Original Article

Silencing of *Pseudomonas aeruginosa* Integron Class I Gene by Short Interference RNA

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Abstract

Background: The elevation of global resistance of *Pseudomonas aeruginosa* to various antibiotic agents represents a public health threat. In the field of bacterial cellular engineering, gene silencing emerges as an important technology capable of reducing the expression of antibiotic resistance genes. Specifically, short interference RNA (siRNA) technology holds promise in addressing bacterial infections linked to antibiotic resistance. This study focuses on the application of siRNA to target the Integron Class I (intI) gene in *P. aeruginosa* as a potential strategy to attenuate antibiotic resistance issues. **Objectives:** The primary objectives of this research involved the identification of the intI gene in *P. aeruginosa* clinical isolates, the design and introduction of constructed siRNA to induce gene silencing, and the assessment of alterations in gene expression using RT-PCR. The study aims to clarify the efficacy of siRNA-mediated silencing in downregulating the expression of the intI gene associated with antibiotic resistance. **Materials and Methods:** A total of 30 clinical isolates of *P. aeruginosa* were included in the study. DNA extraction was performed to identify the intI gene, and a custom siRNA sequence was designed against intI. The siRNA constructs were introduced into *P. aeruginosa* isolates via Gold Nanoparticles (AuNPs). Gene expression changes were quantified using RT-PCR. **Results:** The study found that the intI gene was present in all (100%) *P. aeruginosa* isolates. Importantly, the results demonstrated a significant reduction in the expression level of the intI gene when targeted with siRNA, indicating the efficacy of siRNA in downregulating the gene associated with antibiotic resistance. **Conclusion:** In conclusion, the widespread presence of the intI gene in *P. aeruginosa* is closely linked to antibiotic resistance. The study highlights the substantial capability of siRNA to downregulate intI gene expression, presenting a promising avenue to inhibit antibiotic resistance *in vitro*. This research underscores the potential of siRNA technology as a targeted approach to combat antibiotic resistance in *P. aeruginosa*.

Keywords: Gene expression, integron, P. aeruginosa, silencing, siRNA

Introduction

A significant public health problem is the emergence of antibiotic-resistant bacteria, which can cause illnesses that can increase mortality by delaying or failing to deliver antimicrobial medication.[1,2] A common nosocomial pathogen that develops antibiotic resistance is *Pseudomonas aeruginosa*. Due to its multiple resistance mechanisms, this bacterium can cause infections that are difficult to treat.[3,4] Therefore, caution is warranted as these resistant strains may develop resistance to all available antimicrobial agents.[5,6] Integrons are particularly important in Gram-negative pathogens; they have a crucial role in facilitating the acquisition and dissemination of antibiotic resistance features among a wide range of Gram-negative clinical isolates. Consequently, they actively contribute to the evolutionary process of antibiotic resistance in bacterial populations.[7,8] A novel recombination-independent technique has emerged that selectively modifies gene expression profiles at the post-transcriptional level while preventing from creating any alterations to the DNA. This is accomplished by expressing antisense RNA to silence (turn off) the target mRNA *in vivo*.[9] The

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process of gene silencing, also known as knockdown, is comparatively simpler and more efficient when compared to gene disruption, or knockout, methodologies. The technique of gene silencing through the use of small interfering RNA (siRNA) is a highly effective method employed to reduce the expression of genes. SiRNA molecules possess the ability to selectively identify and attach to mRNA sequences that are complementary to them. The activation of this particular binding event initiates the process of mRNA degradation, leading to a decrease or suppression of gene expression.[10] Numerous diseases, including infections and cancers, have been the subject of intensive research into the potential therapeutic applications of siRNA-mediated gene silencing. The use of siRNA-mediated gene silencing as a method to inhibit antibiotic resistance genes or genes linked to pathogenicity in the field of antibiotic-resistant bacteria is one interesting area for research. This strategy can either reduce the harmful effects of bacteria or improve the efficacy of antibiotics.[11,12] The objective of this study was to develop siRNA targeting the Integron Class I (intI) gene in *P. aeruginosa* and examine the impact of intI silencing on the antibiotic susceptibility of cultured bacteria. Results from this study provide new insight into how gene-targeting by siRNA molecules could be used to treat bacterial infections, especially those caused by *P. aeruginosa*.

