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Evaluation of the Pathogenesis of *Pseudomonas aeruginosa*'s Flagellum Before and After Flagellar Gene Knockdown by Small Interfering RNAs (siRNA)

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A B S T R A C T

Background: *Pseudomonas aeruginosa* possesses a polar flagellum made up of flagellar subunits, which are encoded by fliC gene. Flagella have important roles in the motility, chemotaxis, and establishment of *P. aeruginosa* in the acute phase of infections. The inhibition of flagellar expression may be a promising therapeutic approach to prevent the pathogenesis. The gene-silencing effect of siRNA may be useful for this strategy.
Objectives: The current study investigated the efficacy of siRNA on the expression of flagellin, because it is an important protein in the initial stages of *P. aeruginosa* infections.
Materials and Methods: The current research designed and synthesized 21 bp siRNA duplexes against *P. aeruginosa* flagella. Quantitative RT–PCR was performed to determine whether the siRNAs inhibit the expression of the flagellin mRNA in vitro. The efficacy of siRNA was determined by the motility test and in a murine model of hematogenous pulmonary infection.
Results: In quantitative RT–PCR,it was shown that the siRNA significantly inhibited the expression of the flagella mRNA. FilC gene knockdown by the siRNA resulted in a significant decrease in the expression of the flagellar mRNA in the siRNA group as compared with that of the control ($P < 0.05$). In the motility test,the motility was inhibited in the siRNA group more effectively than in the control group. In the murine infection model, a significant decrease in the number of viable bacteria was detected in the siRNA group when compared with the control (7.87 ± 0.54 in the former versus 4.69 ± 0.35 log cfu/mL in the latter mean ± SD, $P < 0.05$).
Conclusions: The development of delivery systems into bacteria with an efficacy compatible to that in human use could be a key for the potential utility of siRNA for the prophylaxis and treatment of *P. aeruginosa*-induced hematogenous pulmonary infections in humans.

Keywords: fliC; siRNA; Pathogenesis; Pulmonary Infections; Murine Model; *Pseudomonas aeruginosa*

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Implication for health policy/practice/research/medical education:
The development of delivery systems into bacteria with an efficacy compatibleto the one in human use could be a key for the potential utility of siRNA for the prophylaxis and treatment of P. aeruginosa-induced hematogenous pulmonary infections in humans.

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1.Background

Pseudomonas aeruginosa is the most common pathogen responsible for nosocomical and community-acquired infections at various body sites including the lower respiratory tract, urinary tract, cornea, and surgical or burn wounds (1, 2). *P. aeruginosa* has some advantages that help to establish acute or chronic infections under various host conditions (3). One of these advantages is its flagella. Polar flagella provide mobility and chemotaxis, and they also facilitate the adherence to cells and non-living surfaces, which confer the ability to colonize and invade throughout the early phases of infection (4).

Flagella play a critical role in the initial stages of respiratory tract infection, as shown by comparing the virulence of fliC mutants in a neonatal mouse model of pneumonia. There was no mortality in the fliC mutants (2). *P. aeruginosa* flagella are suggested to act in pathogenesis by tethering and adhering to epithelial cells through binding and interaction with epithelial membrane components. However, flagella are also very immunogenic, which renders them susceptible to the host clearance mechanisms and facilitating phagocytic clearance (5, 6). Consequently, it is not surprising that flagella have been considered as smart drugtargets for immunotherapy. On the other hand, efforts are underway to develop antimicrobials from classes of compounds for which specific resistance traits do not exist in nature. Therefore, new therapeutic options for *P. aeruginosa* infections can be explored.

RNA-mediated interference (RNAi), originally as an antiviral mechanism, was discovered in plants, and it was subsequently found in Caenorhabditis elegans, Drosophila and vertebrates (7, 8). RNAi is an evolutionarily conserved system and a powerful tool to silence gene expression (9, 10). To knock-down gene expression by RNAi, two major methods of mRNA silencing exist. The first method utilizes the construction of plasmid DNA that expresses shRNA (short hairpin RNA). The second method is based on siRNA (small interfering RNA), a readily processed 21 to 22 nucleotide sequence that associates with a multiprotein complex known as the RNA-induced silencing complex (RISC). This ultimately targets homologous mRNA,and destroys it after transfection into the cell, based on complementary base pairing (11). siRNAs are projected to provide an interesting therapeutic advantage for curing genetically dominant disorders,cancers, neurological disorders, and viral and bacterial infections (11).

