Research Article

Cloning, Expression, and Purification of *Pseudomonas aeruginosa* Flagellin, and Characterization of the Elicited Anti-Flagellin Antibody

Bahador Behrouz,[1] Nour Amirmozafari,[2,*] Nima Khoramabadi,[3] Mahboobeh Bahroudi,[1] Parisa Legaee,[2] and Mehdi Mahdavi[4]

[1]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran
[2]Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, IR Iran
[3]Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran
[4]Department of Immunology, Pasteur Institute of Iran, Tehran, IR Iran

[*] *Corresponding author*: Nour Amirmozafari, Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, IR Iran. Tel: +98-2188058649, E-mail: namirmozafari@yahoo.com

Abstract

Background: *Pseudomonas aeruginosa* is an important opportunistic human pathogen that causes serious infections in immuno-compromised hosts. The single polar flagellum is an important factor in both virulence and colonization.

Objectives: As flagellin is the major component of the flagellar filament, the main aims of the present study are to identify, clone, express, and purify the recombinant type B flagellin (r-B-flagellin) of *P. aeruginosa*, as well as to evaluate the functional activity of the rabbit polyclonal antibody raised against this r-B-flagellin.

Materials and Methods: In the current experimental study, the r-B-flagellin gene was isolated from the *P. aeruginosa* PAO1 strain by PCR. It was cloned into the pET-28a vector and then transformed into the *E. coli* BL21 strain. Next, r-B-flagellin was overexpressed and affinity purified by Ni-NTA agarose-affinity chromatography, followed by on-column resolubilization. Polyclonal antisera against the recombinant flagellin were raised in rabbits, and the functional activity of the anti-r-B-flagellin antibody was determined by in vitro assays.

Results: The polyclonal antibodies raised against this r-B-flagellin inhibited the motility of the homologous PAO1 strain of *P. aeruginosa*, which significantly decreased the invasion of the PAO1 strain into the A549 cells and also enhanced the opsonophagocytosis of this strain. However, our polyclonal antibody showed little effect on the heterologous PAK strain.

Conclusions: The r-B-flagellin carried antigenic epitopes just like the native flagellin, while the polyclonal antibody raised against it exhibited functional activity.

Keywords: Polyclonal Antibody, Recombinant Flagellin, *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the major colonizing microbial pathogens in cystic fibrosis (CF) patients, and it is a common infectious agent in nosocomial infections, such as patients with severe burns, cancer, transplantations, acquired immune deficiency syndrome (AIDS), and other immunocompromising conditions (1, 2). Due to its resistance mechanisms, the available selection of effective antipseudomonal antibiotics is slowly shrinking as multi-drug resistant *P. aeruginosa* strains emerge (3-5). The development of effective new therapeutic and prophylactic strategies for rapid protection against *P. aeruginosa* infection in immunocompromised patients is therefore vital (5, 6). Pathogenic *P. aeruginosa* strains produce single polar flagella, which are responsible for the motility, adhesion, invasion, and secretion of virulence factors (7). Flagellin, the major compo-

nent of the flagellum, has been classified into two distinct serotypes, type A and type B (8). However, flagellin can also be differentiated by molecular size (8) and genetic analysis (9), as well as encoded by the *fliC* gene (8). Type B flagellin comprises a homogeneous group of proteins, whereas the heterogeneous type A flagellin is divided into several subtypes (9). Most of the structural and functional features of the flagella are determined by the N- and C-terminal conserved regions, while the antigenic or serological variation is found in the central portion of flagellin (7, 10). As an antigenic protein, flagellin elicits a strong NFκB-mediated inflammatory response via signaling through toll-like receptor 5 (TLR5) (11). Additionally, flagellin is a strong inducer of cellular and humoral immune response (12). Several animal studies have demonstrated the importance of motility in the invasive virulence of *P. aeruginosa* (13-15). In the animal model of *P. aeruginosa* infection, flagellin mutants show a decrease in virulence with a reduced ability to in-

vade deeper tissues (16). Further, more than 95% of clinical *P. aeruginosa* isolates are flagellated. For these reasons, flagellin is an important antigen for mounting an immunologic response in *Pseudomonas* infections.

2. Objectives

The aims of this study are to determine the immunogenicity and functionality of recombinant type B flagellin (r-B-flagellin) as a possible antigen candidate for a vaccine against *P. aeruginosa* infection in burn wounds, as well as to determine the protective effects of the anti-r-B-flagellin antibody in vitro.

3. Materials and Methods

3.1. Bacterial Strains, Vector, and Cell Line

In the current experimental study, the *P. aeruginosa* strains PAO1 (type B flagellated strain) and PAK (type A flagellated strain) were obtained from Shahid Beheshti University of Medical Sciences, Tehran, Iran. *Escherichia coli* TOP10F´ and *E. coli* BL21 (DE3) were used as bacterial hosts for preservation and expression. Further, pET28a (+) (Novagen Inc., Madison, WI, USA) was used as the expression plasmid. The A549 cell line was purchased from the Pasteur institute (Tehran, Iran).

