Recombinant Production and Immunogenicity Assessment of *Pseudomonas aeruginosa* Exotoxin A and PilA Fragments in a Murine Model

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Abstract

Introduction: Exotoxin A and pili are key virulence factors of *Pseudomonas aeruginosa*, and their neutralization is a promising strategy to prevent or attenuate infections caused by this pathogen.

Objective: To evaluate the immunogenicity of recombinant exotoxin A and pilin A (PilA) fragments as conserved antigens against different *P. aeruginosa* serotypes.

Materials and Methods: Genomic DNA was extracted from *P. aeruginosa* using the phenol–chloroform method, and *exoA* and *pilA* genes were amplified by PCR with specific primers. Purified PCR products were cloned into the pGEM vector and transformed into *Escherichia coli* DH5α. After confirmation by restriction digestion with BamHI and XhoI, the inserts were subcloned into the pET-26b expression vector and introduced into *E. coli* BL21 (DE3). Recombinant proteins were expressed, purified, and analyzed by SDS-PAGE, which showed bands of approximately 21–23 kDa for EXO and PILI. Expression of pET-26b/PilA in *E. coli* BL21 (DE3) yielded about 12% (v/v) protein after 5 h of IPTG induction. Purified proteins were injected into BALB/c mice with experimental burn injuries. Humoral immune responses were assessed by antigen-specific ELISA, and protein expression and antigenicity were confirmed by dot blot and Western blot.

Results: Immunization with recombinant exotoxin A and PilA induced robust antigen-specific antibody responses in mice, indicating strong immunogenicity.

Conclusion: Recombinant exotoxin A and PilA produced in this study showed promising potential as vaccine candidates against *P. aeruginosa*. Immunization with these conserved components may offer an effective strategy for preventing infections caused by multiple *P. aeruginosa* serotypes.

Keywords: Exotoxin A, Immunogenicity, Pili P, *Pseudomonas aeruginosa*, Vaccine.

1. Introduction

Pseudomonas aeruginosa is a common environmental Gram-negative bacterium belonging to the family *Pseudomonadaceae*. It is aerobic, motile, non-spore-forming, and ubiquitous due to its ability to survive in a wide range of environments. Its global distribution reflects substantial genetic and physiological flexibility in response to environmental changes. *P. aeruginosa* is also one of the most important human opportunistic pathogens associated with nosocomial infections. In 2017, the World Health Organization classified *P. aeruginosa* as a critical priority pathogen, and the development of new treatments against this bacterium is considered essential [23]. *P. aeruginosa* can evade host innate immunity and is highly resistant to a wide spectrum of antimicrobial agents, which makes its infections particularly difficult to treat [32]. Although the discovery of new antibiotic drugs may offer temporary solutions, resistance to newly introduced agents can rapidly emerge [24]. Consequently, increasing attention has been directed toward anti-virulence strategies that focus on attenuating bacterial pathogenicity rather than killing the bacteria [20]. Because these approaches do not directly affect bacterial survival, they are thought to impose lower selective pressure and thus may reduce the emergence of resistant strains [14].

P. aeruginosa employs at least five secretion systems (types I, II, III, V, and VI) to deliver a wide variety of toxins and hydrolytic enzymes into the extracellular milieu and host cells. Among these, the type III secretion system (T3SS) is crucial for subverting host defenses through the injection of four cytotoxic effectors (ExoU, ExoT, ExoS, and ExoY) [17]. Exotoxin A (ETA) is considered the most potent toxin secreted by *P. aeruginosa*; it inhibits host protein synthesis via its ADP-ribosyltransferase activity, ultimately leading to irreversible cell death [10]. The bacterium also expresses a broad range of outer membrane proteins (OMPs) involved in nutrient uptake, antibiotic resistance, and adhesion, many of which remain poorly characterized [19].

Motility and adhesion structures are key virulence determinants of *P. aeruginosa*. Its unipolar flagellum is essential for host colonization and is primarily responsible for swimming and swarming motility, closely linked to chemotactic signaling. Type IV pili (T4P) are polar, retractable appendages that play a critical role in the initiation of infection by mediating surface attachment and twitching motility [18]. Flagella, T4P, and other adhesins are major contributors to the development of robust *P. aeruginosa* biofilms, which exhibit high levels of tolerance to antibiotics, disinfectants, and host immune responses, posing a significant challenge for treatment [29]. Exopolysaccharides such as alginate, Psl, and Pel are important components of the biofilm matrix, further impairing bacterial clearance and promoting chronic, highly resistant infections.

Extensive research on the pathogenic mechanisms of *P. aeruginosa* has led to the identification of numerous virulence factors that can serve as potential vaccine targets. These

include lipopolysaccharide, exotoxin A, ribosomal components, flagella, pili, high-molecular-weight polysaccharides, alginate, outer membrane proteins, multicomponent DNA vaccines, and T3SS proteins [2]. Among them, exotoxin A is one of the most extensively studied antigens. ETA consists of several functional domains: domain I mediates binding of the toxin to its cellular receptor and is subdivided into Ia (amino acids 1–252) and Ib (amino acids 365–404), while domain III (amino acids 405–613) is the enzymatic domain with ADP-ribosyltransferase activity that inhibits protein synthesis and leads to cell death [26]. It has been shown that antibodies directed against exotoxin A can significantly increase the survival of infected hosts [8]. Therefore, the development of anti-exotoxin A antibodies is of considerable interest for the prevention and treatment of *Pseudomonas* infections.

