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Sirna-Mediated Gene Silencing of Efflux Pump Genes in Multidrug-Resistant Pseudomonas Aeruginosa Isolated from Iraqi Patients

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Abstract

Background: *Pseudomonas aeruginosa* is an important opportunistic bacterium responsible for multidrug-resistant infections in hospitalised patients. The *Mex*AB-*Opr*M efflux pump system is a major contributor to antibiotic resistance by the expulsion of antimicrobial drugs from bacterial cells. Therefore, siRNA-mediated gene silencing is being developed as a promising method for resistance-related gene silencing and improving therapeutic efficacy.

Objective: To explore the prevalence and antibiotic resistance profile of Pseudomonas aeruginosa infection clinical isolates and assess the silencing of efflux pump genes (mexB and oprM) that are linked to multidrug resistance and virulence by siRNA.

Methods: Twenty-nine P. aeruginosa strains were recovered from 200 clinical specimens. The identification was achieved by morphological characteristics, biochemical tests, and with the aid of the Vitek 2 Compact system. The antimicrobial susceptibility was tested using 13 antibiotics by the Kirby-Bauer disk diffusion method. Production of AmpC β-lactamase was phenotypically determined, and efflux pump activity was determined by the ethidium bromide cartwheel method. The genes, mexB and oprM, were detected by conventional PCR. A 21-bp siRNA duplex was designed that targets these efflux pump genes, and quantitative RT-PCR was used to assess the level of gene expression prior to and following siRNA treatment.

Results: The highest level of antibiotic resistance was seen with ticarcillin-clavulanate and gentamicin (89.7%), and the lowest was with piperacillin-tazobactam (24.1%) of the 29 isolates. Sixty-seven percent (12/12) of the isolates produced AmpC β-lactamase. None of the 9 (45%) isolates that were found to be positive by phenotypic testing for efflux pump activity were characterized as multidrug resistant. The 9 isolates (45%) identified as efflux pump positive, none were classified as MDR. All the selected isolates were positive for the mexB and oprM genes by conventional PCR. After siRNA transfection, qPCR showed a dramatic down-regulation of the expression of both genes when compared to the control. The fold change of mexB was found to be 2.107 to 0.0006, and that of oprM was 1.521 to 0.0032, respectively, which means the gene silencing was effective.

Conclusion: This study shows the high prevalence of antibiotic resistance and virulence mechanisms associated with efflux pumps in clinical isolates of P. aeruginosa. siRNA-mediated knockdown of mRNA expression of mexB and oprM genes was found to be highly efficient, suggesting that

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siRNA-based therapy could be an effective approach for the treatment of multidrug-resistant P. aeruginosa infections.

Keywords: Pseudomonas aeruginosa, Antibiotic susceptibility test, AmpC, Efflux pump, siRNA, RT-PCR.

1. Introduction

The bacterium *Pseudomonas aeruginosa* is gram-negative with a bacillus shape and a size ranging from 0.5 to 3.0 μm [1], [2]. Natural habitats like water and soil aren't the only places *P. aeruginosa* thrives; it also does well in hospitals, ventilators, and other artificial situations [3]. A variety of diseases, including those of the respiratory system, burns, and wounds, can be caused by the bacteria *Pseudomonas aeruginosa* [4]. Can grow at several temperatures; however, it grows best at 37°C [5], [6]. *P. aeruginosa's* phenotypic characteristics, such as its distinct odor, β-hemolysis, and colour of the colonies [7]. A variety of extracellular pigments are produced by *P.aeruginosa*, including as pyoverdine, pyocyanin, pyorubin, and pyomelanin. These pigments range in colour from yellow-green to brown-black [8].

The mortality rate for *P. aeruginosa* infections caused by multidrug-resistant strains (44.6%) is nearly twice as high as the mortality rate for non-multidrug-resistant strains (24.8%) [9]. The majority of these resistance mechanisms are either genetically or intrinsic chromosomally in *P. aeruginosa* [10]. Intrinsic antibiotic resistance; β-lactamase production, efflux systems, and antibiotic-inactivating enzymes are some of the processes that have been shown to contribute to *Pseudomonas* drug resistance [11].