2. Objectives

The current study investigated the inhibitory effects of siRNA on *P. aeruginosa* flagella *in vitro* and *in vivo*.

3. Materials and Methods

3.1. Bacterial Strain

P. aeruginosa 8821M was kindly provided by Dr. Oulia (Shahed University, Iran). The bacteria were stored at –70°C in brain heart infusion (BHI) broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% (v/v) glycerol and 5% (w/v) skim milk (Yukijirushi Co., Tokyo, Japan) untilused.

3.2. siRNAs

The sequences of siRNA were designed against *P. aeruginosa* 8821M flagella by established methods (Gene Script software) (12). Sequences of all the tested siRNAs are shown in *Table 1*. All the RNA oligonucleotides were synthesized by Bionner Co (South Korea). These oligonucleotides were deprotected according to the manufacturer's instructions. The resulting siRNA duplexes were analyzed for completion of duplex formation by gel electrophoresis. Two sets of siRNAswere used for the experiments and siRNA-1 was found more effective than siRNA-2. Therefore, thefurther experiments utilized siRNA-1 (7, 8).

Table 1. Sequences of the Synthetic and ScrambledsiRNAs Used in This Study

SiRNA	Sequence	Start	GC, %
siRNA-1		*666*	47.37
Sense	*5′-ACGUCAAGGUCGACAUGAATT -3′*		
Antisense	*5′- UUCAUGUCGACCUUGACGUTT -3′*		
siRNA-2		566	57.89
Sense	*5′- GGCACCTACTTCACGGCTA -3′*		
Antisense	*5′-UAGCCGUGUUGUAGGUGCC-3′*		
siRNA- scramble		---	33.3
Control sense	5´- ACAGAAGAUAUAGGAGUGATTTT -3´		
ControlAntisense	5´- UCACUCCUAUAUCUUCUGUTTTT -3´		

3.3. RNA Extraction and RT–PCR

P. aeruginosa 8821M was cultured on a brain heart infusion agar-based (BBL Microbiology Systems) sheep blood agar plate for 24 h at 37°C. Bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3000xg, 4°C and 10 min). The microorganisms were resuspended in cold sterile saline and diluted to 1 × 106 cfu/mL, as estimated by turbidometry. *P. aeruginosa* 8821M was cultured in trypticase soy broth with the targeted siRNA or with a scrambled siRNA as a control (20, 40 pico mol, and 2µ mol, respectively).

The siRNAs were added every 20 min (according to the generation time of the bacteria). After 6 h, RNA was extracted from bacteria with a FastRNA Kit-BLUE(BIO 101, Carlsbad, CA, USA), and RT-PCR was performed to determine the level of mRNA according to the kit instructions. Oligonucleotide primers for PCR were designed according to the sequence for *P. aeruginosa* 8821M flagella (sense, 5'- TGAACGTGGCTACCAAGAACG-3'; antisense, 5'-TCTGCAGTTGCTTCACTTCGC-3'). Rpls gene controls were used as a housekeeping gene (sense, 5'-AAGCGCATGGTCGACAAGA -3'; antisense, 5'- CTGTGCTCTTGCGGTTGTGA -3'). PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide (Figure 1).

3.4. Effects of siRNA on Flagella Production

P. aeruginosa 8821M was cultured with targeted or scrambled siRNA. The siRNAs were added every 20 min. After 4 h, the flagella were assayed by Quantitative Real-time PCR and motility inhibition test.

3.4.1. Quantitative Real-Time PCR

For real-time quantitative PCR, 500ng of RT product was used in a whole volume of 15µl consisting of 7.5 µl of SYBR Green Premix 2X (Takara, Shiga, Japan) and 10pM of mix primer in a Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia). Thermocycling conditions were heat-held at 94C for 1 min, followed by 40 cycles of denaturation at 94C for 20sec, annealing at 57C for 30 sec, and extension at 72C for 30 sec. The amount of each gene expression depends on the cycle at the threshold (Ct), in which the fluorescence density in the PCR microtube rises above the background and is normalized by rpls gene as the endogenous reference gene. The rpls gene controls were used to standardize the quantification of RNA samples. The rpls gene levels for each condition were measured, and flagella/ rpls ratios were compared (*Figure 1*).

The siRNAs were added every 20 min. After 4 h, RNA was extracted from bacteria, and RT-PCR was performed to determine the level of mRNA (A, C). The rpls gene bands for each condition were measured (B) and flagellar/ rpls gene ratios were compared (C). Data are expressed as mean ± SD (n = 5).