To evaluate the immunogenicity of exotoxin A, recombinant DNA technology is commonly used to generate defined toxin fragments, which are subsequently purified and characterized to ensure their structural integrity and functional properties [4]. These fragments are then administered to animal models, typically mice, to assess humoral and cellular immune responses [22]. In addition to exotoxin A, PilA (the major pilin subunit of type IV pili) is another critical virulence factor of *P. aeruginosa*. PilA mediates adhesion to host cells and subsequent colonization and has also been proposed as a promising vaccine candidate. The immune response to PilA-based antigens can be evaluated by measuring the production of specific antibodies as well as T cell-mediated responses, including T cell proliferation and cytokine production following stimulation with PilA fragments [32]. By analyzing these responses in animal models, the protective potential of exotoxin A and PilA fragments against *P. aeruginosa* infection can be determined, providing a basis for the development of novel vaccines or immunotherapies [30].

Despite significant progress, further research is required to optimize immunization protocols, evaluate long-term immune responses, and assess the efficacy of these antigens in human clinical trials. In this context, investigating the immunogenicity of exotoxin A and PilA fragments and their ability to induce protective immunity is of particular importance. The aim of the present study was to prepare recombinant fragments of exotoxin A and PilA and to evaluate their immunogenicity in an animal model.

2. Material and Methods

2.1. Preparation and Cultivation of Bacteria

First, a pair of primers for domains I and II (binding region to receptor and transporter) of exotoxin A and pili A nucleotide sequences were designed based on the exoA gene sequence of *P. aeruginosa* recorded in the NCBI database. The designed primers were checked using Gene Runner software, ensuring adherence to the principles and conditions of designing suitable primers, such as avoiding loops, dimers, hairpins, ensuring compatibility and closeness of Tm and % G+C, appropriate length, etc.

Then, the stock of *P. aeruginosa* bacteria was prepared from the research department of Razi Vaccine & Serum Research Institute-Khorasan razavi (Mashhad).

2.2. DNA Extraction

Genomic DNA extraction was carried out using the phenol-chloroform method. In this

technique, cells were lysed using a lysis buffer containing sodium dodecyl sulfate. Proteinase K was used for enzymatic digestion of proteins and non-nucleic acid cellular components. In the next step, a combination of phenol and chloroform/isoamyl alcohol was added to denature the proteins. After centrifugation, the phenol collected at the bottom of the tube, and the DNA was separated into the aqueous phase. Subsequently, the DNA was washed twice with 70% ethanol to precipitate it [9]. The quantity and quality of the extracted DNA were assessed through methods involving optical absorption measurements at 260 and 280 nm, electrophoresis on agarose gel, and PCR reactions for *ExoA* and *PilA* with DNA Genomics. The PCR reactions were conducted in a 25 µl volume under the specified conditions.

2.3. Primer design

The Domains I and II of exotoxin A (non-toxic parts) and type A pili were amplified using the polymerase chain reaction (PCR) method with specifically designed primers. *ExoA* gene primers are registered in the NCBI Gene Bank with various sizes and codes. The gene in question was identified with the code JX026663.1 for the *P. aeruginosa* strain ATCC 25619 exotoxin A (*toxA*) gene, complete coding sequence.

Pili P gene primers with the code EF418191.1 for the *P. aeruginosa* isolate CF72 PilA (*pilA*) gene, complete coding sequences, were retrieved from the GenBank site as the most comprehensive gene registered. Subsequently, domains I and II of exotoxin A (non-toxic parts) and type A pili were amplified through the polymerase chain reaction (PCR) method using specifically designed primers. For exotoxin A, the forward primer was 5′CCGAGGAAGCCTTCGAC3′ and the reverse primer was 5′GCCGTCGCCGAGGAACTC3′. For *Pili A*, the forward primer was 'AATCCATGGCCTTGACCGTCAACACC3' and the reverse primer was 5'ATAAAGCTTGATGCCGACGCTGATG. The PCR cycles and timings for these primers were determined based on the program outlined in Table 1 & 2. Subsequently, the PCR reaction product was electrophoresed on a 1% agarose gel. Both selected genes were compared separately using BLAST software. Identical parts were then evaluated to identify antigenic regions using CLC 5.5 software and online tools [9].

Table 1. Thermal program of polymerase chain reaction for fragment amplification [Pili]

primary Denaturation	**94**	**4 min**
secondary Denaturation	94	1 min

Annealing	53	1 min
Primary Extension	72	1 min
secondary Extension	72	5 min
Cycle	31	

Table 2. Thermal program of polymerase chain reaction for fragment amplification [Exo]

primary Denaturation	**94**	**4 min**
secondary Denaturation	94	1 min
Annealing	58	1 min
Primary Extension	72	1 min
secondary Extension	72	5 min
Cycle	31	

2. Materials and Methods

2.1. Bacterial Strain and Preparation

Primers for domains I and II (receptor-binding and translocation regions) of exotoxin A and for the *pilA* gene were designed based on *P. aeruginosa exoA* and *pilA* nucleotide sequences available in the NCBI database. The designed primers were evaluated using Gene Runner software to ensure compliance with standard primer design criteria, including the absence of secondary structures (loops, dimers, hairpins), appropriate and compatible melting temperatures (Tm), suitable GC content, and optimal primer length.

A stock culture of *P. aeruginosa* was obtained from the Research Department of Razi Vaccine and Serum Research Institute, Khorasan Razavi (Mashhad, Iran).

2.2. Genomic DNA Extraction

Genomic DNA was extracted using the phenol–chloroform method. Briefly, bacterial cells were lysed in a lysis buffer containing sodium dodecyl sulfate (SDS), and Proteinase K was added for enzymatic digestion of proteins and other non-nucleic acid components. A mixture of phenol and chloroform/isoamyl alcohol was then added to denature proteins. After

centrifugation, the organic phase (containing phenol) settled at the bottom of the tube, while DNA remained in the aqueous phase. DNA was precipitated and washed twice with 70% ethanol [9].