3.2. Amplification and Cloning of the fliC Gene

Specific primers were designed for the *fliC* sequences of the PAO1 *P. aeruginosa* strain from the national center for biotechnology information (NCBI) (GenBank Accession No: NC-002516.2): forward 5'-CTCGGATCCCACTCAGCGCAACC-3'; reverse 5'-ACGAAGCTTGCAGCAGGCTCAG-3'. *BamHI* and *HindIII* restriction sites were incorporated at the 5' terminus of the forward and reverse primers, respectively. The amplifications were carried out using *Pfu* DNA polymerase (Fermentas, Vilnius, Lithuania) as previously described by Goudarzi et al. (17). Briefly, predenaturation was carried out at 94°C for 1 minute, followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The purified *fliC* fragment was digested and ligated into the *BamHI-HindIII* sites of the pET-28a vector, which provided six histidine (His) residues at the C-terminus of the expressed protein. The construct was subsequently verified by PCR, restriction analysis, and DNA sequencing.

3.3. Expression and Purification of the r-B-Flagellin

The purification of the r-B-flagellin was performed as previously described (18) using the QIAexpressionist™ system (QIAGEN, Hilden, Germany), while the expression of the r-B-flagellin was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO, USA) to the cultures. The induced level of r-B-flagellin was determined by 12.5% (w/v) SDS-PAGE (Bio-Rad, Hercules, CA, USA), followed by Coomassie Brilliant Blue (G-250) staining. After 4 hours of induction, the bacterial cultures were pelleted and dissolved in a lysis buffer of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF at pH 8.0. The suspension was subsequently centrifuged, and the cells in the supernatant were lysed in buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, and 8 M urea; pH 8) and centrifuged at 18,000 g for 30 minutes at 25°C to remove the debris. Ni-NTA agarose (QIAGEN) was added to the lysate and incubated on a rotary shaker at room temperature for 1 hour. After applying the mixture to a column, the resin was washed with buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, and 8 M urea; pH 6.3). Urea was removed stepwise using washing buffers with decreasing urea concentrations (8, 6, 4, 2, 1, and 0 M urea). The r-B-flagellin was finally eluted with buffer E (50 mM NaH_2PO_4, 300 mM Tris-HCl, and 250 mM imidazole; pH 4.5). In order to remove imidazole, the protein solution was dialyzed against phosphate buffered saline (pH 7.4) overnight. The total amount of purified solubilized protein obtained from 1 L of bacterial culture was quantitatively measured using a nanodrop 2000 spectrophotometer (thermo scientific, USA).

3.4. Polyclonal Antibody Production

Three-month-old female New Zealand white rabbits (Razi vaccine and serum research institute, Karaj, Iran) were immunized with 100 μg of the r-B-flagellin in complete Freund's adjuvant (1:1; Sigma, USA), which was administered subcutaneously, and boosted twice with 100 μg of the purified flagellin in incomplete Freund's adjuvant (1:1) at weeks 2 and 4. Ten days after the final injection, the anesthetized animals were exsanguinated by cardiac puncture, and serum samples containing the polyclonal antibody against the flagellin protein were collected. Anti-r-B-flagellin immunoglobulin G (IgG) was separated from the whole serum by precipitation with 35% ammonium sulfate.

3.5. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed as previously described (19). Briefly, microtiter plates were coated with 100 μL of r-B-flagellin (1μg per well) in the coating buffer (PBS, pH 7.4) and then incubated at 4°C overnight. Between incubation

steps, the plates were washed three times with phosphate buffered saline with tween 20 (PBS-T). Blocking was carried out using PBS containing 1% (w/v) bovine serum albumin (BSA) at 37°C for 2 hours. Afterward, serial dilutions (from 1:100 to 1:204,800) of the immune sera were added to the plates. A horseradish peroxidase (HRP)-labeled sheep anti-rabbit IgG (Sigma) diluted at 1:10,000 was used as a secondary antibody, and 3, 3, 5, 5'-tetramethylbenzidine (TMB; Sigma) was used as a substrate solution. Following a 30 minute incubation, the reaction was stopped by adding 100 μL of 2N H_2SO_4, and the optical density was read at 450 nm.