Pseudomonas aeruginosa's antimicrobial resistance is largely due to its bacterial multidrug efflux pumps, of which six have been determined: *Mex*CD-*Opr*J, *Mex*EF-*Opr*N, *Mex*XY-*Opr*M *Mex*AB-*Opr*M, *Mex*JK-*Opr*M, and *Mex*VW-*Opr*M. The constitutively produced *Mex*AB-*Opr*M efflux system is particularly interesting since it contributes to the organism's well-known natural resistance to various antimicrobials [12], [13]. The Intrinsic and mutational multidrug resistance are both centered on *Mex*AB-*Opr*M [14].

One effective treatment strategy is the siRNA gene silencing because it spares bacteria from the selection pressure that is frequently linked to the emergence of resistance. This tool emphasizes the proof of principle for its application and positions it as a significant supplement to traditional treatment choices for infections caused by *P.aeruginosa*[15]. Therapeutic applications of small interfering RNAs (siRNAs) have significant potential in cancer treatment, neurological disorders, bacterial and viral infection, and inherited diseases [16].

This study investigates the ability of siRNA to target and suppress antibiotic resistance-related gene expression and pathogenicity in *Pseudomonas aeruginosa*. Specifically, it focuses on the *mex*B and *opr*M genes, which participate significantly in efflux, the pump system of the *Mex*AB-*Opr*M. One of the major contributors to MDR is the efflux system containing *Mex*AB and *Opr*M, which is responsible for the active export of many antibiotics from bacterial cells. This study aims to reduce antimicrobial resistance and pathogenicity by inhibiting the expression of these efflux pump genes.

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2. Methods section

2.1 Bacterial isolation and identification

Pseudomonas aeruginosa of samples (200) isolated from October 2025 to December 2025 clinical Samples procured from Baquba hospital (Baquba Teaching Hospital/Al-Batoul Teaching Hospital). Morphological and biochemical characteristics, such as oxidase, Catalase, triple Sugar Iron, and Indole test, were employed to identify *P. aeruginosa*. The identification of *P. aeruginosa* was verified using the Vitek-2 compact system (Biomerieux, France).

2.2 Antimicrobial susceptibility test

Antimicrobial susceptibility testing followed the guidelines established by the European Committee for Antimicrobial Susceptibility Testing. The antimicrobial agents tested in this study were 13 Antimicrobial agents belonging to 7 different classes. The antibiotic includes Penicillin + β-lactamase suppression Piperacillin/Tazobactam-PIT (100) (Piperacillin-PI (100), and Ticarcillin/Clavulanic-TCC (75)); Fluoroquinolones (Ciprofloxacin-CIP (5), Norfloxacin-NX (10) and Levofloxacin-LE(5); Aminoglycosides (Gentamicin-GEN (10) and Tobramycin-TOB (10)); Carbapenems (Meropenem-MRP (10) and Imipenem-IPM (10)); Cephalosporins (Ceftazidime-CAZ (30), and Cefepime-CPM (30)); and Monobactams (Aztreonam-AT (30)) [17]. After the A 0.5 McFarland bacterial suspension was used. Measuring the optical density with a spectrophotometer. Was a cotton swab utilized to disseminate the bacterial solution, place the antibiotic discs on top of the Muller-Hinton agar, and leave them to incubate. The results were analysed in accordance with the CLSI standards (2025) [18]. Multidrug resistance index (MDRI) of each bacterial isolate was calculated [19], [20].

As: MDRI =Number of antibiotics resistant /Number of antibiotics used

High levels of antibiotic exposure may be contaminating the environment if the MARI value is more than 0.2, whereas low levels of antibiotic usage are indicated by a MARI value of ≤0.2. High levels of antibiotic exposure may be contaminating the environment if the MARI value is more than 0.2, whereas low levels of antibiotic usage are indicated by a MARI value of ≤0.2.