The concentration and purity of the extracted DNA were determined by measuring absorbance at 260 and 280 nm. DNA integrity was evaluated by agarose gel electrophoresis and by PCR amplification of *exoA* and *pilA*. PCR reactions were performed in a final volume of 25 µl under the conditions described below.

2.3. Primer Design and PCR Amplification

Domains I and II of exotoxin A (non-toxic regions) and *pilA* were amplified by polymerase chain reaction (PCR) using specific primers. The *exoA* gene sequence was obtained from GenBank under accession number JX026663.1 (*P. aeruginosa* strain ATCC 25619 exotoxin A [*toxA*] complete coding sequence). The *pilA* gene sequence was obtained under accession number EF418191.1 (*P. aeruginosa* isolate CF72 PilA [*pilA*] complete coding sequence).

For exotoxin A, the primers used were:

- Forward: 5′-CCGAGGAAGCCTTCGAC-3′
- Reverse: 5′-GCCGTCGCCGAGGAACTC-3′

For *pilA*, the primers used were:

- Forward: 5′-AATCCATGGCCTTGACCGTCAACACC-3′
- Reverse: 5′-ATAAAGCTTGATGCCGACGCTGATG-3′

PCR cycling conditions and times for each primer pair are summarized in Tables 1 and 2. PCR products were analyzed by electrophoresis on 1% agarose gel. The amplified *exoA* and *pilA* sequences were compared with reference sequences using BLAST software. Conserved regions were then analyzed to identify antigenic epitopes using CLC 5.5 software and relevant online tools [9].

2.4. Cloning and Subcloning of *exoA* and *pilA* Fragments

The *exoA* and *pilA* gene fragments were initially cloned into the pTZ57R vector and transformed into *Escherichia coli* DH5α using a T/A cloning strategy according to the manufacturer's instructions (Ins T/A Clone™ PCR Product Cloning Kit).

To screen for recombinant pTZ57R-*exoA* and pTZ57R-*pilA* clones, white colonies growing on LB agar plates containing ampicillin, X-Gal, and IPTG were selected and re-streaked on LB agar with ampicillin to obtain pure cultures. Positive colonies were verified by colony PCR and restriction enzyme digestion using BamHI and XhoI.

For subcloning into the expression vector, the recombinant plasmid pTZ57R-*exoA* (carrying the *exoA* and *pilA* inserts) and the expression plasmid pET-22b(+) were digested separately

with EcoRI and SalI. Digested products were analyzed by agarose gel electrophoresis, purified, and ligated. The ligation mixtures were then transformed into *E. coli* BL21(DE3), used as the expression host.

2.5. Expression of Recombinant Proteins

Recombinant protein expression was induced and analyzed by SDS-PAGE. A single confirmed recombinant colony was inoculated into LB broth containing ampicillin and incubated at 37°C with shaking. When the culture reached an optical density of approximately 0.5 at 600 nm, 1 ml was collected and centrifuged at 9,000 rpm for 3 min; the resulting pellet was used as the non-induced control sample [12].

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the remaining culture at a final concentration of 1 mM, and incubation was continued for an additional 2 h. Subsequently, another 1 ml sample was collected and centrifuged at 9,000 rpm for 3 min; the pellet was used as the induced sample. Protein expression was evaluated by SDS-PAGE.

To optimize expression conditions and maximize recombinant protein yield, expression was further assessed at different time points (1, 2, 3, 4, and 24 h post-induction), using IPTG concentrations of 0.125, 0.25, 0.5, 1, 2, and 3 mM, and incubation temperatures of 28°C and 37°C.

To determine whether the recombinant proteins were expressed in soluble form or as inclusion bodies, 100 ml of induced culture was centrifuged at 7,000 rpm for 10 min. The cell pellet was resuspended in 3 ml of lysis buffer and subjected to sonication [16]. Sonication was performed 10 times for 20 s each at 80% power while maintaining the samples on ice. The lysate was then centrifuged at 8,000 rpm for 10 min. The supernatant (soluble fraction) and pellet (insoluble fraction) were collected separately and analyzed by SDS-PAGE.

2.6. Extraction and Purification of Recombinant Proteins and Immunization

Following expression, bacterial cells were lysed and the cell wall disrupted (heat shock and mechanical lysis), and the supernatant was collected by centrifugation at 5,000 rpm for 10 min [12]. Proteins were precipitated from the cytoplasmic fraction using ammonium sulfate. Solid ammonium sulfate was slowly added to the clarified lysate under gentle stirring on a magnetic shaker to reach 75% saturation. After complete dissolution, the mixture was centrifuged at 11,000 rpm, and the resulting pellet containing total proteins, including recombinant exotoxin A and PilA, was collected [27].

A non-recombinant *E. coli* BL21 culture was processed in parallel as a negative control and analyzed by SDS-PAGE. Following purification, recombinant exotoxin A and PilA proteins were formulated for immunization by mixing with an appropriate amount of ammonium hydroxide as an adjuvant [16].

Polyclonal antibodies were generated in mice. Four mice per group were immunized subcutaneously with the purified recombinant exotoxin A or PilA proteins in three injections

administered at 1-week intervals. As additional controls, two mice were injected with heat-killed *P. aeruginosa* (treated with chloroform), and two mice received *E. coli* BL21 lysate as a negative control. In the fourth week, mice were bled, and blood samples were allowed to clot and then centrifuged at 4,000 rpm for 30 min. The sera containing antibodies were collected and stored.

The quantity and quality of antibodies raised against the recombinant proteins were evaluated by indirect ELISA.

2.7. Western Blot Analysis

Proteins separated by 15% SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry or wet transfer system. Membranes were blocked with 3% (w/v) skim milk in phosphate-buffered saline (PBS) and then washed three times for 10 min each with PBST (PBS containing 0.1% Tween-20) [15].

