved and immunologically relevant antigens [9-11].

Recently, immunoinformatics and reverse vaccinology approaches have enabled the rational design of multi-epitope vaccines that combine immunodominant regions from multiple antigens to enhance immune coverage while reducing unnecessary or reactogenic components [12]. In a previous study, a multi-epitope vaccine construct derived from conserved regions of PcrV and OmpE was designed and experimentally evaluated in a rabbit model, demonstrating promising immunoreactivity [13].
However, comprehensive evaluation of vaccine-induced immune responses in murine models remains essential,

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particularly for assessing systemic and mucosal immunity, which plays a critical role in protection against UTIs. Moreover, adjuvant effects, such as those mediated by aluminum-based compounds, require careful experimental validation in relevant animal models [14].

Therefore, the present study aimed to experimentally evaluate the systemic and mucosal immunogenicity of a previously designed multi-epitope vaccine candidate (PcrV.OmpE), administered alone or formulated with alum, in BALB/c mice. This study focuses on humoral immune responses, including serum IgG and IgA, as well as mucosal antibody in urine, to provide further evidence supporting the vaccine's potential against *P. aeruginosa*-associated UTIs.

MATERIALS AND METHODS

Vaccine Antigen Preparation

The recombinant multi-epitope vaccine construct, previously designed based on conserved immunogenic regions of PcrV and OmpE, was expressed in *E. coli* BL21 (DE3) using a pET28a expression vector. Protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) and the recombinant protein was purified under denaturing conditions using Ni-NTA affinity chromatography. Purity and molecular weight of the protein were confirmed by SDS-PAGE and Western blot analysis [13].

Animals and Experimental Design

Female BALB/c mice were used in this study. Animals were randomly divided into four experimental groups, each consisting of 12 mice: (i) mice immunized with the multi-epitope vaccine alone, (ii) mice immunized with the multi-epitope formulated with alum, (iii) control mice receiving phosphate-buffered saline (PBS), and (iv) control mice receiving alum alone. The animal Studies were performed according to the Ethical Number: IR.IAU.QODS.REC.1401.005.

Immunization Protocol

Mice were immunized using the subcutaneous route, following a prime-boost regimen. Each animal received an initial immunization followed by two booster doses at defined intervals (days 14 and 28 after the first vaccine dose). The animals were immunized with 50 µg of the multi-epitope construct alone or admixed with 200 µg of alum adjuvant. Blood samples were collected from the tail veins of the injected mice two weeks after each immunization (days 15, 30, and 45) and sera were obtained after centrifugation. In addition, urine samples were collected two weeks after the final booster dose for evaluation of mucosal immune responses. All samples were stored at −20°C until further use.

Evaluation of Humoral Immune Responses

Serum samples were analyzed to detect the total IgG and IgA antibodies specific to the multi-epitope using Enzyme-Linked Immunosorbent Assay (ELISA). Urinary samples were similarly assessed to measure the level of mucosal IgG and IgA responses. Briefly, the recombinant multi-epitope was diluted with coating buffer (0.1 mM $NaHCO_3$, pH=7.4) and 96-well ELISA plates (Greiner, Germany) were coated with the diluted protein (10 µl/ml). After overnight incubation at 4°C, the plates were washed and blocked with PBS containing 3% bovine serum albumin (BSA; Sigma) for 2 h. Then, the serum samples prepared in 1% BSA were added to the plates for 2 h. The plates were washed and HRP-conjugated goat anti-mouse IgG antibody (Sigma, USA) in 1:30,000 dilution was added to the plates. Thereafter, the reactions were stopped with 0.5 M sulfuric acid and 100 µl of tetramethylbenzidine (TMB) substrate was added to the plates and the adsorption (O.D. 450 nm) of reactions was read by a microplate reader (Biotek, USA). To determine IgA response in serum and urine samples, coating and blocking the microtiter plates were similar to the total IgG. Afterwards, serum and urine samples were added to the plates. The plates were washed and secondary antibody specific for each IgG1, IgG2a, and IgA was added in 1:1000 dilution to the wells. After incubation of the plates for 60 min and washing, the reactions were read after addition of TMB at O.D. 450 nm.

Statistical Analysis

Statistical significance between the values in the immunized mice was determined by Student's t-test and One-way ANOVA to compare vaccinated and control groups, as well as to evaluate the effect of alum formulation. All graphic analyses were depicted using Prism version 6 (GraphPad Software, Inc.). $P<0.05$ was considered as significant and each value was regarded as the mean±SD.