Materials and Methods

DNA extraction and amplification

The study included a total of 30 clinical isolates of *P. aeruginosa* where DNA was extracted from isolates by using a commercially available extraction kit (Presto Mini gDNA Bacteria Kit, Taiwan), following the manufacturer's instructions. The designed primers by Primer3Plu (https://www.primer3plus.com/index.html) used in this work were obtained from Macrogene Company in Korea [Table 1]. They were used following their manufacturer's instructions to identify the intI gene of *P. aeruginosa*. The PCR mixture was prepared for each primer, with a final volume of 20 µL per reaction. The procedure employed was based on the instructions provided by the manufacturer of the Master Mix AccuPower PCR PreMix (Bioneer, Korea). Each PCR reaction mixture consisted of 1 µL Forward Primer (10 pmole), 1 µL Reverse Primer (10 pmole), 10 µL master mix, 5 µL de-ionized water, and 3 µL DNA. DNA amplification was conducted in a temperature gradient thermal cycler with conditions as follows: DNA denaturation at 95°C for 5 min, followed by annealing temperatures of 58°C for 30 s, with an extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis in a 1.5% W/V agarose gel in TBE buffer (1X) and then visualized by Gel Documentation.

Table 1: Designed primers used in this study's conventional PCR and Multiplex-RT-PCR

Target gene	Primer name	Oligonucleotide primer sequence (5'-3')	Amplicon size (bp)
integI	F	GGGATGCGAACCACTTCAT	311
	R	CGCATACGCTACTTGCATTACA	
integI (RT)	F	GCCGTAGAAGAACAGCAAGG	180
	R	CGAACCGAACAGGCTTATGT	
recAS	F	GCGGTGAAAGAAGGTGATGA	120
	R	GTAGATGCCCTTGCCGTAAA	
Reference		**Designed in this study**	

bp = base pair; F = forward sequence; R = reverse sequence

Design and conjugate siRNA to gold nanoparticles

The siRNA sequence was designed by this study against IntI were synthesized by Macrogene Company (Korea) to have a complementary sequence to the target mRNA. siRNA constructs were delivered to IntI via Gold Nanoparticles (AuNPs) (purchased from Vira Carbon Nano Materials Co. Ltd., Iran) after conjugating in salt solution. The siRNA exhibited a length of 34 base pairs; the provided siRNA sequence was 5' ATGAACAGGTATAACGGATCTGCCAAA CCTGACT -3.' Lyophilized siRNA was dissolved in nuclease-free water to give a final concentration of 100 pmoles/µL. Conjugated gold nanoparticles for the design of siRNA involved the preparation of different concentrations of sodium chloride (NaCl); concentrations including 3, 2, 1.5, 1, and 0.5 M were used. Firstly, 3 mL of gold nanoparticles were mixed with 2 mL of 3M NaCl in a 10 mL plan tube and incubated in a shaker incubator for 30 min at 60°C. Afterward, add 20 µL of siRNA that was prepared by dissolving in nuclease-free water to give a final concentration of 100 pmoles/µL and return it back to shaker incubation for 10 min. Finally, add 500 µL every 30 min of residual concentrations from NaCl and keep them under the same incubation conditions. The red-safe-stained bands of the siRNA-AuNPs conjugates were directly visualized by the gel imaging system.