Blocked membranes were incubated with rabbit polyclonal primary antibodies diluted 1:1000 in PBST for 1.5 h at room temperature. After incubation, membranes were washed three times with PBST (10 min each) and then incubated for 1 h with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:5000 in PBST [1]. After final washes, protein bands were visualized using a suitable chromogenic or chemiluminescent substrate.

3. Results

Cultures grown on cetrimide agar produced characteristic colonies of *P. aeruginosa* (Figure 1). Smears prepared from single colonies and examined by Gram staining showed Gram-negative bacilli with no detectable contamination.

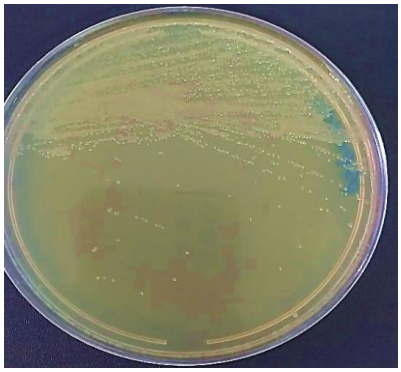

Figure 1. Cultivation of *Pseudomonas aeruginosa* bacteria

3.1. Molecular Analysis of *exoA* and *pilA* Genes

The nucleotide sequences of exotoxin A (*exoA*) and pilin A (*pilA*) from *P. aeruginosa* were retrieved from the NCBI (GenBank) database and supplemented with information from previous studies. Both genes were analyzed separately using BLAST to identify homologous

regions, and conserved segments were examined with CLC 5.5 software and online tools to predict antigenic regions.

Selection criteria included correct open reading frame and translation, predicted secondary structure, antigenicity profiles, and hydrophobicity patterns of the encoded proteins. In addition, enzyme recognition sites, potential proteolytic cleavage sites, and repetitive gene segments were evaluated (Figures 2).

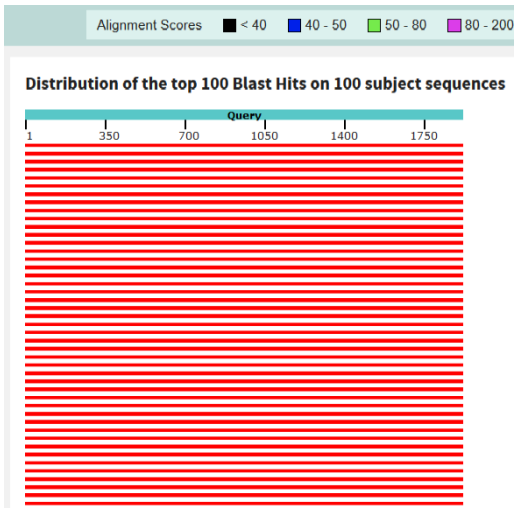

Figure No 2. shows the sequence blast results of *Pseudomonas aeruginosa* exotoxin A at NCBI

3.2. PCR Amplification and Initial Analysis of *exoA* and *pilA*

PCR amplification of the selected *exoA* and *pilA* fragments produced bands of the expected sizes when analyzed on 1% agarose gel electrophoresis, confirming successful amplification of the designed fragments and the absence of non-specific products (Figure 3).

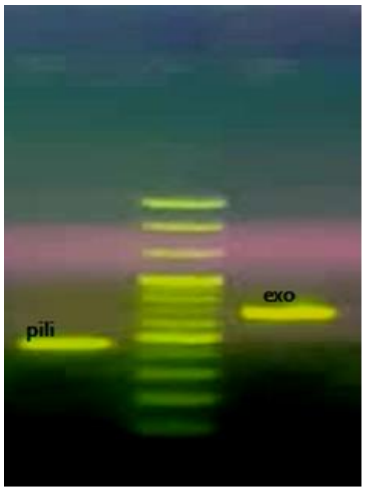

Figure 3. shows the results of the band analysis of the designed fragments after PCR on a 1% agarose gel (1. pili-ladder-exo)

3.3. Cloning of *exoA* and *pilA* Fragments into pTZ57R

Following ligation into the pTZ57R vector and transformation into *E. coli* DH5α, colonies were grown on LB agar containing ampicillin, IPTG, and X-Gal. After incubation and refrigeration of plates to improve color distinction, both blue and white colonies were observed. White colonies, indicative of successful insertional inactivation, were selected for further screening (Figure 4).

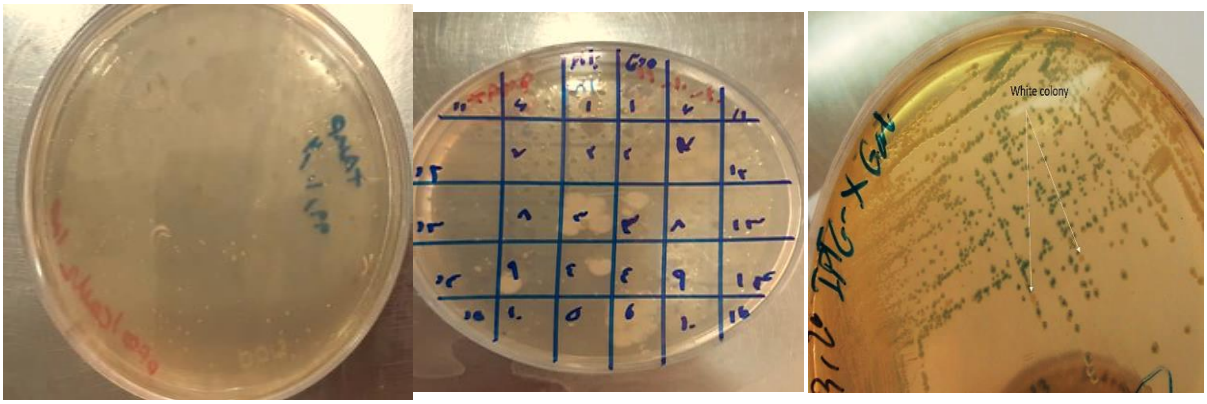

Figure 4. Formation of white colonies containing cloned plasmid among blue bacteria

The orientation of the inserted fragments was examined using M13 universal primers in combination with fragment-specific primers. Out of approximately 40 white colonies screened, only a subset contained inserts in the correct orientation for subsequent subcloning into the pET expression vector. As predicted, correct orientation was confirmed when the forward M13 primer paired with the reverse *exoA* or *pilA* primers yielded amplification products of approximately 564 bp and 519 bp, respectively (Figure 5).

