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Impact of siRNA-Mediated Gene Silencing on IntII Gene Expression for Clinical Isolated *Pseudomonas aeruginosa*

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Abstract: Integrons hold significant importance in gram-negative bacteria, which are known for being susceptible to acquiring antibiotic resistance. The aim of this study was to identify Integron class II (intII) genes and induce silencing in the intII gene by designing small interfering RNA (siRNA) molecules specifically targeting this gene in *Pseudomonas aeruginosa* and demonstrating a silencing effect on antibiotic resistance. The DNA was extracted from 30 clinical isolates of *P. aeruginosa* to identify the intII gene, and a specific siRNA was designed and attached to gold nanoparticles (AuNPs) for delivery into bacterial cells. The replica plate method and RT-PCR techniques were applied to assess the impact of silencing. The findings indicated the presence of the intII gene in 10% of the isolates, and the process of joining siRNA and AuNPs was very effective. Phenotypically silencing that was induced by siRNA showed significant differences between transformed (sensitive) and non-transformed (resistant) colonies, but in contrast, gene expression of the intII gene was increased after being treated with siRNA. The study concluded that siRNA-AuNPs could be a potential therapeutic agent for *P. aeruginosa* infections. Based on the findings, treatment with siRNA showed a very high significant difference between transformed (susceptible) and non-transformed colonies (resistance), and the pre-treatment with siRNA dramatically reduced the bacteria load.

Keywords: siRNA, silencing, Integron, Antibiotic resistance, *P. aeruginosa*.

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Introduction

Integrons are distinct genetic components that have the capacity to group and express drug resistance genes. They consist of several "cassettes" that each include genes for antibiotic resistance. It significantly contributes to bacterial multi-antibiotic resistance development. As a result, bacteria can adapt and survive in the presence of antibiotics. They can capture and express resistance genes (1,2). Integrons play a major role for gram-negative bacteria like *P. aeruginosa*, which are known for having the ability to become resistant to antibiotics. They play an important role in the spread of antibiotic resistance traits across a variety of clinical isolates with gram-negative bacteria. So, they actively contribute to the evolution of antibiotic resistance in bacterial populations (3,4). Due to the potential clinical implications, the relationship between integrons and multidrug resistance (MDR) in *P. aeruginosa* is extremely concerning. MDR *P. aeruginosa* strains pose a significant challenge in healthcare settings, limiting treatment options and increasing the

risk of treatment failure (5). The overuse of broad-spectrum antibiotics in the burn ward and Intensive Care Units (ICUs) by creating selective pressure on bacteria likely led to the emergence of MDR strains (6,7). Research findings indicate that class 1 and 2 integrons are widely present in *P. aeruginosa* isolates obtained from clinical environments across different geographical areas. These integrons have the ability to confer resistance to regularly prescribed therapeutic medications, including β-lactamases, fluoroquinolones, and even carbapenems (8). The emergence of *P. aeruginosa* has become a significant public health problem. So, attention is required because these resistant strains may show resistance to all available antimicrobials or show susceptibility only to toxic ones such as colistin or polymyxins leaving no choices for the healthcare team in the treatment of severe infections associated with MDR *P. aeruginosa* (9,10). Gene silencing by siRNA (small interfering RNA) is a powerful technique used to downregulate gene expression, RNA molecules that can specifically target and bind to complementary mRNA sequences. This binding triggers the degradation of the targeted mRNA, resulting in reduced or silenced gene expression (11). Gene silencing by siRNA has been investigated as a potential therapeutic approach for various disorders, including cancers and infections. In the context of antibiotic-resistant bacteria, siRNA-mediated gene silencing could be explored as a strategy to downregulate antibiotic resistance genes or genes involved in virulence, potentially restoring the effectiveness of antibiotics or reducing the pathogenicity of bacteria (12,13). By reducing the expression of antibiotic-resistance genes or genes involved in bacterial virulence factors by gene silence technology, we can attenuate the pathogenicity of bacteria and potentially enhance the effectiveness of host immune responses and sensitivity to antibiotics (14). The use of siRNA to induce gene silencing in bacterial cells, particularly in the context of antibiotic resistance, is still an area of active research. This study aimed to identify intII genes and induce silencing in this gene by siRNA molecules specifically targeting the intII gene in *P. aeruginosa* and demonstrating a silencing effect on antibiotic resistance.