Silencing of IntI gene by conjugated siRNA

To introduce the conjugated siRNA to bacterial isolates, we selected only five isolates (P.A6, P.A11, P.A24, P.A32, and P.A61) for silencing, which were selected based on their biofilm formation ability and showing high resistance to the antibiotic as illustrated in Table 2. We cultured the *P. aeruginosa* isolates and incubated them overnight at 37°C on a cetrimide agar plate. The bacterial suspension that was prepared in Brain heart infusion broth and adjusted to 0.5 McFarland was then incubated for 24 h at 37°C. After incubation, 200 µL of bacterial suspensions are inoculated into 7.020 mL of conjugated salt solution that was previously prepared and incubated in a shaker incubator for 24 h at 37°C.

RNA extraction and real-time RT-PCR

RNA was isolated from the sample using TRIzol Reagent according to the protocol described by the manufacturer's instructions. The total RNA was reversely transcribed to complementary DNA (cDNA) through newly designed primers [Table 1]. The experimental protocol involved the addition of an 18 µL aliquot from each extracted total RNA sample into a fresh PCR tube. Subsequently, a ProtoScript reaction mix was employed, which consisted of dNTPs, buffer, and other crucial constituents, with a volume of 10 µL allocated for each individual sample. Next, 2 µL of MuLV Enzyme was introduced into the reaction for each sample, along with an aliquot of 2 µL of hexamer. The PCR amplification was carried out under the following thermal cycling conditions: Initial denaturation at 95°C for 3 min, followed by 40 cycles (denaturation, annealing, and extension) of 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s. The quantification of qRT-PCR data involved determining the difference in Ct values between the target gene and the reference housekeeping gene. The study employed the ΔΔCt method to measure the relative changes in gene expression levels, as outlined in the work of Schmittgen and Livak.[13]

Statistical analysis

The Duncan test was used to compare the mean levels of integrons and controls. Other $P \leq 0.05$ was detected as significant. The present data were programmed by the SPSS v. 24.0 statistical software program.

Table 2: Selected isolates for screening of *P. aeruginosa* integron genes

Isolated numbers	Susceptibility to antibiotic	Biofilm intensity
P.A 24	*MDR	Strong biofilm former
P.A32	MDR	Strong biofilm former
P.A 6	MDR	Moderate biofilm
P.A 61	*XDR	Strong biofilm
P.A 11	*PDR	Moderate biofilm

MDR = multi-drug resistance, XDR = extensively drug-resistant, PDR = pan-drug resistance

Ethical approval

Ethical approval was obtained from a local ethics committee at the College of Science, University of Baghdad, according to document No. EC/1313 on May 15, 2022.

Results

DNA extraction and IntI gene detection

Efficient extraction of whole-genome DNA was performed on overnight cultures of 30 selected isolates of *P. aeruginosa*. The PCR results obtained in this investigation demonstrated the presence of IntI in all *P. aeruginosa* isolates, with a 100% percentage of detection [Figure 1]. Also, gel electrophoresis was used to examine the conjugation of siRNA-AuNPs. Where gels with conjugated siRNA-AuNPs appeared as fluorescent bands in gel wells, unconjugated gold nanoparticles were migrated in opposite directions to normal DNA migration while free siRNA with minimal size cannot be recognized.

Effect of siRNA treatment on the expression of the IntI gene

In the present study, a quantitative RT-PCR assay analyzed the mRNA expression of the intI gene by comparing the treated isolates with siRNAI–AuNPs and untreated isolates. For each sample, the assay was performed three times, and the mean of each of the outcomes was used to determine the amount of a particular gene's expression in that sample. The results indicated a decrease in gene expression in the intI gene of the isolates P.A24, P.A11, P.A32, and P.A61 after being treated with siRNAI–AuNPs. In contrast, the results show an increase in the gene expression of isolates (P.A6) after silencing by siRNA. The determination of gene expression fold change was performed utilizing the relative quantification approach based on the delta-delta Ct method [Table 3]. The outcome showed significant differences ($P < 0.050$) among levels of Integron Class I within bacterial isolates and controls. The bacterial isolates P.A11, P.A24, 32, and 61 scored lowest levels (0.807 ± 0.030, 0.137 ± 0.012, 0.087 ± 0.003 and 0.090 ± 0.012) than controls (1.000 ± 0.000); in contrast, the bacterial isolate P.A6 scored the highest level of intI (1.803 ± 0.140) than controls (1.000 ± 0.000) [Table 4].