3.6. Immunoblotting

The r-B-flagellin was electrophoresed by SDS-PAGE with 12.5% poly-acrylamide mini-gels and then transferred onto PDVF membrane (Hi-bond Amersham biosciences, piscataway, NJ, USA) using a semi-dry blotting apparatus (Labconco, Kansas City, MO, USA). The lanes on the membranes were cut to 3 mm wide strips, which were destained with distilled water and then washed with TBS-T composed of 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 0.05% Tween 20. The strips were blocked for 1hour with TBS buffer containing 1% (w/v) skim milk. The monoclonal anti-His tag antibody (Sigma) was tested in order to evaluate its ability to recognize this r-B-flagellin, with an HRP-conjugated anti-His (Sigma) diluted at 1:10,000 used as a secondary antibody. After undergoing adequate washes, the strips were developed using an enhanced chemiluminescence substrate.

3.7. Invasion Assay of the P. aeruginosa Strains

To determine the ability of anti-r-B-flagellin IgG to inhibit the invasion of the P. aeruginosa strains into the A549 cell line, a gentamicin protection assay was used as previously described (20). The P. aeruginosa strains (10^7 CFUs) were mixed with different concentrations (10, 50,100, 150, 200, and 250 μg/mL) of anti-r-B-flagellin IgG, and then incubated on a rotary shaker at room temperature for 1 hour. Next, this neutralized bacterial mix was added to the A549 cells (5x105 cells per well in a 24-well plate, in triplicate) and incubated at 37°C in a 5% CO_2 humidified incubator for 1 hour. For the quantification of the intracellular bacteria, 200 μL of gentamicin (100 μg/mL) was added to each well for 1 hour. Afterward, the cells were lysed with 0.5% (v/v in PBS) Triton X-100 (Sigma) (250 μL per well) and aliquoted onto LB agar (Invitrogen, USA) plates. The average invasion percentage was calculated as: [100× (number of bacteria recovered/number of bacteria inoculated)].

3.8. Opsonophagocytic Activity Assay

The opsonophagocytic assay was performed as previously described (21), with the following adaptations. Bacterial cultures were grown in tryptic soy broth (12) and incubated at 37°C until an OD_{650} of 0.2 was reached. The isolated mouse peritoneal macrophages were counted and resuspended at a concentration of 2×10^9 in a medium of RPMI-1640 (Gibco, Darmstadt, Germany), with 10% heat-inactivated fetal bovine serum and fresh infant rabbit serum as a complement source. Heat-inactivated rabbit serum (1:10 dilution) was used as an opsonic antibody. The opsonic activity of the immune sera was compared to that of the sera obtained prior to vaccination. Control tubes containing 100 mL RPMI medium/fetal calf serum rather than the antibody, complement, or macrophages were run with each assay. After 90 minutes of incubation, a 50 μL portion was removed, diluted in PBS containing 0.05% Tween 20, and then plated onto the LB agar medium (Sigma). This experiment was performed in triplicate for each quantity. The opsonic activity of the immune sera was calculated as follows:

Opsonophagocytic activity = [1- (CFU of immune serum at 90 minutes/CFU of pre-immune serum at 90 min)] ×100.

3.9. Motility Inhibition Assay

The motility inhibition assay was performed as previously described (22). Briefly, three Petri dishes were filled with 10 mL of motility agar (LB with 0.3% agar) mixed with either polyclonal or pre-immune rabbit serum diluted at 1:20. Twenty microliters of each P. aeruginosa cell suspension (OD600 = 0.2) was dispensed into the central well of each plate. The plates were then incubated at 37°C. The mean diameter of the bacterial spread with sharp and less distorted rings was measured after 18 hour incubation.

3.10. Statistical Analysis

For all statistical analyses, SPSS 21.0 software was used (SPSS Inc., Chicago, Illinois, USA). All results were analyzed using a one-way ANOVA and GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, USA). The ELISA data were analyzed using the post-hoc test (LSD). The analyses of the mean opsonic activity triplicate samples of serum were compared using Student's t-test (StatView). Linear regression was used to examine the correlation between dose and invasiveness studies, and the results were displayed as mean $\pm$ standard deviation (SD). For each set of various assays, appropriate positives and controls were included in order to minimize any potential bias in our data analysis, and p values of < 0.05 were considered to be statistically significant.