Materials and methods

DNA extraction and Amplification

The investigation encompassed a collective of 30 isolates of *P. aeruginosa* that were obtained from clinical sources. The DNA has been isolated from the isolates using the utilization of a commercially available extraction kit. The Presto™ Mini gDNA Bacteria Kit from Taiwan was utilized in accordance with the instructions provided by the manufacturer. The designed primer by Primer3Plu (https://www.primer3plus.com/index.html) used in this work was obtained from Macrogene Company in Korea (Table 1) was used in accordance with their manufacturer's instructions to identify the intII gene of *P. aeruginosa* 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). The estimation of the PCR reaction mixture is presented in Table (2). The gradient thermocycler in Table (3) was run using the optimal PCR program.

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)
intII	F	GATTTTGATAATGGCTGCATCA	433
	R	GCCCTAAGAGTTCTTGCACAGT	
intII (RT-PCR)	F	CGCCTAATCCCAGCAATAAA	159
	R	GCTGGAGGGAAAGACAAAC	
recA	F	GCGGTGAAAGAAGGTGATGA	120
	R	GTAGATGCCCTTGCCGTAAA	

*** bp = base pair, F = forward sequence, R = reverse sequence.**

Table (2): PCR component calculation.

Components	Volume in 1 sample(µl)
Master Mix	10µl
Nuclease Free Water	5µl
DNA	3µl
Forward primer	1µl
Reverse primer	1µl
Total volume	20µl

Table (3): Program of PCR for detection intII gene in *P. aeruginosa*

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	30
Extension	72	00:30	30
Final extension	72	07:00	1

Conjugated siRNA to gold nanoparticles and introduced to bacteria cell

The siRNA sequence was designed by this study against intII synthesized by Macrogene Company (Korea). siRNA constructs were administered to intII using Gold Nanoparticles (AuNPs) manufactured by Vira Carbon Nano Materials (VCN) Co. Ltd., Iran. The conjugation process was accomplished in a salt solution. The siRNA demonstrated a length of 32 bp. The provided siRNA sequence was 5'-AACAGTCCATTTTTAAATTCTATA CGCACGGA -3'. Conjugated gold nanoparticles to designed siRNAII to silencing *P. aeruginosa* intII gene involved preparation different concentration from sodium chloride (NaCl) to increase the efficiency of conjugation, the concentration including (3, 2, 1.5, 1, and 0.5 M). Briefly procedure outlined below:

3ml of gold nanoparticles (100 ppm) mixed with 2 ml of 3M NaCl and incubated in 60°C for 30 min.

1. Then add 20 µl from siRNA that was prepared by dissolved in a nuclease free water to give a final concentration of 100 picomol/µl and return it back to shaker incubation for 10 min.
2. Then add 500 µl from residual concentrations of NaCl every 30 min and keeping them under the same incubation condition.
3. Silencing of intII gene by conjugated siRNA was obtain by add 200 µl of 0.5 McFarland bacterial suspensions to conjugated salt solution and incubated in shaker-incubated at 37°C for 24 h.

Replica plate method

This approach involves the transfer of bacterial colonies from one plate to another while preserving the original arrangement of colonies, to

monitoring the cells in a silencing suspension that subjected to silencing. The identification of these cells can be achieved through transfer each single colony appear on cetrimide agar plate (bacteria cultured after silencing) to three MH agar plate (one MH agar plate free from antibiotic consider master plates and other MH agar plates with different type of antibiotic) selected antibiotic was add to media in the form as solution with different concentration depending on the antibiotic type according to CLSI 2020 (15). Type of antibiotic that added depending on the selected isolates for silencing and their antibiotic resistance before silencing illustrated in Table (4). Counting the number of colonies that inhibited on each MH agar plate with antibiotic to determine the silencing frequency.

Table (4): Antibiotics powder and solutions used in this study.

No.	Family of antimicrobial agent	Antibiotics	Con. µg\ml
1	B-lactam combination agent	Piperacillin-tazobactam	100\10
2	Cephems (parenteral including cephalosporin)	Ceftazidime	30
3	Aminoglycosides	Amikacin	30

RNA extraction and real time RT-PCR

RNA was isolated from 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 primer (Table 1). To conduct the experiment, 18 µl of each extracted total RNA sample was transferred to a fresh PCR tube. ProtoScript, containing dNTPs, buffer, and other essentials, was then used. volume of 10µl allocated for each individual sample. Next, 2 µl of MuLV Enzyme and 2 µl of hexamer were added to each sample in the process. The PCR amplification was performed using the following thermal cycling parameters: an initial denaturation step at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 30 seconds. The difference in Ct values between the target gene and the reference housekeeping gene quantified qRT-PCR data. The ΔΔCt approach was used to quantify gene expression levels (fold change) described by (16).