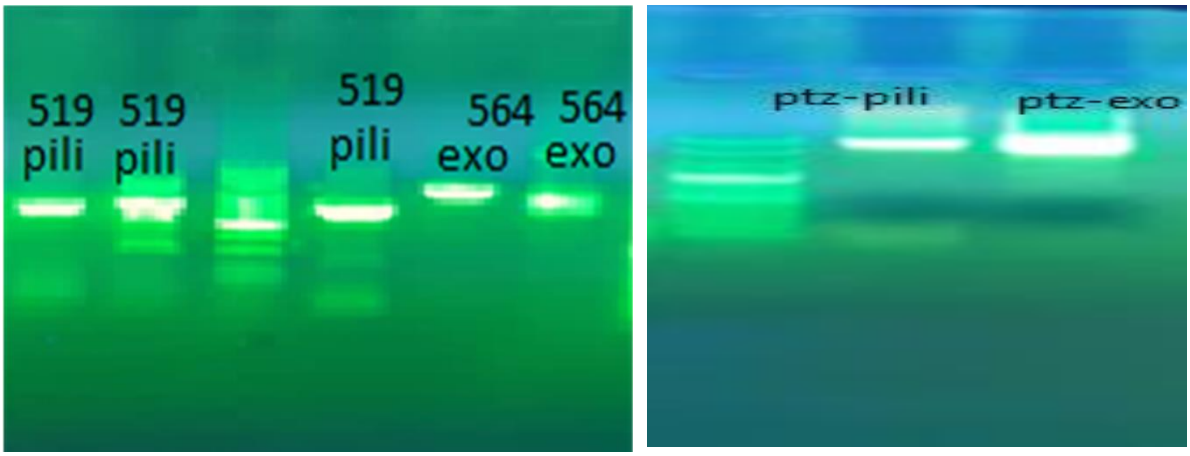

Figure 5. Agarose gel electrophoresis of plasmids extracted from pTZ57R clones containing *exoA* and *pilA* inserts with correct orientation.

3.4. Subcloning into the pET22a(+) Expression Vector

Recombinant pTZ57R-*exoA* and pTZ57R-*pilA* plasmids were digested with the appropriate restriction enzymes, and the released inserts were separated and purified from agarose gels (Figure 7). In parallel, the pET22a(+) expression vector was linearized with EcoRI and SalI to generate compatible ends (Figure 8). The purified *exoA* and *pilA* fragments were then ligated into the digested pET22a(+) vector for expression.

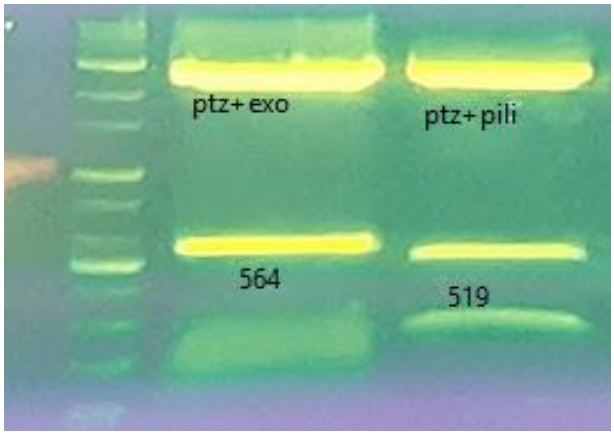

Figure 6. Restriction digestion of pTZ57R plasmids carrying *exoA* and *pilA*, showing released fragments and their size confirmation on 1% agarose gel.

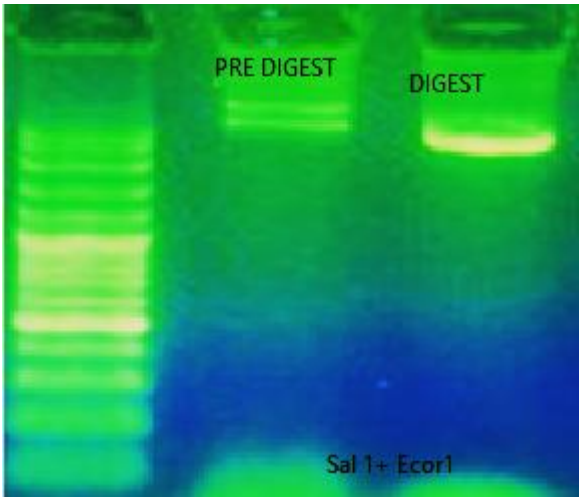

Figure 7. Restriction digestion of the pET22a (+) expression vector with EcoRI and SalI, demonstrating complete linearization and appropriate fragment size.