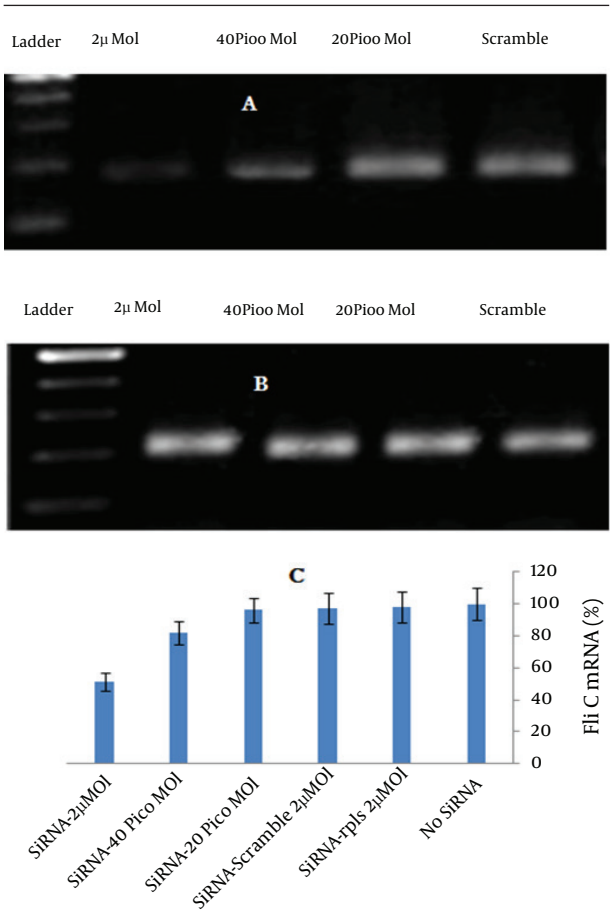


Figure 1. Effects of siRNA Treatment on the Expression of Flagellar mRNA. *P. aeruginosa* Was Cultured With Targeted or Scrambled siRNAs.

3.4.2. Motility Inhibition Test

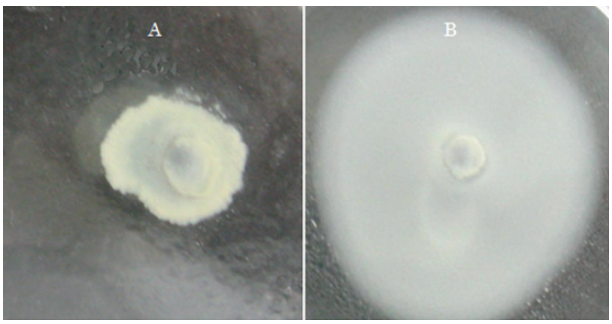
To confirm the effect of siRNA, two plates were filled with 10 ml of motility agar (LB with 0.3% (w/v) agar, soft agar). Then 25µL of cell suspensions of *P. aeruginosa* strain 8821M (OD600 = 0.2), affected and not affected by siRNAin PBS,were inoculated into the central well (5 mm in diameter) of each plate. For each assay, triplicate plates were examined. The plates were incubated at 37C. The mean diameters of bacterial spreading with sharp and less spreading rings were measured after incubation for 24 h (13) (*Figure 2*).

3.5. Dimension of the Integration of 32P-Labelled siRNA by Bacteria

To confirm the entrance of the siRNA into thebacteria, ^{32}P labeled siRNA was used. ^{32}P siRNA was prepared as described previously (7). Bacteria (1×10^6 cfu/mL) were incubated with siRNA (30 pmol) for 4 h; then the medium was removed, and the bacteria were washed five times with PBS and centrifugation. The washed bacterial pellets were solubilized by addition of 780 mL PBS, 10 mL of 10%

SDS, and 10 mL of chloroform. After centrifugation (3000 g, 4C and 10 min), the supernatant was collected as the cytoplasmic fraction. The pellet, which contained the membranes and cell walls, was resuspended in 100 mL of distilled water. The integrated radioactivity in the different fractions was measured with a scintillation counter. For the control group, bacteria were washed immediately after treatment with radiolabelled siRNA, and cell fractions were prepared as described above (*Figure 3*).