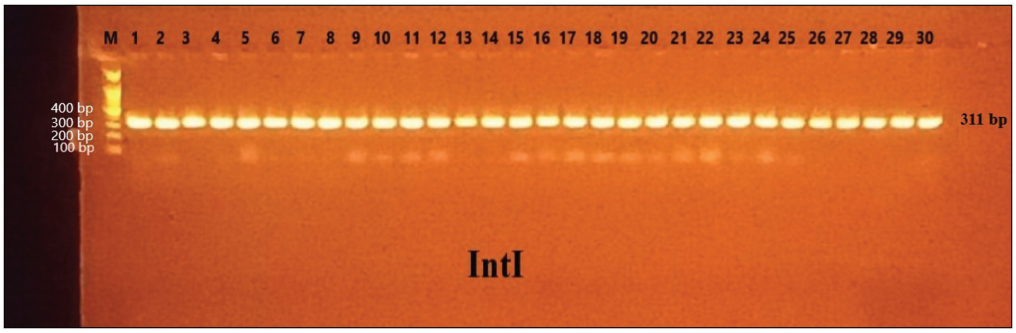

Figure 1: The agarose gel electrophoresis analysis of intI gene. The electrophoresis was performed on 1.5% agarose gels, which were then stained with red-safe dye. The electrophoresis process was conducted for a duration of one hour at a voltage of 100 V

Table 3: Fold of gene expression of intI gene depending on ΔΔCt method

Sample	CT Treatment	CT Untreated	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2 ^ -ΔΔCt		
Control	26.78	18.39	0	0	0	1	1	1
24	26.78	18.39	−5.46	−8.33	2.87	0.136786713	0.124787	0.148787
11	25.48	18.22	−7.96	−8.27	0.31	0.806641759	0.836642	0.776642
61	29.43	21.87	−3.42	−6.89	3.47	0.090245575	0.078246	0.102246
32	28.19	22.24	0.44	−3.08	3.52	0.087171479	0.090171	0.084171
6	25.82	23.48	−3.48	−2.63	−0.85	1.802500925	1.942801	1.662201

Table 4: Gene expression of Integron Class I

	No.	Mean	Std. Deviation
Control	3	1.000[b]	0.000
P.A 6	3	1.803[a]	0.140
P.A 11	3	0.807[c]	0.030
P.A 24	3	0.137[d]	0.012
P.A 32	3	0.087[d]	0.003
P.A 61	3	0.090[d]	0.012

Small, different letters refer to significant differences ($P < 0.05$)

[a]Group labeled with "a" is significantly different from groups labeled with any other letter in the same column

[b]Group labeled with "b" is significantly different from groups labeled with any other letter in the same column, except for those labeled with "a"

[c]Group labeled with "c" is significantly different from groups labeled with any other letter in the same column, except for those labeled with "a" or "b"

[d]Group labeled with "d" is significantly different from groups labeled with any other letter in the same column, except for those labeled with "a", "b", or "c"

