Original Article

Effect of Gold-conjugated Nanoparticle siRNA on Efflux Pump MexB Gene Expression in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is a prevalent nosocomial infection associated with significant mortality rates. Microorganisms, such as *P. aeruginosa*, possess the capacity to acquire a significant level of resistance to many drugs. The MexAB-OprM system, which exhibits significant levels of expression, is considered one of the most prominent efflux pumps associated with multi-drug resistance. **Aim:** The aim of this study was to reduce the gene expression of efflux pump in *P. aeruginosa.* In this study we used gold-conjugated nanoparticle siRNA against MexB gene, then we determined the phenotypic change in efflux pump activity and the level of gene expression after the gene silencing method. **Materials and Methods:** This study examined seven strains of *P. aeruginosa* isolated from patients admitted to various hospitals in Baghdad, which identify by the Vitek compact 2 system; then we have created a 34-bp single-strand siRNA loaded on gold nanoparticles (AuNPLs). The Ethidium Bromide-Cartwheel Test (EtBr-CW) method was used to assess changes in the phenotype of efflux pump activity in response to siRNA. The expression of MexB mRNA was examined using RT-PCR to determine if the siRNA has impacted on efflux pump activity. **Results:** The results of this study demonstrate that the utilization of siRNAs loaded on AuNPLs led to a reduction in phenotypic efflux pump activity in six isolates, with just one isolate exhibiting persistent active (positive) efflux pump activity. The mRNA expression level of the MexB gene exhibited a decrease in fold change $2^{-\Delta\Delta CT}$ in *P. aeruginosa* clinical isolates (67, 49, PDR5, 44, 5, and 66) when examined *in vitro*. The specific fold change values observed were 0.104, 0.031, 0.029, 0.169, 0.747, and 0.005, respectively. **Conclusion:** Since the efflux pump is one of the mechanisms for antibiotic resistance in *P. aeruginosa*, targeting the MexB gene using gold-conjugated nanoparticle siRNA may be a creative way to lessen *P. aeruginosa* resistance to a variety of antibiotics.

Keywords: Efflux pump, Gene expression, *Pseudomonas aeruginosa*, silencing gene, siRNA

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen causing severe infections. Its antibiotic resistance, innate and acquired, poses a major challenge in preventing and treating infections caused by this microorganism.[1-3]

Several mechanisms of antibiotic resistance have been associated with *P. aeruginosa*; one of them is the production of bacterial efflux pumps and porins are linked to innate resistance, as they move substrates from the bacteria's interior to the cell surface.[4-6] The efflux pumps are divided into five families: resistance-nodulation-cell-division (RND) families, major facilitator subfamily (MFS), small multidrug regulator (SMR), energy-dependent ATP-driven pumps are the ATP-binding cassette (ABC) family, and multidrug and toxic compound extrusion (MATE).[7,8]

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MexAB-OprM is one example of the RND type efflux pump. These crucial RND-type efflux pumps, which are constitutively produced in wild-type bacteria, are in charge of the organisms' inherent resistance to the majority of antibiotics.[3,9]

The MexA-MexB-OprM pump, an essential excretory system component, can be inhibited or eliminated. MexA, MexB, OprM, and MexR are encoded in the same operon, with MexR upstream but with a different transcription pattern. MexA connects OprM and MexB, while MexB detects and mediates antibiotic expulsion. MexR is an auto regulator of MexR and a repressor of the MexAB-OprM system.

The MexAB-OprM expression in *P. aeruginosa* strains is growth phase-dependent, peaking in the late log phase or early stationary phase. N-butyryl-L-homoserin lactones increase MexAB-OprM expression, triggering virulence factors and suggesting quorum sensing control.[10,11]

Efflux pump suppression occurs when energy, expression regulator, assembly of its component parts, or outer pores block causing antibiotic efflux.[12,13]

Gene silence in bacteria occurs when a gene is inhibited, preventing translation or transcription, either experimentally to manipulate gene expression for scientific or therapeutic objectives, or spontaneously, affecting gene expression. RNA interference (RNAi) is a novel molecular strategy for gene expression inhibition, initially discovered as an antiviral mechanism in plants but evolved into a potent gene silencing technique in eukaryotes. For prokaryotes, however, such a powerful RNA silencing weapon still needs to be constructed.[14]

Messenger RNA (mRNA) is degraded by siRNA, affecting gene expression regulation; siRNA pathways in bacteria are less understood than eukaryotes. SiRNAs play a crucial role in bacterial cells' defense against foreign RNA molecules, targeting them through complementary base pairing. The RNase III enzyme breaks down the double-stranded RNA route, allowing further degradation by other enzymes.[15]

SiRNAs, 21-23 bp long, are effective exogenous agents for gene manipulation and low toxicity. While promising for antibacterial strategies, few studies have reported success, but one showed successful inhibition of *Staphylocoagulase* gene in *Staphylococcus aureus*.[16]

Nanoparticles are essential in biomedical applications due to their thermal stability, low size, and low toxicity. Gold nanoparticles (AuNPs) are a popular choice for targeted transport, bioimaging, gene delivery, medication administration, and therapeutic applications. Researchers have developed methods to functionalize gold and other nanoparticles using oligonucleotides or modified DNA.[17-19]

The purpose of this study was to create single-strand siRNA targeting the MexB gene in *P. aeruginosa,* analyze efflux pump activity and gene expression, and provide clinical insights on bacterial gene silencing.