2.3 Phenotypic Detection of β-lactamase production

Detection of AmpC production

Employed for the purpose of detecting inducible AmpC synthesis. An isolated sample was spread out on a Mueller-Hinton agar plate; its turbidity was the 0.5 McFarland standard. Two 30-gram discs of cefotaxime and one 30-gram disc of cefoxitin were spaced 20 millimetres apart from one another. Screening for AmpC-lactamase was performed on isolates that exhibited a blunting of the cefotaxime zone of inhibition next to the cefoxitin disc [21].

2.4 Phenotypic detection of Efflux pump activity

The EtBr-agar cartwheel conducted for the morphological detection of efflux pumps involved preparing dilutions of all bacterial isolates using sterile physiological saline and measuring turbidity with the McFarland Standard instrument. This study was conducted on bacterial isolates exhibiting antibiotic resistance characteristics by employing the cartwheel agar-EtBr method, utilizing tryptic soy agar medium and ethidium bromide dye at varying doses as specified in [22], [8].

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2.5 Polymerase chain reaction (PCR) Amplification

The DNA template was prepared by the Commercial genomic DNA extraction kit (MBEPTECH, China), and Final volume for multiplex PCR mixture was (5µl template DNA,12.5µl of Green Master Mix 2x, 1µl primers for each forward and reverse primer (3 primers), and finally, 5.5µl nuclease- free water), placed in a thermocycler for polymerase chain reaction after being briefly stirred by vortex. The conditions for PCR were as follows: 3 minutes at (95) °C, thirty cycles each of 30 s at (95) °C, 30s at (52,55) °C, 1 min at (72) °C, and a three-minute extension at (72) °C. For complete detection, use ethidium bromide staining and agarose gel electrophoresis (1% agarose, 5v/cm2 for60 min)

2.5.1 Detection of mexB and oprM genes by (PCR)

A polymerase chain reaction was deployed in order to identify the 16S rRNA gene as well as the mexB and opeM genes for 12 P. aeruginosa isolates; the primer used in the current study is illustrated in Table 1.

2.5. 2 Primers Preparation

An initial concentration of 100 picomol/µl was achieved by dissolving and diluting lyophilised oligonucleotide primers in nuclease-free double-distilled water (amount according to manufacturer's recommendation), and then further dilution in the same water was used to reach around 10 picomol/µl. All primers used in this study were subjected to this process, as shown in Table 2. The specifications of all genes provided by Alpha DNA Company, USA

Table 1: The primers applied to identify the genes in the present research.

Name of primers	Sequence (5`-3`)	Annealing Temperature. (°C)	Size (bp)	Reference
mexB	AGGTCCAGGTGCAGAACAAG	52	185	Study design
	CGGGTCCTGGATGTTGGAAA			
oprM	TTCGGGTTCCTGGTTGTTCC	55	191	study design
	GCAACTGCTCGGTGAAGGTA			
16s RNA	TCCTACGGGAGGCAGCAGT	60	448	[23]
	GGACTACCAGGGTATCTAATCCTGT T			

2.5.3 Small interfering RNA (siRNA) designation:

The mexB and oprM genes of the identified P. aeruginosa isolates served as templates for the design of small interfering RNAs (siRNA). Using NCBI BLAST and the bioinformatics tool Gen Script siRNA Target Finder Table, homology screens were performed on siRNA to identify sequence homologous genes, as shown in Table 2.

Table 2: Designed nucleotide sequences of small interfering RNAs (siRNAs) used in this study.

siRNA	Sequences (5^*—3^*)
mexB	GGUGCAGAACAAGCUGCAACU
	UUGCAGCUUGUUCUGCACCUG
oprM	GGCGCGAGUUCUUCCGCGACC
	UCGCGGAAGAACUCGCGCCAG

The study's experimental design and stages for quenching the efflux pump of *Pseudomonas aeruginosa* production in *vitro* by using the siRNA gene-silencing technique.