3.5. Construction of Recombinant pET22a (+)-*exoA* and pET22a (+)-*pilA* and Transformation into *E. coli* BL21(DE3)

Ligation mixtures containing *exoA* or *pilA* inserts and digested pET22a (+) were transformed into *E. coli* BL21(DE3). Transformants were selected on LB agar plates containing ampicillin (with or without IPTG/X-Gal, depending on the screening strategy). A sufficient number of white colonies were obtained, and putative recombinant clones were analyzed by colony PCR using T7 promoter and terminator primers specific to the pET22a (+) vector.

Most screened colonies yielded amplification products corresponding to the predicted *exoA* and *pilA* insert sizes, indicating a high rate of successful cloning into the expression vector

3.6. Expression and Analysis of Recombinant EXO and PILI Proteins

Recombinant protein expression was induced in *E. coli* BL21(DE3) harboring the pET22a (+)-*exoA* or pET22a (+)-*pilA* constructs using IPTG. Cell lysates were analyzed by SDS-PAGE. Both recombinant EXO and PILI proteins were detected as prominent bands with molecular weights of approximately 21–23 kDa, consistent with the predicted sizes (Figure 10).

Expression of r-PilA in *E. coli* BL21(DE3) harboring pET26b/*pilA* was also evaluated after 5 h induction in the presence and absence of IPTG, confirming IPTG-dependent overexpression. Lane M contained the molecular weight marker; representative lanes showed *pilA*- and *exoA*-expressing samples alongside controls.

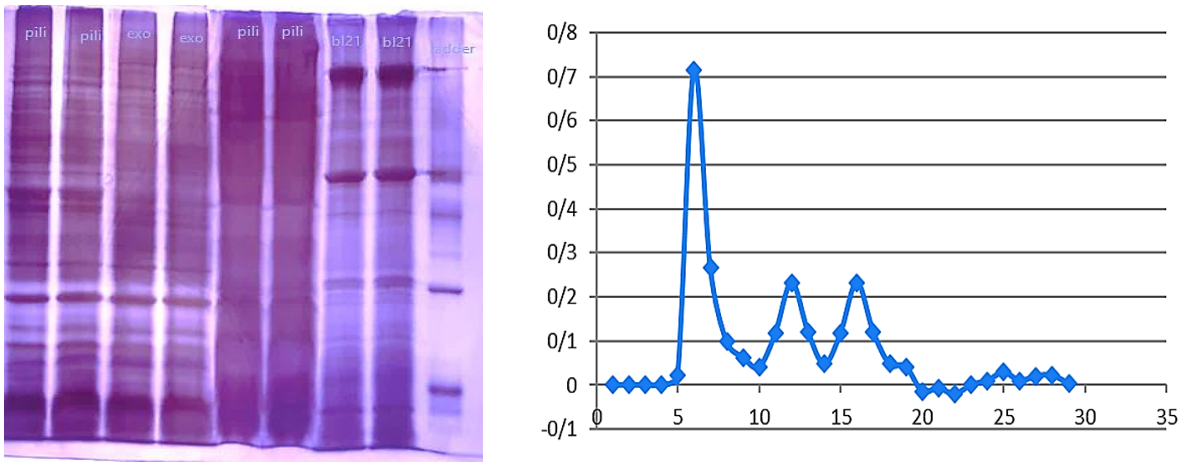

Figure 8. Molecular weight of produced proteins

Crude recombinant proteins were obtained by cell disruption, followed by ammonium sulfate precipitation. The partially purified preparations were then subjected to size-exclusion chromatography on Sephadex G-50 columns to remove low-molecular-weight contaminants and further enrich the target proteins (Figures 11 and 12). The purification strategy aimed to obtain peptide preparations with minimal contaminating proteins suitable for immunological assays.

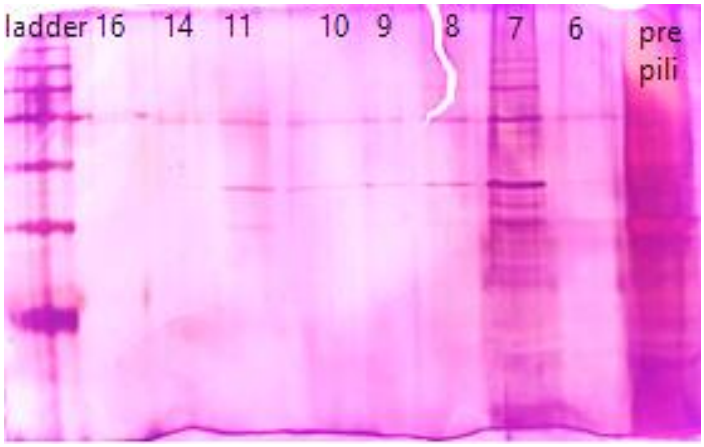

Figure 9. SDS-PAGE pattern indicating the approximate molecular weight of the purified recombinant proteins (highlighted bands).

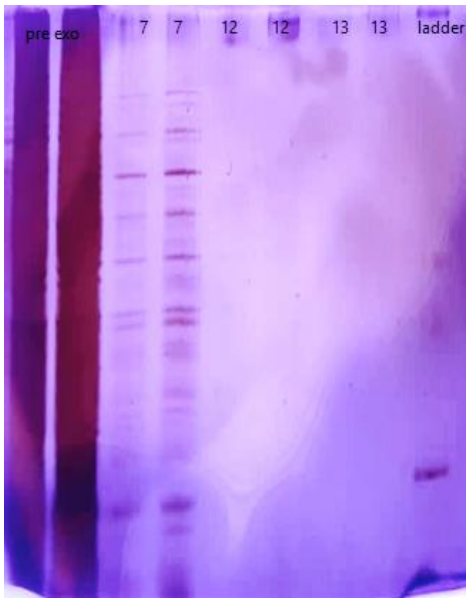

Figure 12. SDS-PAGE gel showing the purification steps of EXO and PILI proteins following ammonium sulfate precipitation (CS-AS) and Sephadex G-50 gel filtration.