4. Results

4.1. Cloning and Expression of the r-B-Flagellin

The electrophoresis of the PCR product resulted in a single band with an approximate size of 1,500 bp, which confirms the amplification of the *fliC* gene (Figure 1). The existence of the insert (*fliC*) in the recombinant vector (pET-28a/*fliC*) was also proven by digestion using *BamHI and HindIII* restriction enzymes (Figure 2). Finally, DNA sequencing confirmed the identity and orientation of the *fliC* gene in the recombinant construct (data not shown). *Escherichia coli* BL21 (DE3) was transformed with recombinant plasmid pET28a/*fliC* containing a T7 promoter as the expression host. Protein production was induced with IPTG (1 mM). SDS-PAGE gel detected approximately 53 kDa proteins after staining with Coomassie Blue G-250 (Figure 3). The product was then purified by Ni-NTA affinity chromatography under denaturing conditions (Figure 3). Significantly high amounts of the recombinant protein were obtained via the denaturing procedures. This purification approach yielded 58 mg of highly purified r-B-flagellin from one liter of the induced culture. Recombinant protein and recombinant plasmid pET28a/*fliC* were detected in a purified form in the crude cell lysate of the induced cultures using the anti-His monoclonal antibody (Figure 4).
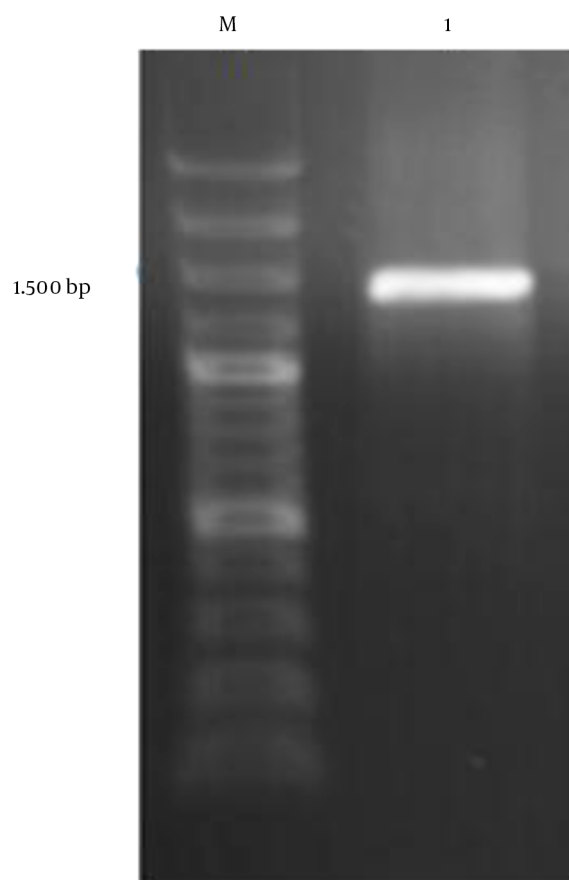
4.2. Production of Rabbit Anti-r-B-Flagellin IgG

Polyclonal antibodies against the r-B-flagellin were produced in rabbits. The anti-r-B-flagellin IgG-rich fraction was obtained from the serum via ammonium sulfate precipitation. The specificity of the anti-r-B-flagellin IgG was verified by indirect ELISA using purified r-B-flagellin (Figure 5). The total IgG in serum dilutions of 1:100 to 1:12,800 from immunized rabbits was significantly higher than that from non-immunized rabbits ($P < 0.01$). No significant difference ($P > 0.05$) was observed between the immunized and non-immunized serum at dilutions of 25,600 to 204,800 (Figure 5).

4.3. The Anti-r-B-Flagellin IgG Effects on In Vitro Invasion

The inhibitory effects of anti-r-B-flagellin IgG on the invasion of the A549 cells by PAO1 (Figure 6) decreased in a dose-dependent manner. Further, PBS and normal rabbit serum (NRS) demonstrated 100% and 92% invasion, respectively. As shown in Figure 6, in the presence of 250 and 200 μg/mL of the anti-r-B-flagellin IgG, the relative invasion values against the PAO1 *P. aeruginosa* strain were 32.94 and 34.43%, respectively ($P < 0.01$ versus results for the NRS group). This antibody had a slight inhibitory effect on the heterologous strain at 250 μg/mL (65.80% invasion; $P < 0.05$ versus the results for the NRS group).

Figure 1. Analysis of the PCR Product Following the Amplification of the *fliC* Gene

Lane M, DNA size marker; and lane 1, PCR product resulting from the amplification of the *fliC* gene.