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The experiment was conducted on the efflux pump by *P.aeruginosa*. These isolates were determined by the EtBr-CW test protocol as previously described.

2.5.4 Preparation of the Bacterial Suspension

The bacteria were cultured overnight in a suitable growth medium to obtain active cells. Subsequently, a bacterial suspension equivalent to 1×10^6 McFarland standard was prepared. The siRNA solution was then prepared by adding nuclease-free distilled water into an Eppendorf PCR tube, followed by gentle mixing using a vortex mixer to ensure proper dissolution. The bacterial suspension was exposed to the siRNA by adding 10 µL of siRNA at 20-minute intervals over a period of 4 hours. After the exposure period, the bacterial culture was further incubated for an additional 4 hours to allow for effective gene silencing.

2.6 Real time-PCR Screening Test

Two of the P. aeruginosa isolates were selected for the siRNA experiment, as Pa8 (pa26). Analysis was applied before and after treating isolates within the quenching design experiment, in addition to the 16S rRNA housekeeping gene. Implemented P.aeruginosa 2 isolates with siRNA (treated (T) and untreated control (C))

The TRIzol Reagent was used to extract RNA from the sample in accordance with the manufacturer's instructions. The total RNA was then subjected to reverse transcription using complementary DNA (cDNA) through primer (Table 1). To conduct the experiment, 10 µl of each extracted total RNA sample was transferred to a fresh PCR tube. ProtoScript, containing dNTPs, buffer, and other essentials, were then used. Volume of 4µl allocated for each individual sample. Next, by adding 6µl of nuclease-free distilled water, the volume was brought up to 20µl.The reaction mixture was subjected to reverse transcription under the following conditions: 25°C for 10 minutes, followed by 42°C for 15 minutes, and enzyme inactivation at 85°C for 5 seconds.

The PCR amplification was done with these temperature settings: denaturation at 95°C for 30 seconds, annealing at 52°C, 55°C, or 60°C for 30 seconds, and extension at 72°C for 30 seconds. Every cycle was 45 to 50 seconds long. Difference in Ct values between target genes and the reference housekeeping gene quantified qRT-PCR data. The ΔΔCt approach was used to quantify gene expression levels (fold change) described by [24].

2.7 Statistical analyses

Correlation was investigated and the findings were displayed using the R studio ggplot2 program. Stack charts are created through the use of R Studio's Chart Builder and the ggplot2 package [25]. Analysis of Gene Expression using Livak Method.

3. Results and Discussion Section

3.1 Isolation and Identification of P. aeruginosa

From a total of 200 samples from wounds, sputum, and burns, 159 (79.5%) showed positive growth, while 41 (20.5%) showed negative growth in the culture medium, When Pseudomonas aeruginosa isolates were cultured on MacConkey agar, they appeared as pale communities whose aroma is reminiscent of grapes , they can't break down lactose. Finally, the diagnosis was confirmed using the VITEK2 compact system, with 29 isolates (14.5%) belonging to Pseudomonas aeruginosa bacteria,

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disseminate among wounds, sputum, and burns. The proportion of bacteria isolated from wounds was 44.8%, close to the percentage obtained by researcher [26], where the percentage of isolates from wounds was 42.1%. The percentage of isolates from sputum was 34.5%, close to the percentage obtained by researcher [27], where the percentage of isolates from sputum the percentage obtained by researcher 33,33%, and the percentage of burns was 20,7%, which is similar to [28], where the percentage of burns isolates was 21%.

3.2 Antimicrobial susceptibility test

The following antimicrobials were shown to have a high rate of resistance among P. aeruginosa isolates: Ticarcillin-clavulanate and Gentamicin (89.7%) Piperacillin, Aztreonam and Tobramycin (44.8%), followed by Cefepime (37.9), Ceftazidime, Imipenem, Ciprofloxacin and Meropenem (34.5%), In contrast, the rates of resistance were lower to Levofloxacin and Norfloxacin (31%) and the lowest resistance was to Piperacillin-Tazobactam (24.1%), as shown in Fig. 1.