Figure 2. Motility Inhibition Assay



To confirm the effect of siRNA, two plates were filled with 10 ml of motility agar (LB with 0.3% (w/v) agar, Soft agar) 25μL of a cell suspension of *P. aeruginosa* strain 8821M (OD600 = 0.2) exposed (A)and no exposed (B)to siRNAin PBS was inoculated into the central well (5 mm in diameter) of each plate. The motility of the bacteria was inhibited by the specific siRNA (A) as compared them with negative control (B)

3.6. Laboratory Animals

Six-week-old pathogen-free male Balb/C mice (25–30 g body weight) were purchased from PasteurInstitute (Tehran, Iran). All animals were housed in a pathogen-free environment at the Laboratory Animal Center. The Ethics Review Committee for Animal Experimentation at our institution approved all of the experimental protocols described in this study in advance. Animal experiments in this study were done in compliance with Baqiyatallah University of Medical Sciences institutional guidelines.

3.7. Inoculum

The method of inoculation was described previously (14, 15). Bacteria were pre-incubated with siRNA (2 μM) or scrambled siRNA (2 μM) for 6 h, then they were suspended in endotoxin-free sterile saline and harvested by centrifugation (3000 g, 4°C, and 10 min). The collected microorganisms were resuspended in cold sterile saline and diluted to 2–4 $\times 10^9$ cfu/mL, as estimated by turbidimetry. The suspension was warmed to 45°C, after which 10 mL of the suspension was mixed with 10 mL of 4% (w/v) molten Noble agar (Difco Laboratories, Detroit, MI, USA) at 45°C.

The agar–bacterium suspension (1.0 mL) was placed in a 1.0 mL syringe, and the suspension was rapidly injected into 49 mL of rapidly stirred ice-cooled sterile saline using a 26-gauge needle. This resulted in solidification of the agar droplets into beads of ~200 μm in diameter. The final concentration of agar was 0.04% (w/v), and the final number of bacteria was 2-4 $\times 10^7$ cfu/mL. Mice were injected in the tail vein with 0.20-0.25 mL of the bacteria–agar beads per mouse (10 mL/g of body weight). Before the bacteria were embedded in the agar beads, their numbers were verified by inoculating duplicates of serial dilutions onto blood agar plates and counting the cfu after incubation for 48 h at 37°C (7, 8). 4 groups were used for examination, (bacteria+siRNA, bacteria+ scrambled siRNA, naked siRNA, and bacteria + siRNA injected separately)

3.8. Bacteriological Examinations

Each group of animals was sacrificed by cervical dislocation 72 h after infection. After exsanguination, the lungs were dissected and removed under aseptic conditions. Organs used for bacteriological analyses were homogenized and cultured quantitatively by serial dilution on blood agar plates.

3.9. Statistical Analysis

Bacteriological data were expressed as means ± SD. Differences between groups were examined for statistical significance using an unpaired T-test. A P value of < 0.05 was considered statistically significant (*Figure 4*).

4. Results

4.1. Motility, siRNA Treatment, and Expression of Flagellar mRNA

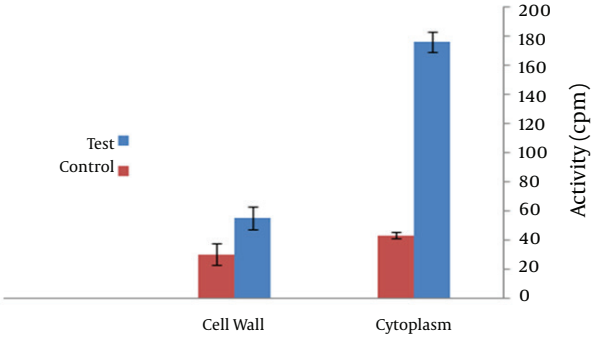
In the motility test, siRNA inhibited the motility of *P. aeruginosa*in siRNA group in comparison with that of the control group (*Figure 1*). To determine that whether siRNA can control the flagellin production in *P. aeruginosa*, expression of the flagellar mRNA level was evaluated by RT-PCR after addition of the siRNA to the bacteria. A high dose (2 μM) of siRNA significantly inhibited the mRNA expression. In addition, compared with 2 μM of the scrambled (control) siRNA, 2 μM of siRNA significantly inhibited the production of flagella in *P. aeruginosa*. It was concluded that mRNA expression was dose-dependently inhibited by siRNAs. Finally, there was an important reduction in flagellar mRNA expression in the siRNA group ($P < 0.05$).

4.2. Absorption of Labeled siRNA by Bacteria

To confirm that the siRNA were transferred to the bacteria, we measured the amount of ^{32}P incorporated into the bacteria following the transfection with ^{32}P-labelled siRNA. Intracellular incorporation was determined after washing and eliminating the cell wall and membrane.