4.4. Opsonophagocytic Activity

To evaluate the functional activity of anti-r-B-flagellin IgG in enhancing the phagocytosis of the *P. aeruginosa* strains, opsonophagocytosis assays were used. The number of viable PAO1 cells decreased by over 44% following incubation with immune sera diluted to 1:10 when compared to the decrease following incubation with PBS ($P < 0.001$ versus the results for the NRS group). The antibody raised against the r-B-flagellin also induced the partial killing of PAK (12.12% at a dilution of 1:10; $P < 0.01$ versus the results for the NRS group) (Figure 7). These data indicate that the anti-r-B-flagellin IgG acts as a good opsonin for killing the homologous PAO1 strain and as a slight opsonin for the heterologous PAK strain. In the presence of normal rabbit serum (as negative control), the number of viable bacterial cells was slightly reduced (by approximately 4% in all strains).
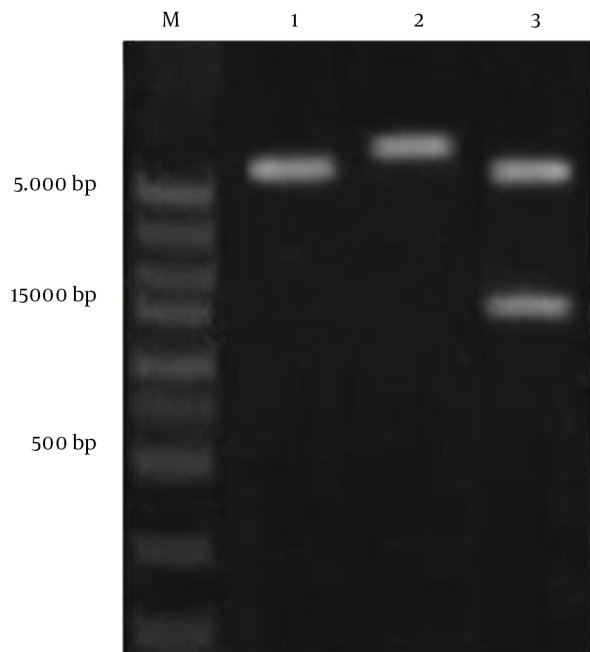
4.5. Motility Inhibition

The rabbit anti-r-B-flagellin IgG was tested for its ability to inhibit the motility of the *P. aeruginosa* strains using a motility inhibition assay. The anti-r-B-flagellin IgG was able to inhibit the motility of PAO1. The antibody showed some cross-reactivity to the type A flagellin, since it slightly inhibited the motility of the PAK strain. The non-immunized rabbit serum was unable to inhibit the motility of any of the strains, indicating that this serum would not have a protective effect (Figure 8).

5. Discussion

As an opportunistic pathogen affecting humans and one of the major causes of nosocomial infections (23), several antigens of *P. aeruginosa* have been proposed as possible vaccine targets (24-26), particularly flagellin, since they are highly conserved and have only two major serogroups (27). In this study, the r-B-flagellin was successfully cloned, expressed, and characterized. The flagellin gene was isolated from the *P. aeruginosa* PAO1 genome and then cloned in a pET-28a expression vector. When the recombinant vector was induced, SDS-polyacrylamide gels revealed an ap-

parent molecular mass of 53 kDa, which is slightly larger than that of the type A flagellin (45 kDa). PCR and sequencing analysis revealed that our specific primers amplified a 1,489 bp length fragment that is in accordance with the B type of flagellin.
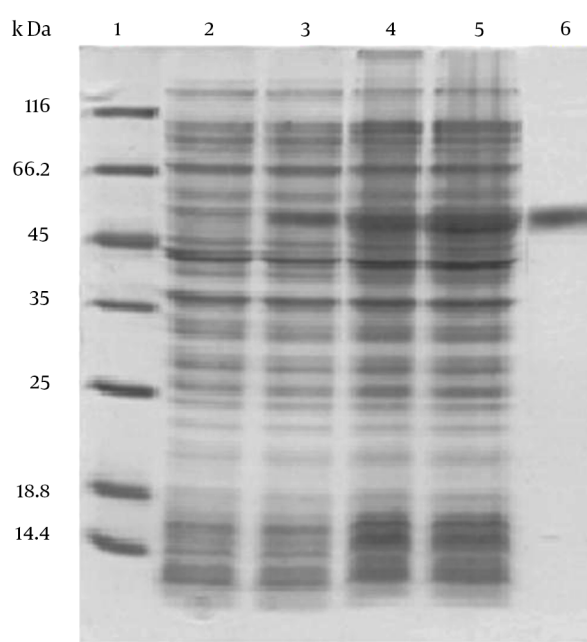
Winstanley et al. found that the PCR amplification of 37 *P. aeruginosa* clinical isolates using CW46 (N-terminal) and CW45 (C-terminal) primers generated products of 1.02 kb (type A flagellin) or 1.25 kb (type B flagellin), respectively (28). Brimer and Montie used these same primers for the amplification of the main portion of the *fliC* gene in order to compare the PCR products of different strains (29). They found that the 1,200 bp fragments were associated with type A flagellin. Due to the positioning of the oligonucleotide primers on the flagellin gene, not all of the *fliC* sequences were amplified by this procedure. Wintenberg et al. cloned and expressed plasmid DNA containing the structural gene of the flagella from *P. aeruginosa* PAO1 into *E. coli* using the triparental mating method (30). Goudarzi et al. have recently isolated the *fliC* gene of *P. aeruginosa* strain 8821M, which they cloned into a pET-28a expression vector (17). In their study, recombinant type A flagellin was expressed as inclusion bodies, which were subsequently