Pseudomonas aeruginosa is resistant to beta-lactam antibiotics because it produces β-lactamase enzymes, which deconstruct the β-lactam ring. It also has efflux pumps and can alter the outer membrane's permeability. Moreover, it is resistant to anti-aminoglycosides because it produces enzyme modifiers like phosphotransferase and N-acetyl transferase, and has resistance genes on its plasmid or chromosomes. While *P.aeruginosa* develops resistance to fluoroquinolones as a result of DNA gyrase mutations or by blocking the enzyme's activity, it inhibits DNA synthesis. This consideration was in agreement with our result and many other studies like [29-33].

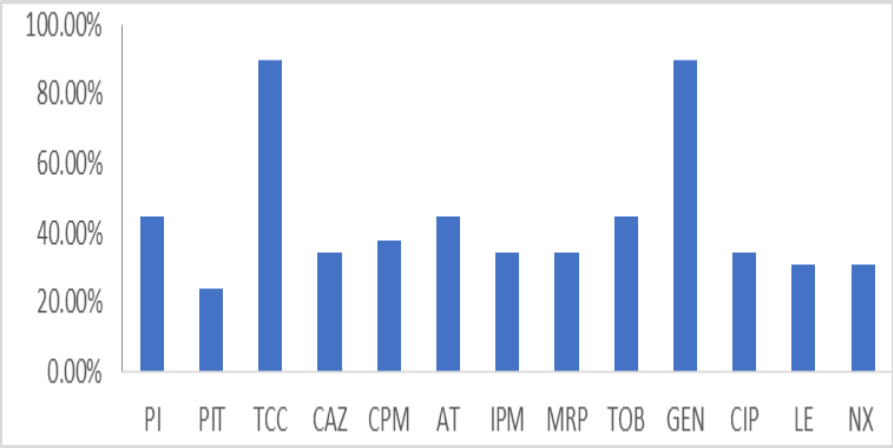

Fig. 1 Antimicrobial susceptibility patterns of P. aeruginosa isolates

The multiple antibiotic resistance index (MDRI) was determined for each isolate based on the antibiotic susceptibility results. The MDRI values ranged from 0.07 to 1, with the number of isolates showing values greater than, equal to, and less than 0.2 (Table 3). The variation in MDRI values observed among the isolates indicates that they originated from environments with different levels of antibiotic exposure. Lower MDRI values (<0.2) suggest limited exposure to antibiotics, while higher values (>0.2) indicate high-risk sources such as hospital settings where antibiotics are frequently used. The presence of borderline values (0.2) reflects moderate exposure and possible early development of resistance. Overall, this variation highlights the diversity of resistance patterns among the isolates.

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Table 3: AMR Index distribution in 29 bacterial isolates showing resistance stratification.

Isolate NO.	Resistant Count out of 13 Antibiotics
1	count:13| index:1
2	count:3| index:0.23
3	count:2|index:0.15
4	count:1|index:0.07
5	count:1|index:0.07
6	count:2| index;0.15
7	count:2|index:0.15
8	count:7| index:0.5
9	count:2| index:0.15
10	count:13| index:1
11	count:1|index:0.07
12	count: 2| index:0.15
13	count:13| index:1
14	count:2| index:0.15
15	count:1| index:0.07
16	count:12| index:0.9
17	count:3|index:0.23
18	count:3|index:0.23
19	count:3| index:0.23
20	count:2| index:0.15
21	count:2|index:0.15
22	count:13|index:1
23	count:13|index:1
24	count:2|index:0.15
25	count:3|index:0.23
26	count:13|index:1
27	count:13|index:1
28	count:10|index:0.76
29	count:10|index:0.76

3.3 Phenotypic Detection of Ambler class C beta-lactamase (AmpC)

The 29 isolates were tested for AmpC enzymes using a disc antagonism test. Those that demonstrated a blunting of the cefotaxime inhibition zone next to the cefoxitin disc or lower resistance to each of those antibiotics were found to be positive for AmpC β-lactamase production. [34]. The results were 12(41.4%) of the isolates had the ability to produce AmpC enzymes, which was lower than 22(19.2%) found by the local study conducted by [35], while 17(58.6%) isolates did not have the ability to produce AmpC enzymes, Fig. 2.