3.7. Antibody Production, Western Blotting, and Cross-Reactivity

Following immunization of mice with purified EXO and PILI recombinant proteins, sera were collected and evaluated by Western blotting and ELISA. Western blot analysis of purified EXO and PILI preparations using their corresponding specific antisera demonstrated strong, specific recognition of the respective recombinant proteins. Cross-reactivity assays were performed using antisera raised against EXO, PILI, and whole *P. aeruginosa* antigens.

The results indicated that each antibody preparation reacted strongly with its homologous antigen and showed limited cross-reactivity with heterologous antigens. Sera raised against whole *P. aeruginosa* displayed detectable reactivity with both EXO and PILI proteins. Notably, *P. aeruginosa* antigens exhibited stronger reactivity with anti-PILI antibodies than with anti-EXO antibodies, suggesting differential immunodominance of surface-exposed pili compared to exotoxin A.

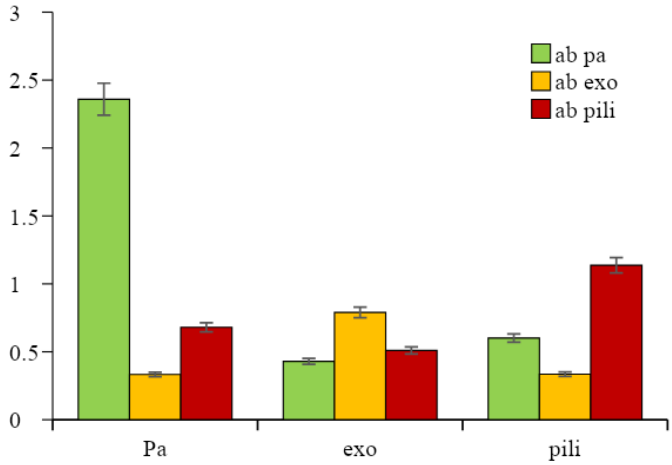

Table 3. Cross-reactivity of anti-EXO and anti-PILI antibodies with different antigens.

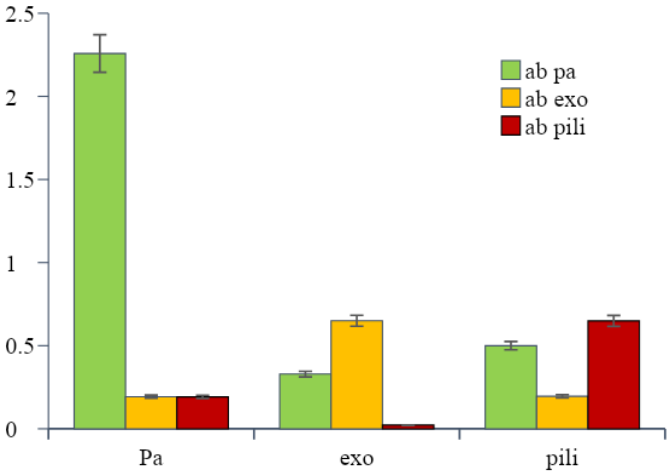

Table 4. Comparison of specific antibody responses to EXO, PA, and PILI antigens within each immunized group.

4. Discussion

Pseudomonas aeruginosa is a leading cause of pneumonia and other severe infections and possesses numerous plasmid- and chromosome-encoded antibiotic resistance determinants, which make antimicrobial therapy particularly challenging [24]. Despite the introduction of new antibiotics, mortality associated with *P. aeruginosa* infection has not significantly decreased. Therefore, alternative approaches, such as immunotherapy and immunoprophylaxis, are being actively explored as complementary or replacement strategies. Several virulence factors of *P. aeruginosa* have been proposed as vaccine candidates, among which exotoxin A (ETA) is of particular interest because its neutralization can significantly reduce the severity of infection. Active and passive immunization against *P. aeruginosa* is increasingly considered a promising strategy in view of the widespread intrinsic and acquired resistance to antibiotics [1]. This is especially relevant in critically ill patients, such as burn patients, for whom rapid and effective control of infection is essential; in such cases, immunization may represent an optimal approach for both prevention and treatment.

The selection of appropriate antigenic targets is a key step in rational vaccine design. Adhesins, including type IV pili (T4P), play a pivotal role during the early stages of infection by mediating attachment to epithelial cells and promoting subsequent colonization and invasion [6]. In the present study, we focused on the feasibility of producing recombinant exotoxin A and PilA under optimized conditions. We systematically evaluated parameters affecting protein expression, including induction time, incubation temperature, and IPTG concentration, and demonstrated that both antigens can be produced at high levels in a recombinant system, supporting their potential utility for large-scale production and downstream immunological applications.

A substantial body of evidence indicates that pili represent highly attractive vaccine candidates [11]. Due to their central role in colonization, T4P have been widely studied as targets for antibody-mediated inhibition. Several studies based on monoclonal antibody binding have shown that the C-terminal domain of the pilin subunit contributes to receptor recognition and bacterial adherence to epithelial cells [20]. In addition, antibodies directed against receptor-binding domains (RBDs) on pili have been shown to block pilus-mediated adhesion, providing a strong rationale for pilus-based vaccine design [31]. Because adhesion and colonization are critical steps in pathogenesis, we designed and expressed recombinant, domain-specific PilA and exotoxin A constructs to evaluate their production and immunogenic potential.