Results and discussion

DNA extraction and intII gene detection

Whole-genome DNA from overnight cultures for 30 clinical isolates of *P. aeruginosa* were extracted efficiently by Presto^{TM}Mini gDNA Bacteria Kit Quick Protocol (Geneaid). PCR was performed on extracted DNAs to detect intII gene (433 bp) that was found in three isolates only (P.A.67, P.A68, P.A69) from a total of 30 isolates of *P. aeruginosa* isolates with percentage (10%), all of the genes were detected from burn infections rather than other infections (Table 5). Integrons are a section of dsDNA that are involved in bacterial evolution and versatility. Class I resistance integrons are the most prevalent, followed by class II and class III. Integrons have received attention recently for their potential contribution to the transmission and spread of resistance factors (17,18). Nearly identical findings to our investigation of *P. aeruginosa* isolates showed in, only five (3.81%) contained a class 2 integron(19). In one study the prevalence of class 2 integrons was reported at 29% of *P. aeruginosa*

isolates were obtained from clinical specimens such as wounds, blood, urine, and other samples (20). In another study by Abd-Elmonsef and Maxwell Class 2 integrons were not detected (21). Therefore, this percentage of intII gene consider normal when compared to these studies.

Table (5): Frequency and percentage of Integron-positive samples for *P. aeruginosa* isolates based on clinical samples

Clinical sample	No. of samples	intII positive No. (%)	intII negative No. (%)
Burn	11	3 (27.27%)	8 (72.72%)
Wound	7	0	7 (100%)
UTI	3	0	3 (100%)
Blood	2	0	2 (100%)
Ear Infection	4	0	4 (100%)
Sputum	3	0	3 (100%)
Total	30	3 (10%)	27 (90%)

Detection the conjugated of gold nanoparticles to siRNA by gel electrophoresis.

siRNA-gold nanoparticles conjugates were investigated in detail by gel electrophoresis experiment. Gels with conjugated siRNA-AuNPs appeared as fluorescent band in gel wells because the heavy weight of nanoparticles remine in the well, while unconjugated gold nanoparticles were migrated in opposite direction to normal DNA migration because there different to normal DNA charge, while free siRNA with very small size cannot be recognized because their fast migration through the gel, therefore only conjugated siRNA-AuNPs can be detected as fluorescent band when compared to DNA ladder that was used as identifier for normal migration process (Figure 1).

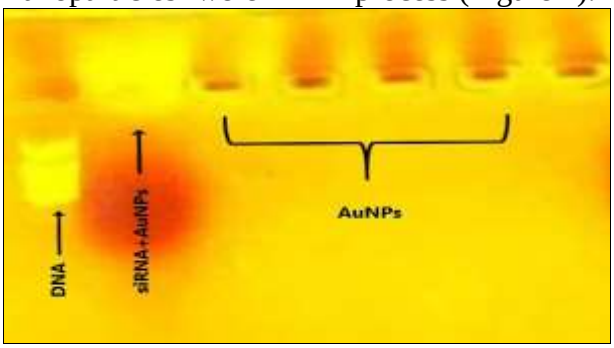

Figure (1): Agarose gel electrophoresis of siRNA-AuNPs conjugates on 1.5% agarose gels stained with red safe stain and run for one hour at 100 V

Frequency of the transformed *Pseudomonas aeruginosa* silenced by siRNA.

The results of silencing intII gene by siRNA showed the disappearance of colonies when incubated in presence of antibiotics (Figure 2). Where the P.A 67 isolate showed 100 %, 10 % and 6 % of colonies transformed from resistance to sensitive for Amikacin, Ceftazidime and Piperacillin\ tazobactam respectively. While P.A 68 isolate showed transformation of 100 %, 4 % and 10 % to the same antibiotics. P.A69 isolate showed higher resistance percentage compared to the other, where the frequency of transformed colony on plate with Amikacin and Ceftazidime was less than what appeared on the P.A67 and P.A68 isolates, where the results showed the transformation to

sensitive were 36% for Amikacin and 24% for Ceftazidime, in accordance with the Table (6). This phenotype alteration was transient, and the silencing forfeited upon subculturing. Results of this study showed there is a very high significant difference (*P<0.001****) between transformed (susceptible) and non-transformed colony (resistance). Also, we note that Pre-treatment with siRNA dramatically reduced the load of bacteria, where all the colonies showed poor growth on antibiotic plates, this can be attributed to the effect of siRNA on bacteria cells. These results similar to those obtained before by (12). This can be a spot light on using siRNA to prevent or eliminate bacterial infections.