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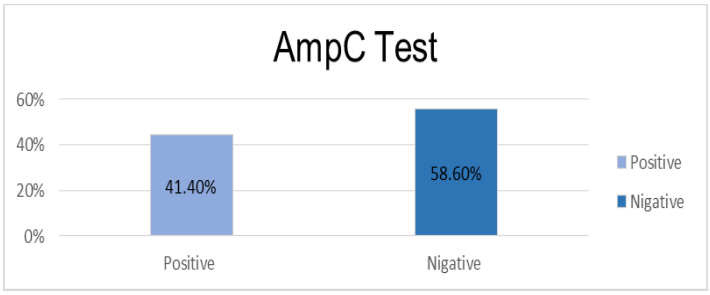

Fig. 2 Distribution of the Pseudomonas aeruginosa isolates (n=29) according to AmpC production.

3.4. Detection of Efflux Pump

P. aeruginosa 20 isolates in this study submitted the ethidium bromide cartwheel method to explain efflux pump activity, with results depending upon the stain concentration as a reference for phenotypic detection. The findings indicate that there are (11) inactive strains (negative for efflux pump activity) across all concentrations of ethidium bromide. On the other hand, there are (2) isolates positive for efflux pump in all concentrations. Five strains show positive efflux pump at the concentration of (1, 1.5, 2, 2.5), and there is (2) isolates that were positive at the concentration of (1.5, 2, 2.5), this result was shown in Table (4) and Fig. (3). The negative result exhibits fluorescence under UV light due to the retention of ethidium bromide within the cells, whereas the positive result does not glow as it cannot retain ethidium bromide. As a percentage our study recorded (11) isolates (55%) were negative for efflux pump activity and (9) isolate (45%) were positive for efflux pump activity this result was disagree with [36] who recorded (36%) of *P. aeruginosa* isolates to have efflux pump activity, and also highly disagreed with study in Egypt that proven all isolates of *P. aeruginosa* were 100% production efflux pump [37], This discrepancy may be due to the difference in the sample size.

Table 4: Results of phenotypic analysis of efflux pumps in P. aeruginosa utilizing varying doses of ethidium bromide dye in tryptic soy agar

No. of isolates	%	Ethidium bromide concentration				
		0.5	1	1.5	2	2.5
2	45%	+	+	+	+	+
2		-	-	+	+	+
5		-	+	+	+	+
11	55%	-	-	-	-	-