Figure 4. Immunoblotting Results
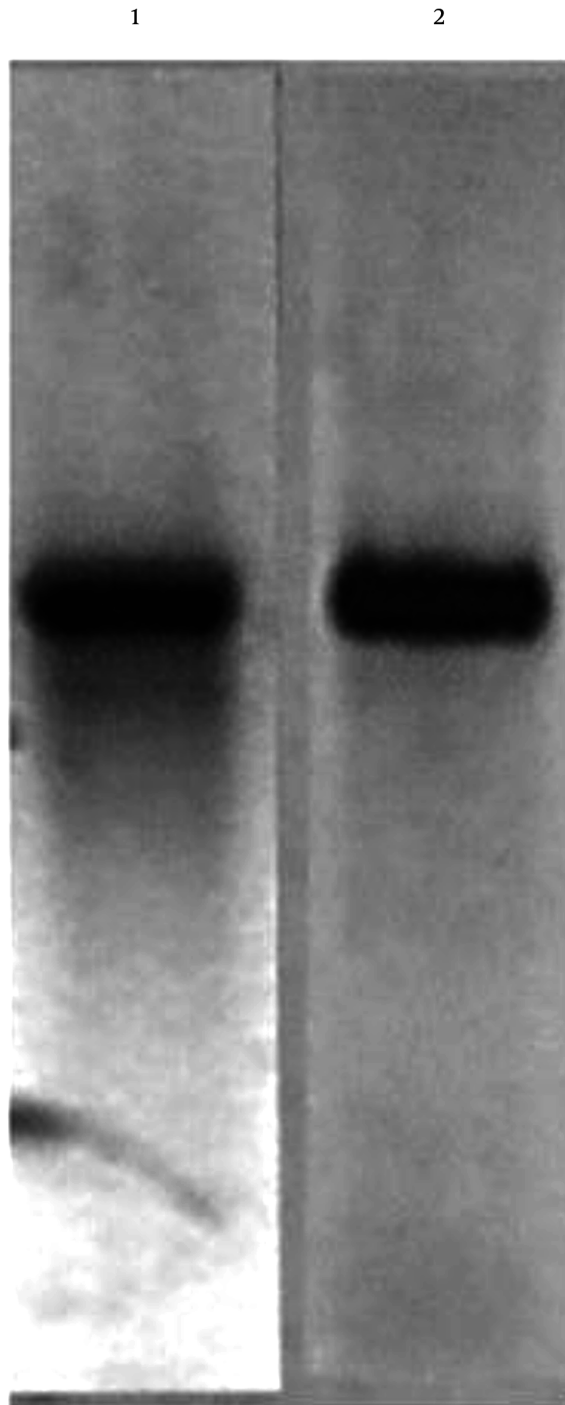
Lane 1, crude cell lysate of 4 h-induced bacteria detected by the monoclonal anti-His tag antibody; and lane 2, purified r-B-flagellin detected by the monoclonal anti-His tag antibody.

Figure 5. An indirect ELISA was used to determine the specific antibodies against r-B-flagellin produced in rabbits
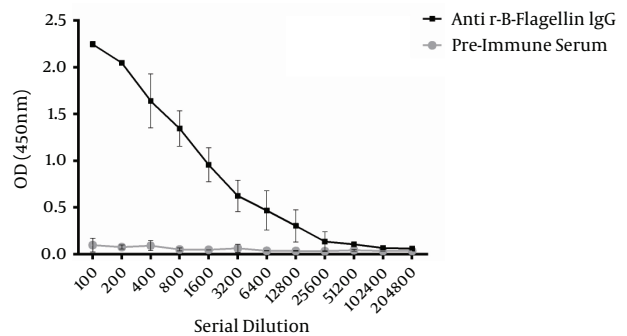

Different dilutions of immune sera (1:100 to 1:204,800) versus non-immunized sera. Results were accepted as significant at $P < 0.05$.

Figure 6. The Inhibitory Effects of Anti-r-B-flagellin IgG on the Invasion of *P. aeruginosa* Strains Into A549 Cells
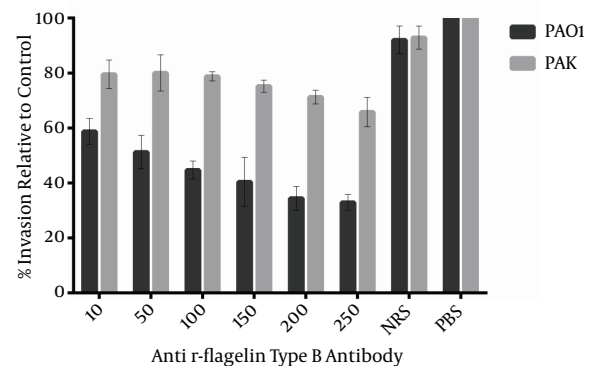
The *P. aeruginosa* strains were incubated with different amounts of anti-r-B-flagellin IgG (10-250 μg/ mL). NRS and PBS were used as negative controls. The bars are presented as the mean of triplicate independent experiments $\pm$ SD. Results were accepted as significant at $P < 0.05$.