RESULTS

Expression and Purification of the Recombinant Protein

The multi-epitope vaccine protein was successfully expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA affinity chromatography. SDS-PAGE and Western blot analyses confirmed the presence of a distinct protein band corresponding to the expected molecular weight of approximately 48 kDa, indicating successful expression (Fig. 1) and purification suitable for immunization studies.

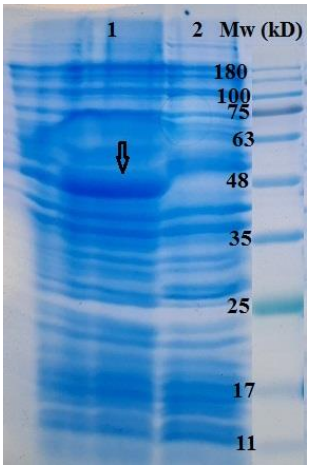

Fig. 1. Evaluation of protein expression by SDS-PAGE. Lane 1: induced clone (0.5 mM IPTG), Lane 2: Un-induced clone, and Mw: Protein marker.

Systemic Humoral Immune Responses

Following subcutaneous immunization, mice receiving the multi-epitope protein exhibited a gradual increase in serum IgG levels. After the first immunization, IgG responses were detectable but did not differ significantly from the control groups ($P>0.05$). In contrast, booster immunizations led to a significant enhancement of IgG responses in the vaccinated mice as compared to the control mice ($P<0.05$). The third immunization induced the highest antibody levels, indicating effective immune memory induction (Fig. 2A). Notably, mice immunized with the multi-epitope protein formulated with alum showed significantly

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higher IgG levels compared to mice receiving the multi-epitope protein alone (P<0.05) (Fig. 2A). Serum IgA levels followed a similar trend, with a marked increase observed after the final booster dose, particularly in the alum-adjuvanted group (Fig. 2B).

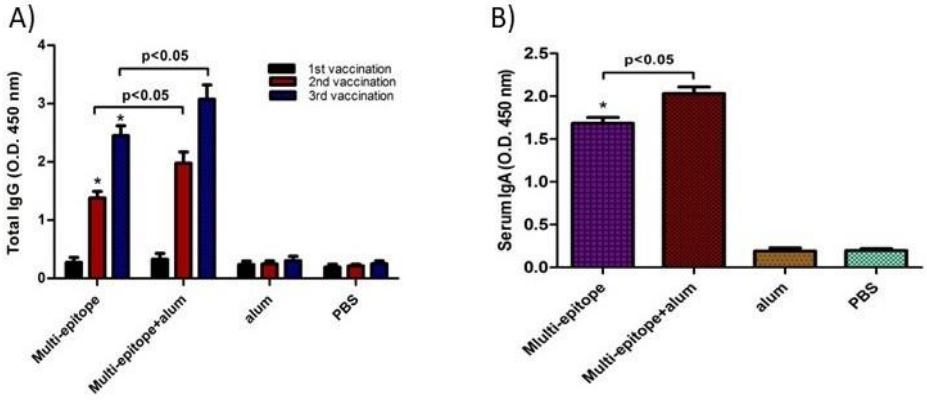

Fig. 2. Comparison of serum antibody levels after the subcutaneous injection. Different groups of mice were vaccinated with either protein alone (Multi-epitope) or the protein admixed with alum (Multi-epitope+alum) and (A) IgG responses were measured two weeks after each injection, as well as (B) IgA two weeks after the third injection. Control groups were injected with PBS and alum. Single asterisks indicate that the serum IgG and IgA levels in these groups were significantly higher than in the control groups (P<0.05). Significant differences for other groups are shown with brackets. Results are presented as mean ± S.D. and represent three independent experimental repeats at a serum dilution of 1:100.

Mucosal Immune Responses

Analysis of urinary samples revealed that the vaccinated mice produced significantly higher levels of IgG and IgA compared to the control animals (P<0.05). Both vaccine formulations including the multi-epitope with and without alum induced mucosal antibody responses, highlighting the ability of subcutaneous immunization to elicit distal mucosal immunity. While alum formulation significantly enhanced urinary IgG responses (P<0.05), differences in urinary IgA levels between vaccine-alone and vaccine-plus-alum groups were less pronounced (P>0.05) (Fig. 3).