The radioactivity in the cytoplasm was more than 2-fold higher for the 4 h pre-treated group than for that of the control group *(Figure 3)*.

Figure 3. Absorption of 32P-labelled siRNA Into Bacteria.

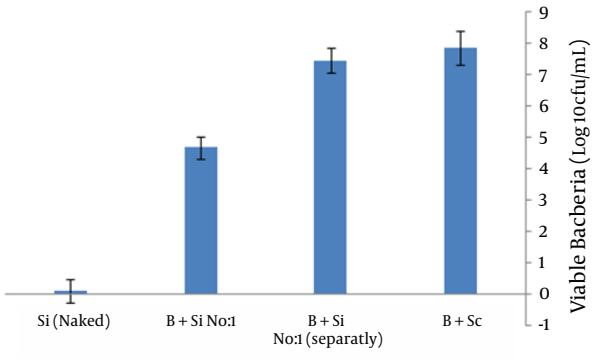


Bacteria were incubated with Bacteria were incubated with 32P-labelled siRNA for 6 h, after which the bacteria were separated into cytoplasmic and membrane/cell wall fractions. The radioactivity in the various fractions is shown for the control bacteria and the bacteria treated for 6 h with siRNA. The radioactivity in the cytoplasmic fraction of the siRNA-treated sample was more than 3-fold higher than in the control sample.

4.3. Effects of siRNA in a Murine Model of Hematogenous Pulmonary Infection

In a murine model of the hematogenous pulmonary infection, the amount of *P. aeruginosa* was 7.87 - 0.54 and 4.69 - 0.35 log cfu/mL (mean ± SD) in control and bacteria+ siRNA groups, respectively, indicating that there was a significant decrease in the number of viable bacteria in the bacteria+ siRNA group ($P < 0.05$). There was no such significant difference in other groups when compared with control (*Figure 4*).

Figure 4. Effects ofsiRNA in a Murine Model of Hematogenous Pulmonary Infection



The number of viable bacteria in The number of viable bacteria in the lung from mice sacrificed after 3 days of infection was significantly fewer in the siRNA-1 group than in the scrambled siRNA group. Bacteriological examinations were performed three times without significant differences among them. B=Bacteria, Si=SiRNA, Sc=Scramble) (*p<0.05)

5.Discussion

The current report indicated that (i) siRNAs can potently inhibit *P. aeruginosa* flagellin production in vitro, and that (ii) the number of viable bacteria was significantly decreased by pre-treatment with siRNA. These findings have important implications for the use of siRNA for prophylaxis and treatment of *P. aeruginosa*-induced hematogenous pulmonary infection. This study is the first report on the importance of siRNA against *P. aeruginosa*. The present study examined the effect of siRNA against a pathogenic factor, because the suppression of pathogenic factors is significant for inhibition of infections (16).

The current study results suggest that siRNA may be a therapeutic choice in addition to the other therapeutic methods against infection by *P. aeruginosa*via inhibition of flagellin production. In particular, it was found that siRNA reduced the motility of bacteria (flagella) and the amount of felagellin mRNA in vitro and considerably lowered the number of viable bacteria in vivo. Therefore, siRNA against flagella may be effective in the treatment of *P. aeruginosa*-induced hematogenous pulmonary infections.

However, the inhibition efficacy of the mRNA expression of *P. aeruginosa* flagellin by siRNA was only approximately 45% compared with the control. Therefore, various devices, to increase the efficacy, are necessary for clinical use, including employment of other vectors such as liposomes for better transfection efficacy. In this regard, lectin-facilitated liposomes, (17), which may allow targeting of specific cell types, may be particularly useful.

Since the number of viable bacteria was significantly lower in the siRNA pre-treated group than in the control group, the injection of naked siRNA into the tail vein of mice was tested, resulting in no effect. Therefore, for in vivo delivery, it may be useful to deliver the siRNA in a cationic liposome (18). It is reported that siRNA can be delivered into the lung by rapid intravenous injection of a large volume of siRNA in PBS (19), but, in the current studyexperiments,siRNA was used to target bacteria rather than human cells. The development of delivery systems into bacteria with an efficacy compatible to the onein human use could be a key for the potential utility of siRNA for the prophylaxis and treatment of *P. aeruginosa*-induced hematogenous pulmonary infections in humans. Future studies are needed to genetically screen and identify new siRNA-target genes and to use this siRNA as a flagella-siRNA complementary strand.

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Authours' Contribution

None declared.

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