Effect of siRNA treatment on the expression of intII gene.

With the exception of isolate P.A68, which had poor growth after period of storage therefore give ineffective RNA extraction, the present investigation demonstrated that the amount of RNA that extracted from isolates (P.A67 and P.A69) before and after treatment with siRNAII-AuNPs was efficient. The main purpose of this step is to measure the gene expression of the intII genes, before and after treatment of *P. aeruginosa* isolates with siRNA-AuNPs in order to prove the role of siRNA in the silencing process of the intII gene. The assay was performed four times for each sample and the average of the values taken into account as the amount of a certain gene expression for that sample. The results indicated there was an increase in the expression of intII gene of isolate (P.A67, P.A69) after treated with siRNA-AuNPs as shown in Tables (7). Results showed significant differences ($p<0.050$) among levels of intII gene within bacterial isolates and controls. The bacterial isolates P.A67 and P.A69 scored highest levels (5.990 ±2.378 and 4.912 ±2.378) than controls (1.000 ±0.000) (Figure 3).

The application of siRNA for the purpose of inducing gene silencing in bacterial cells is a relatively new field of research, requiring more study to fully understood the underlying mechanisms. Nevertheless, several investigations have indicated that siRNA could unexpectedly enhance gene expression in bacterial cells, in contrast to its original purpose of downregulation (22, 23, 24). The underlying causes of this phenomenon remain not much understood, either due to off-target impacts of the siRNA or the existence of undiscovered mechanisms in bacterial cells for RNA processing (23). Off-target effects develop as a consequence of the binding of siRNA to mRNA sequences that were not intended, hence resulting in the unexpected suppression of genes. The increased stability of mRNA may enhance the probability of off-target consequences (25). Also, the accessibility of the target site for siRNA binding can be influenced by the stability of mRNA. The efficacy of gene silencing may be reduced if the stability of mRNA is high, since it can provide challenges for siRNA to effectively access and bind to the intended target site. The process of gene silencing produced by siRNA involves the breakdown of the mRNA molecule that corresponds to the target gene. If the mRNA exhibits a high level of stability and resistance to degradation, it has the potential to remain stable for extended durations, hence decreasing the efficacy of silencing by siRNA mechanisms(22).

Therefore, degree of accessibility, target site, and specific sequence of siRNA are additional influential factors that contribute significantly to the efficacy of gene silencing. It is imperative to acknowledge that not all investigations have reported upregulation in gene expression following siRNA treatment in bacterial cells. As an illustration, a particular study that applied the siRNA techniques to induce silencing showed the high efficacy of siRNA in suppressing the expression of coagulase gene in methicillin resistant *Staphylococcus aureus* within an in *vitro* setting. (12). Furthermore, another study employed siRNA to suppress the activity of genes implicated in the process of invasion by pathogenic bacteria. (24). In general, the use of siRNA for inducing gene silencing in bacterial cells continues to remain under active investigation, requires additional study to fully understand the underlying mechanisms and evaluate the potential for unknown effect.

Table (6): Frequency of transformation on Replica plate after silencing intII gene

Isolate No.	Master plate	Plate with Amikacin			Plate with Ceftazidime			Plate with Piperacillin\ tazobactam		
	Colonies	Resistance	Sensitive	Frequency of transformation	Resistance	Sensitive	Frequency of transformation	Resistance	Sensitive	Frequency of transformation
P.A 67	50	0	50	100 %	45	5	10 %	47	3	6%
P.A 68	50	0	50	100 %	48	2	4%	50	5	10%
P.A 69	50	32	18	36 %	38	12	24 %			
P value		***P<0.001*****			***P<0.001*****			***P<0.001*****		