Discussion

P. aeruginosa, a clinically important *Pseudomonas* species, causes nosocomial infections worldwide. *P. aeruginosa* isolates had higher antibiotic resistance levels, primarily due to the ability of the bacteria to acquire resistance through gene transfer.[14,15] Integrons are a double-stranded DNA segment that holds significant importance in bacterial adaptation and evolutionary processes. They play a crucial role in the dissemination of antibiotic resistance, which is a prominent concern in the field of global public health, especially in Gram-negative pathogens.[16,17] The results of our investigation pertaining to the frequency of class 1 integron-positive isolates are consistent with prior scholarly inquiries done in Iraq, whereby a 100% occurrence of class 1 integron-positive *P. aeruginosa* clinical isolates was reported.[18] An additional investigation demonstrated that 89 (95.7%) of the *P. aeruginosa* strains carried intI genes.[19] The majority of the antibiotic's resistance genes are collectively located in integrons.[20] These findings demonstrated a strong association between the development of antibiotic resistance in *P. aeruginosa* and the presence of the intI gene. Acquiring class 1 integrons exposes bacteria to foreign genes, creating multi-drug-resistant isolates.[21] The utilization of homology-based gene silencing has emerged as a highly effective approach for the suppression of gene expression, facilitating the exploration of gene activities. Numerous gene silencing techniques utilizing antisense or siRNA have been employed for this purpose. The objective of this study was to suppress the expression of the IntI gene using a siRNA-based strategy, building upon prior research.[21–23] This siRNA sequence that is designed against the intI gene, is introduced to bacterial cells via AuNPs. Binding siRNA to AuNPs offers several advantages, including enhanced cellular uptake, protection of siRNA from degradation, and the potential for targeted delivery.[24] Nevertheless, the procedure necessitates meticulous planning and refinement in order to guarantee successful association, transportation, and suppression of genes. Ionic interactions between AuNPs and negatively charged siRNA provide excellent protection of siRNA against extracellular nuclease attack as well as simple cellular absorption through an endocytic route.[25] RT-qPCR stands out among several techniques utilized for gene expression analysis due to its sensitivity, accuracy, and fast results.[26] This method has become widely recognized as an ideal standard for gene expression analysis. In relative PCR, the expression level of the target gene is compared to a reference gene (also called the housekeeping gene) that is assumed to have consistent expression across samples. It does not require a standard curve or known concentrations but rather calculates the relative fold change in gene expression between samples. The data is reported as a fold difference in expression between the target gene and the reference gene.[27] SiRNA typically decreases gene expression. When siRNA molecules are introduced into a cell and bind to the complementary mRNA of a target gene, they trigger a process that leads to the degradation of the mRNA. This degradation prevents the mRNA from being translated into protein, effectively reducing the expression of the target gene.[28] By targeting integron genes with siRNA, it may be possible to downregulate their expression and reduce the spread of antibiotic resistance. SiRNA-mediated gene silencing of specific genes in *P. aeruginosa* has been shown to enhance antibiotic sensitivity.[22] This is considered transient silencing due to the stability of siRNA, rapid degradation of siRNA, and bacterial replication.[29] Therefore, transient silencing is valuable for studying the effects of gene manipulation without making permanent changes to an organism's genome. Consequently, the

suppression of intI gene expression was considered a potentially effective approach to enhance antibiotic susceptibility. The extensive utilization of antibiotics in Iraq for the management of severe infections caused by Gram-negative bacteria, particularly burn infections, induces the emergence of many resistance mechanisms among various pathogens against these antimicrobial agents.[30] Gene silencing technology by using siRNA has an impact on the mRNA expression of the intI gene in clinical isolates of *P. aeruginosa*. By targeting integron genes with siRNA, it may be possible to reduce the spread of antibiotic resistance and the severity of infections caused by this bacterium.

Conclusion

In conclusion, the widespread presence of the intI gene in *P. aeruginosa* is closely linked to antibiotic resistance. The study highlights the substantial capability of siRNA to downregulate intI gene expression, presenting a promising avenue to inhibit antibiotic resistance *in vitro*. This research underscores the potential of siRNA technology as a targeted approach to combat antibiotic resistance in *P. aeruginosa*.

Author contributions

Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing—Original Draft Preparation, Review, and Editing, Project Administration, and Funding Acquisition have been contributed by the first author, and Conceptualization, Visualization, and Supervision by the second author.

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Conflicts of interest

There are no conflicts of interest.

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