solubilized in guanidine hydrochloride (GuHCl), followed by affinity purification with Ni^{2+}-Sepharose resin (17). The expression of recombinant flagellin in a heterogeneous host is associated with misfolding and aggregation into inclusion bodies. As a result of the on-column resolubilization method, urea solubilized inclusion bodies are physically immobilized to Ni-NTA resin, after which the denaturing agent is gradually replaced by a non-denaturing buffer. This protocol greatly helps the internal localization of hydrophobic domains and the efficient superficial positioning of hydrophilic parts in the configured protein. Our results also indicated that using the refolding of inclusion body protein on the Ni-NTA column led to an enhanced yield of soluble proteins.
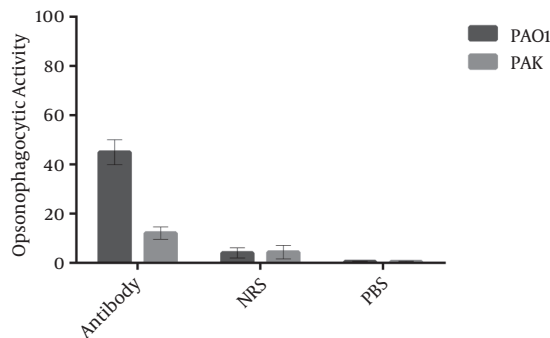
Bacterial strains were incubated with rabbit polyclonal anti-r-B-flagellin IgG and mouse macrophages in the presence of rabbit complement. The bars are presented as the mean of triplicate determinations ± SD. Results were accepted as significant at P < 0.05.

In vitro, flagellin causes human monocytes, murine alveolar macrophages, and epithelial cells to produce NF-κB-dependent pro-inflammatory mediators (31, 32), while *in vivo*, flagellin causes mice to develop hypotension, acute pulmonary inflammation, and vascular hypocontractility similar to septic shock (33). Further, increased levels of circulating monomeric flagellin and its antibody were detected in septic human patients as well as in a murine sepsis model, which strongly supports the critical role of flagellin in gram-negative sepsis (34, 35). The C-terminal and N-terminal domains of pseudomonas flagellin are essential for bacterial motility (33, 34). Thus, we decided to use, for the first time, the whole recombinant type B flagellin molecule as the target antigen for producing effective neutralizing antibodies.

A opsonophagocytic activity assay is used to measure the functional capacities of antibodies. Here, we have shown that the antibodies elicited by active immunization in rabbits were capable of opsonizing bacterial cells and mediating their phagocytic killing. Antibodies raised against r-B-flagellin can enhance the killing of the homologous strain, whereas this antibody had some cross activity against the PAK strain, which indicates the flagellin-type specific phagocytosis of the antibody for the flagella type B strain. This outcome is contrary to the findings of Campodonico's study (24), which demonstrated that anti-flagellin type A exhibits low opsonic killing activity with the homologous PAK strain and no killing activity on the PAO1 strain, while the anti-type B flagellin has no opsonic killing activity against either the homologous PAO1 strain or the heterologous PAK strain. The antibody raised

against the r-B-flagellin was able to inhibit the motility of the homologous strain. Further, this antibody was only partially able to inhibit the motility of the heterologous PAK strain. The mechanism of inhibition is not yet well understood; however, previous studies have suggested that antibodies binding to the flagellar filaments caused bacterial agglutination and disruption of the rotational movements of the structure, which leads to a loss of colony spreading on the plates (22, 36). Anderson and Montie (37, 38) demonstrated that the passive transfer of anti-flagellar antibodies would provide protection in burned mice, and these antibodies have the ability to inhibit the motility of *P. aeruginosa* as well as to improve opsonophagocytosis. Additionally, the anti-r-B-flagellin antibody suppressed the invasion of the A549 cells by *P. aeruginosa* PAO1, while having only a minor effect on the heterologous strain, which indicates the specificity of this antibody for the r-B-flagellin prepared from PAO1. In contrast to a previous study (24), the antibodies raised against r-B-flagellin showed little effect on the heterologous strain (i.e., PAK). The difference in the expression vector and the targeted antigen (whole recombinant type B flagellin) may account for this discrepancy.

Based on several prior studies, protective antibodies should involve different properties that include an enhanced opsonophagocytosis as well as an inhibition of bacterial motility and invasiveness (20, 24, 39). The results described herein confirm the protective actions of the anti-r-flagellin type B antibody.