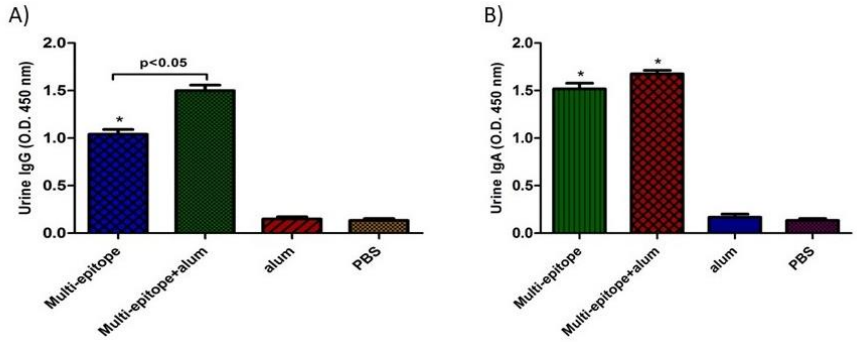

Fig. 3. Comparison of urinary IgG and IgA levels after subcutaneous immunization. Different groups of mice were vaccinated and (A) IgG and (B) IgA responses were measured in the urine samples collected two weeks after the third injection. Single asterisks indicate that the IgG and IgA levels in these groups were significantly higher than in the control groups (P<0.05). Significant differences for other groups are shown with brackets. Results are presented as mean ± S.D. and represent three independent experimental repeats at a urine dilution of 1:2.

DISCUSSION

In the present study, a recombinant multi-epitope vaccine candidate derived from the immunodominant regions of PcrV and OmpE was evaluated for its immunogenicity in a BALB/c mouse model. The results demonstrated that the designed construct was capable of inducing significant systemic and mucosal humoral immune responses, particularly following booster immunizations and formulation with alum.

Following subcutaneous immunization, a gradual increase in antigen-specific IgG levels was observed after the second and third doses, indicating effective priming and boosting of the humoral immune response. The lack of a significant IgG response after the first dose is consistent with typical subunit vaccine behavior and highlights the necessity of booster administrations for achieving optimal antibody titers. Importantly, the alum-adjuvanted formulation elicited significantly higher IgG levels compared to the antigen alone, confirming the role of alum in enhancing antibody-mediated immunity [15, 16].

In addition to the systemic responses, the induction of IgA antibodies in both serum and urine samples represents a notable finding of this study. Since *P. aeruginosa* urinary tract infection is initiated at mucosal surfaces, the ability of a subcutaneously administered vaccine to elicit mucosal antibodies is particularly relevant. Detection of antigen-specific IgG and IgA in urine

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suggests that the immune response was not restricted to the systemic compartment and may contribute to local protection in the urinary tract [17-19].

Compared to the previous studies focusing on single-antigen vaccine candidates, the multi-epitope approach used here offers distinct advantages. Vaccines based solely on PcrV have been shown to induce protective antibodies in animal models; however, antigenic variability and formulation challenges have limited their broader applicability [20, 21]. Similarly, outer membrane proteins such as OmpE have demonstrated immunogenic potential but often suffer from solubility and stability issues when expressed as full-length proteins [13]. In the present study, the use of selected immunodominant epitopes rather than full-length proteins likely contributed to improved solubility and consistent expression, while simultaneously maintaining strong antigenicity.

Moreover, the incorporation of epitopes from two functionally distinct proteins involved in virulence and membrane transport may have broadened the immune recognition spectrum of the vaccine construct. This combined antigenic strategy could reduce the likelihood of immune escape and enhance cross-strain effectiveness, although this hypothesis requires further validation in challenge studies [22].

Despite the promising humoral immune responses observed, this study has certain limitations. Cellular immune responses, including T-helper cell activation and cytokine profiling, were not assessed. Given the established role of Th1- and Th17-mediated immunity in defense against *P. aeruginosa* [23], future studies should evaluate T-cell responses and functional antibody activities such as opsonophagocytosis. Additionally, protective efficacy against live bacterial challenge remains to be determined.

In conclusion, this study demonstrated that a recombinant multi-epitope vaccine based on selected regions of PcrV and OmpE can induce strong systemic and mucosal humoral immune responses in BALB/c mice. The alum-adjuvanted formulation significantly enhanced immunogenicity, particularly in terms of IgG production. These findings support the potential of this rationally designed multi-epitope construct as a promising vaccine candidate against *P. aeruginosa*-associated urinary tract infections. Further studies evaluating cellular immunity and protective efficacy are warranted to advance this candidate toward translational application.

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interest.

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