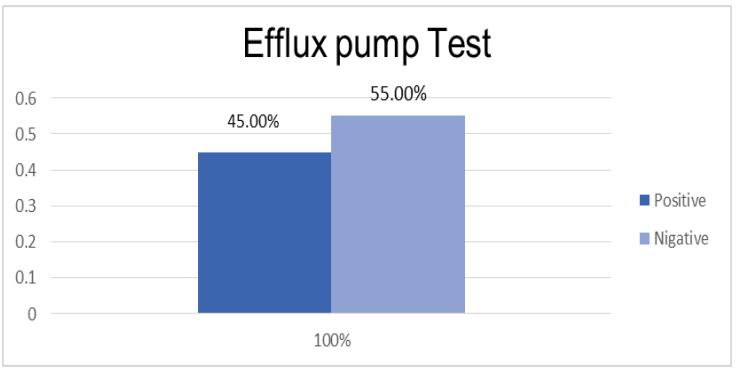

Fig. 3 Efflux pump in the Pseudomonas aeruginosa isolates

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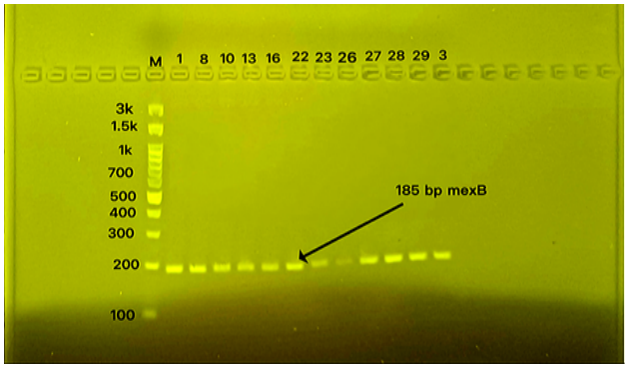
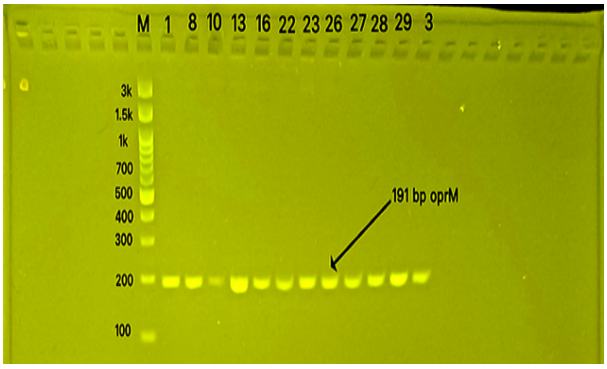
3.5. Detection of mexB and oprM gens by PCR

The polymerase chain reaction (PCR) results for the *oprM* and *MexB* genes in twelve selected strains of *Pseudomonas aeruginosa* revealed the presence of these two genes, responsible for efflux pumps, in all twelve strains. Gene expression levels for both genes were recorded at 100%, indicating a high expression rate in our isolate as displayed in Fig. 4. This high gene expression contributes to increase in the bacteria's ability to resist antibiotics in our isolate and confirms the impact of outflow pumps on the reclassification of clinical strains as susceptible, intermediately susceptible, or resistant, thus supporting the findings of [38]. This isolate, used to detect the *oprM* and *MexB* genes, exhibited higher antibiotic resistance, consistent with the results of [39], [40]. This finding is also highly consistent with those of [41], who reported high resistance of *Pseudomonas aeruginosa* to certain antibiotics.



A: PCR products of *Mex B.*



B: PCR products of *oprM* gene.land 100 pb DNA Ladder; Lanes (12) samples of *P. aeruginosa* isolates (1% agarose gel at 5V/cm^2 for 1 hr)

Fig. 4 Electrophoresis on an agarose gel of PCR results from Pseudomonas aeruginosa isolates.

3.6. Real time PCR

Based on multidrug resistance and efflux pump production ability, two isolates of Pseudomonas aeruginosa were specifically selected. Experiments using small interfering RNA showed that efflux pump activity was successfully suppressed in one of the treated isolates. Real-time PCR screening outcomes for the selected Pseudomonas aeruginosa isolate (ps. 8, ps. 26) before and after siRNA transfection experiment disclosed harmonized results with the phenotypic examination test.

Summarized data of gene expression for all 2 isolates provides variable ranges for each treatment within the fold change calculation. Variability indicates downregulation in gene expression in the treatment group compared to the control group. This means that siRNA has the potential to suppress or "quench" the expression of the targeted gene prospectively involved in the efflux pump in persistent *P.aeruginosa* isolates, as shown in Fig. 5. For isolate (T26), the calculated fold change is approximately 0.0006. This indicates a substantial downregulation in gene expression for the mexB gene within the treatment compared to the control one (Table 5).