This is the first report concerning the overexpression of whole recombinant type B flagellin with a His-tag in a heterologous system. The r-B-flagellin carried antigenic epitopes just like the native flagellin and the polyclonal antibody raised against it had functional activity. We have demonstrated for the first time that targeting the whole recombinant type B flagellin with a specific polyclonal antibody led to opsonic killing activity as well as an immobilization of the pathogen and the inhibition of bacterial invasiveness. Based on these initial results, our future goal is to evaluate the protective efficacy of the anti-r-flagellin type B antibody for use as an alternative to antibiotic treatment in a murine infected burn model.

Footnotes

Figure 8. Evaluation of the motility inhibition of *P. aeruginosa* strains PAK and PA01 with antibodies developed against the r-B-flagellin
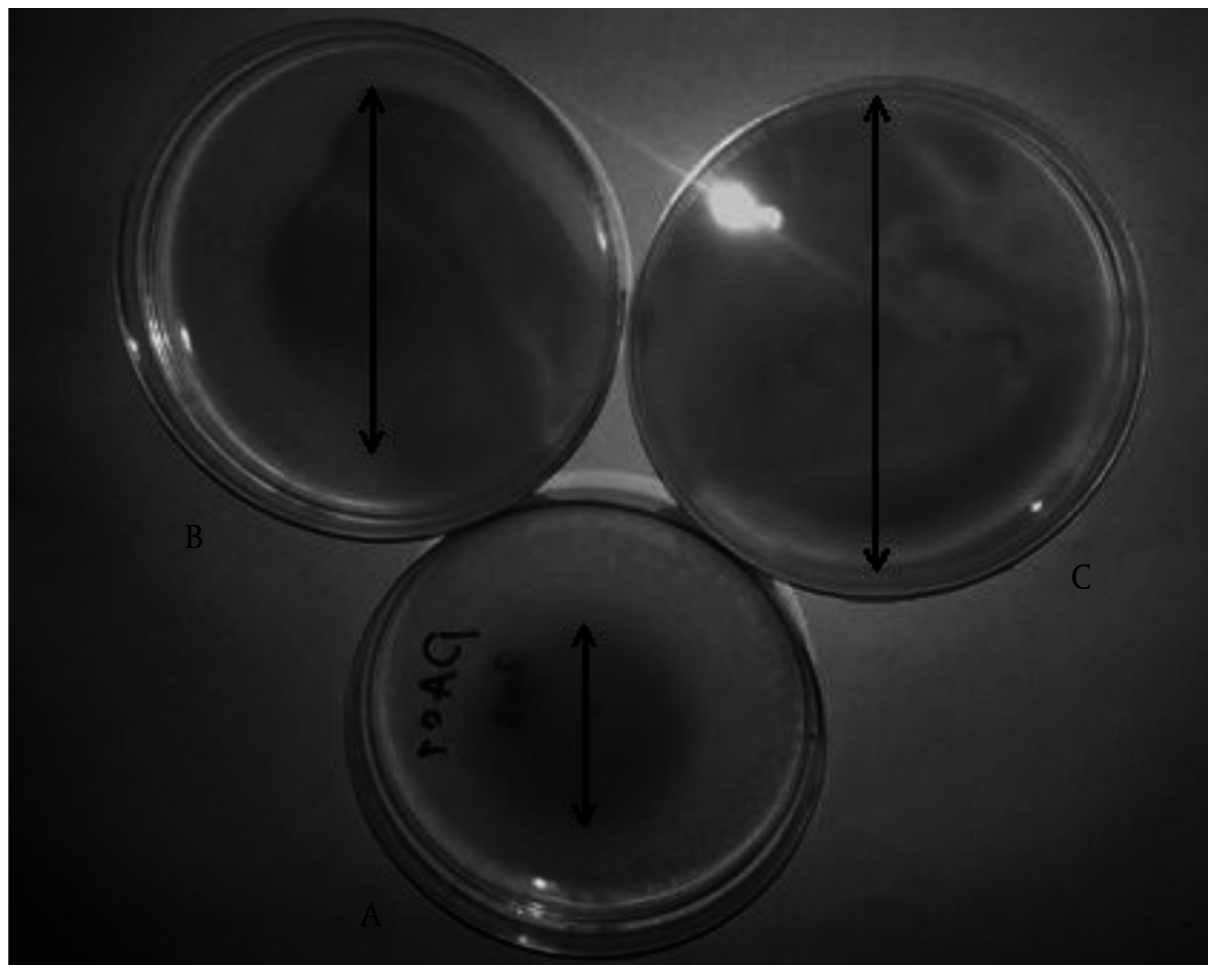
Anti-r-B-flagellin inhibited the motility of *P. aeruginosa* PAO1 (A), with a slight effect on the PAK strain in motility agar (B) (LB with 0.3% w/v agar) when compared with non-immunized rabbit serum (C).

each provided an extensive intellectual contribution; All of the authors read and approved the final manuscript.

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