In this study, we successfully obtained a recombinant form of the *P. aeruginosa* PilA protein and a non-toxic fragment of exotoxin A using *E. coli* expression systems. The importance of exotoxin A as a virulence determinant has been extensively documented in both clinical and experimental models. Interestingly, some earlier animal studies reported that ETA-deficient *P. aeruginosa* mutants may display altered virulence phenotypes compared with parental strains, highlighting the complex interplay between different virulence factors [26]. In a non-toxigenic PAO1 background, cloned *toxA* genes can direct the synthesis of intact exotoxin A [5]. Douglas et al. expressed exotoxin A under the control of the tac promoter in

an expression vector in *E. coli* and reported that the recombinant protein localized predominantly in the periplasmic compartment [7]. Similarly, Lory et al. expressed the *toxA* gene in *E. coli* and, using immunoblotting, showed that exotoxin A mainly accumulated in the cytoplasmic fraction [3]. Expression of other *P. aeruginosa* extracellular enzymes, such as phospholipase C, in *E. coli* has also been investigated; Lory and Tai found that this enzyme was associated with the outer membrane, although its processing from the precursor form was not fully elucidated [28].

Consistent with these previous findings, our results showed that recombinant exotoxin A and PilA were not secreted into the culture medium by *E. coli* but accumulated intracellularly, requiring cell lysis for recovery [28]. Based on bioinformatics and immunoinformatics analyses, BLAST comparisons, and sequence alignments, we designed primers targeting immunologically relevant N- and C-terminal regions of the *exoA* and *pilA* genes. Given the high GC content and structural constraints of these sequences, specific restriction sites were engineered into the primers to facilitate cloning. The amplified fragments were first cloned into the pTZ57R vector and subsequently subcloned into an appropriate expression vector. However, due to the high GC content and associated cloning difficulties, direct cloning in some constructs was not successful, and the sequence had to be synthesized and inserted directly into the expression plasmid. This observation is in agreement with the report by Goval et al. (2018), who showed that unusual sequence composition and elevated GC content can negatively affect replication, cloning, and expression efficiency [6].

In our system, the use of the pET22b(+) vector and the presence of multiple cysteine residues in the C-terminal fusion region facilitated purification by Ni-Sepharose affinity chromatography. Inclusion bodies formed during expression could be efficiently solubilized in 8 M urea. Although initial elution using only a pH gradient was not fully efficient, the addition of 250 mM imidazole markedly improved the recovery of the target proteins. Moreover, the use of *E. coli* BL21(DE3) producing T7 RNA polymerase enabled robust expression of the recombinant constructs upon IPTG induction, in line with previous studies employing T7-based systems for *P. aeruginosa* proteins [26].

Western blot analysis of sera from animals immunized with recombinant exotoxin A and PilA demonstrated the presence of specific antibodies against both antigens. This supports the importance of exotoxin A as a key pathogenic factor and underscores the relevance of neutralizing this toxin as part of an anti-*P. aeruginosa* strategy. The observed antibody responses indicate that the recombinant proteins produced in this study possess immunogenic epitopes capable of eliciting specific humoral responses. SDS-PAGE and Western blotting confirmed that the recombinant PilA migrated at approximately 17 kDa (theoretical 17.68 kDa) under denaturing conditions, and the exotoxin A fragment migrated at approximately 20 kDa (theoretical 20.12 kDa), which is consistent with the predicted molecular masses.

Furthermore, dot blot, indirect ELISA (including cross-reactivity assays), and Western blot analysis using mouse antisera showed that anti-EXO, anti-PILI, and anti-*P. aeruginosa* sera each reacted strongly with their homologous antigens. Limited cross-reactivity was observed among heterologous combinations. In particular, whole-cell *P. aeruginosa* antigens exhibited stronger reactivity with anti-PILI antibodies than with anti-EXO antibodies, suggesting that pili are more prominently exposed on the bacterial surface and may be more immunodominant in natural infection. The detection of specific antibodies against both exotoxin A and PilA in sera underscores the contribution of these factors to *P. aeruginosa* pathogenicity and supports their potential as vaccine targets.

Collectively, these findings indicate that the recombinant exotoxin A and PilA proteins produced in this study are suitable for further immunization experiments. Future work should evaluate their efficacy in active and passive immunization models, explore their potential as components of multivalent vaccines, and assess their utility as antigens in diagnostic assays. Ultimately, clinical studies in human subjects will be required to determine their protective efficacy and safety in the context of *P. aeruginosa* infection.

Conclusion

Exotoxin A and recombinant PilA were successfully expressed in the pET22b (+) vector in *E. coli* BL21(DE3) under IPTG induction and were readily detectable by SDS-PAGE as distinct bands at the expected molecular weights. The optimized expression conditions (induction for up to 5 h at 37°C) enabled high-level production of these recombinant proteins. Our results demonstrate that exotoxin A and PilA can be efficiently produced at laboratory scale and are amenable to purification in a form suitable for immunological studies.

Given their central roles in *P. aeruginosa* pathogenesis and their demonstrated immunogenicity in animal models, this recombinant, non-toxic exotoxin A and PilA constructs represent promising vaccine candidate antigens. Further preclinical evaluation, including detailed immunization and protection studies, is warranted to assess their potential use in active and passive immunization strategies against *P. aeruginosa* infections.

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Ethics

Not Applicable

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests statement

The authors declare that they have no conflict of interest.

Authors' contribution

NNS Conducted data collection and drafted the manuscript. MN Conceptualized and designed the initial study. MM and SD Provided consultation and guidance in drafting the manuscript. MJ Performed data analysis and interpretation of results. All authors reviewed and approved the final version of the manuscript.

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Data Availability

The datasets generated and analysed during the present study are not publicly accessible. They can be obtained only in coordination with the corresponding author, who will provide them upon reasonable request.

AI usage statement

"The authors used ChatGPT (OpenAI, GPT-4, March 2024 release) solely to assist with reference formatting and minor English-language editing. The tool was not employed for data generation, analysis, interpretation or scientific content creation, and all AI-suggested changes were reviewed and approved by the authors."

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