In isolates (T8), fold change is present at 4.4229, indicating increased changes in gene expression between the combined treatment and control isolates. In conclusion, treatment for isolate (T26) and (T8) seems to have a stimulatory effect on gene expression. Fold change for the oprM gene isolate (ps. 8, ps. 26) was recorded at (6.4755, 0.0032), which means the treated sample (T8) exhibits a significant upregulation compared to the control sample. Finally, (T26) shows a decrease compared to the control sample, as shown in Table 6.

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Table 5: Ct values and fold of expression for mexB of P. aeruginosa treated with siRNA

Sample	16sRNA	*mexB*	ΔCT	ΔΔCT	Folding		
Ps.8	19.10	15.67	-3.43	1.08	0.475	1.291	1
Ps.26	19.07	13.49	-5.58	-1.08	2.107		
T8	21.11	14.46	-6.65	-2.15	4.4229	2.2118	1.713634634
T26	19.55	25.65	6.10	10.61	0.0006		
Mean	-4.51						

Table 6: Ct values and fold of expression for oprM of P. aeruginosa treated with siRNA

Sample	16sRNA	*oprM*	ΔCT	ΔΔCT	Folding		
Ps.8	19.10	15.06	-4.04	0.61	0.657	1.089	1
Ps.26	19.07	13.82	-5.25	-0.61	1.521		
T8	21.11	13.77	-7.34	-2.70	6.4755	3.2394	2.97400501
T26	19.55	23.20	3.65	8.30	0.0032		
Mean	-4.65						

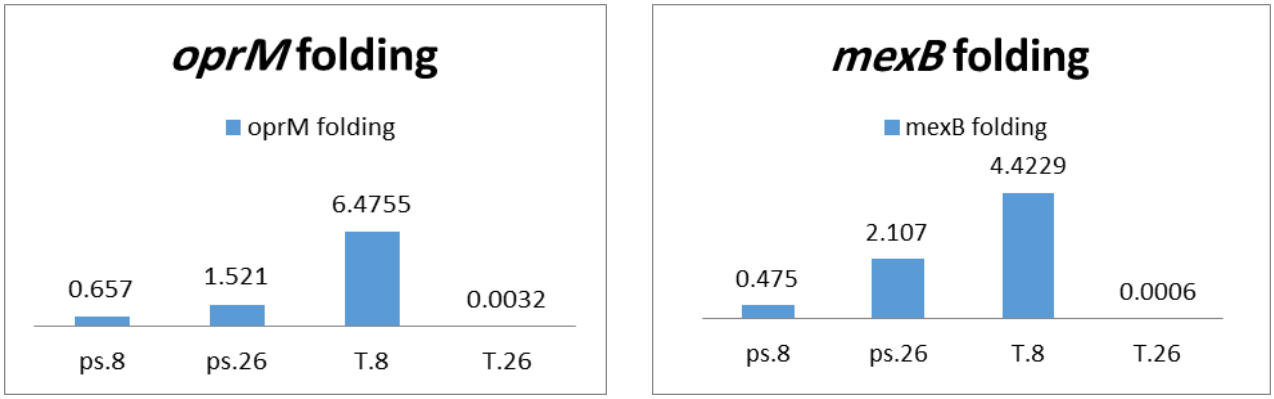

Fig. 5 Fold of expression for oprM and mexB of P. aeruginosa that treated with siRNA

4. Conclusion

The study's characteristics and obtained findings highlight providing evidence of concept for siRNA used and establish it as an excellent addition to conventional treatment options for P.aeruginosa infections. Persister-caused chronic and recurrent infections will continue and probably become more widespread as a result of a rising tide of people lacking enough immune systems and the use of medical devices. New strategies to identify and repress P.aeruginosa resistant protocols have been proposed and recommended. With a better knowledge of how to gene-quench virulence factors repression, gene-silencing with siRNA procedures could be the incoming promised therapeutic dependence protocol.

Conflict of Interest: There is no conflict of Interest.

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