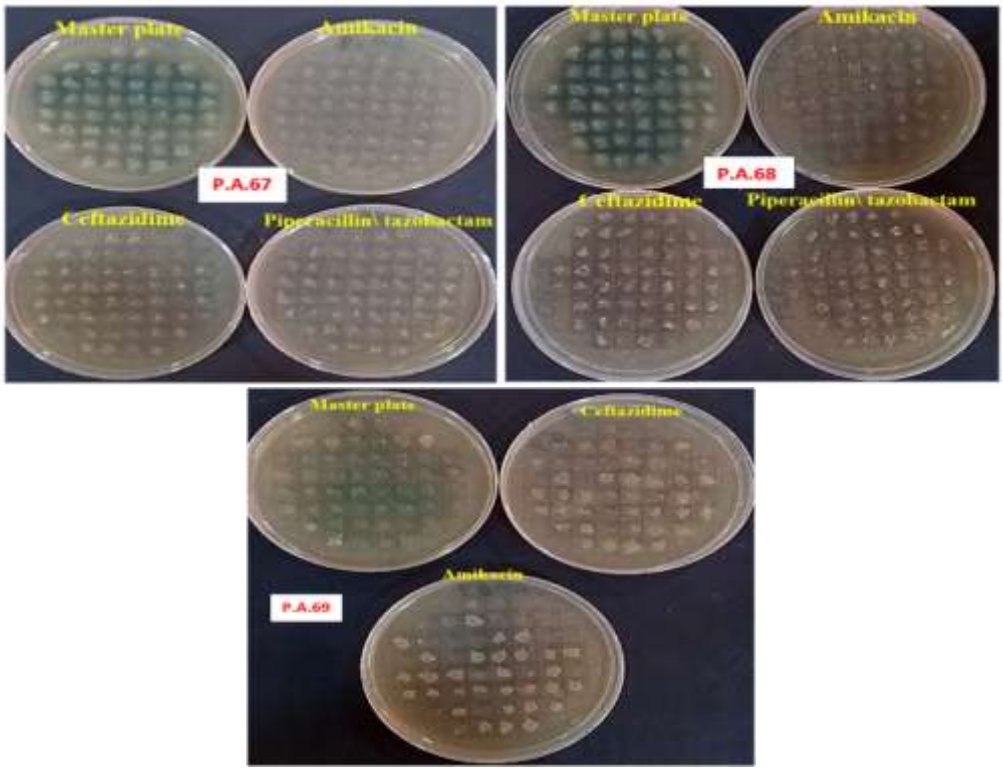

Figure (2): Replica plate method of *P. aeruginosa* isolates after silencing intII gene

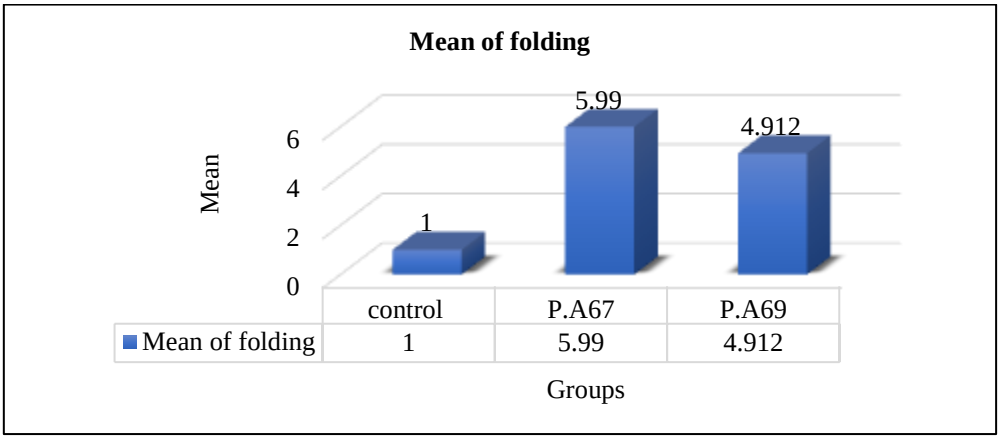

Figure (3): Mean of fold expression change of intII gene.

Table (7): Gene expression of integron class II.

	N	Mean	Std. Deviation
Control	4	1.000 [a]	0.000
67	4	5.990 [b]	2.378
69	4	4.912 [b]	2.378

Small different letters refer to significant different (p<0.05).

Conclusion

The study concluded that siRNA-AuNPs could be a potential therapeutic agent for *P. aeruginosa* infections. Based on the findings where treatment with siRNA showed a very high significant difference between transformed (susceptible) and non-transformed colonies (resistance), and the pre-treatment with siRNA dramatically reduced the bacteria load. Also, the results showed that the treatment with siRNA led to upregulated expression of intII gene in comparison to the control. However, the bacterial isolates P.A67 and P.A69 scored the highest levels than controls. Further research is needed to explore the efficacy and safety of siRNA-AuNPs in clinical settings.

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