Materials and Methods

Bacterial isolates and identification

A total of seven isolates of *P. aeruginosa* were obtained from several hospitals in Baghdad, including Al-Kindi Teaching Hospital, Shaikh Zayed Hospital, and Imam Ali Hospital. The researchers gathered samples from several sources, including the surfaces of burn patients, wound swabs, ear swabs, urinary tract infection (UTI) infections, throat swabs, aspiration fluid, sputum, and urethral swabs. The identification of all bacterial isolates was conducted using morphological techniques, which involved the use of culture media, including cetrimide Agar, as well as biochemical assays including oxidase, catalase, TSI, and Indol.[20-22] The confirmation of the diagnosis for all isolates was conducted using the Vitek-2 compact system, manufactured by Biomerieux in France.

Determination of efflux pump activity by ethidium bromide (EtBr) cartwheel test

The ethidium bromide (EtBr) cartwheel assay was used to test the ability of efflux pumps to get rid of ethidium bromide. To achieve this particular aim, *P. aeruginosa* isolates were cultured in 5 mL of nutrient broth until they attained an optical density (OD) of 0.6 at a wavelength of 600 nm. The OD of the cultures was adjusted using phosphate-buffered saline (PBS) to a value of 0.5 based on the McFarland standard.

On the day of the experiment, tryptic soy agar (TSA) plates with a concentration of 4 mg/L of EtBr were prepared. Bacterial cultures were then swabbed onto these plates and streaked in a radial pattern, starting from the center and extending toward the edges. To shield the plates from exposure to light, they were enveloped in aluminum foil. The EtBr agar plates were incubated overnight at a temperature of 37 °C. Subsequently, they were observed using an appropriate UV light source, such as a UV transilluminator. Isolates exhibiting an absence of fluorescence were indicative of active efflux pump activity, whereas isolates displaying fluorescence were indicative of a lack of efflux pump activity.[23,24]

Detection of the efflux pump genes by conventional PCR

Depending on the manufacturer's instructions, DNA extraction was performed by using a Presto Mini gDNA Bacteria Kit (Geneaid, Taiwan),[25] monoplex PCR assay was performed to amplify efflux pump genes (*MexB)* of *P. aeruginosa* by using EasyTaq PCR SuperMix kit (TransGen, China). Primer stock solution was created using lyophilized primers from (Macrogen, Korea).

Table 1: Primer sequences used in conventional PCR for detection of efflux pump gene *MexB*

Gene	Primer sequence (5'-3')	Annealing temperature °C	Product (size bp)	Reference
Mex B	F: GAAGAACTTCCTCATGGTGGTC R: GAGGGTCTTCACTACCTCATGG	58	634	This study

The specific primers employed in this study are listed in Table 1. The PCR mixture contained 12.5 µL master mix (dNTPs, Taq polymerase MgCl2, and PCR buffer), 1 µL each of forward and reverse primers, 3 µL of DNA and 7.5 µL of free water adjusted to a total volume of 25 µL. After centrifuging, the mixture was transferred to a thermal cycler to start the reaction according to the steps of the suitable program of 32 cycles as the following: for Initial denaturation step 5 min at 95ºC, denaturation step 45 s at 95ºC, Annealing step 45 s at 58 ºC, extension step 45 s at 72 ºC, and Final extension step 5 min at 72 ºC.

The PCR products were examined by agarose gel electrophoresis on 1% agarose stained with ethidium bromide (Himedia, India), and using a DNA ladder of 100 bp as a reference to compare the size of DNA fragments (TransGen, China). Then, agarose gel was visualized using a UV trans illuminator.

siRNA design and synthesis

Single-strand siRNA sequences were designed and synthesized against *MexA* and *MexB* genes in *P. aeruginosa.*

For all siRNAs, the first step is to design siRNA molecules that are specific to the efflux pump target genes to be silenced. In this step, the siRNA sequence must be designed to match the target sequence of the mRNA to be silenced with high specificity and minimize off-target effects. Bioinformatics tools can be used to predict and design effective siRNA sequences. All siRNAs designed for this study by prof. Dr. Ahmed Abduljabbar Suleiman, university of Anbar, college of science, biotechnology department, Iraq.

We synthesized 34 bp single-strand siRNA sequences designed against *MexB gene* 5'-ATAGGCCCA TTTTCGCGTGGGTGATCGCCTTGGT-3'. Once the siRNA sequences were designed, then, siRNA was synthesized by Macrogen, Korea.

Detection of the antibacterial effect of gold nanoparticles against *P. aeruginosa* by using Rezazurin dye.

The determination of the minimum inhibitory concentration (MIC) of gold nanoparticles for the survivability of bacterial isolates was conducted based on the research conducted in reference,[26] with certain modifications. The detection of the antibacterial effect of gold nanoparticles was carried out by microtiter plate using Resazurin dye as follows: overnight cultures of *P. aeruginosa* diluted approximately to 10^4 CFU/ml. Gold nanoparticles with a specific size range of 5–20 nm (Vira carbon nanomaterials, Iran) were diluted 1:2 in nutrient broth of various concentrations (100, 50, 25, 12.5, 6.5, 3, 1.5, 0.75, 0.38, and 0.18 ppm) of gold nanoparticles. Each well containing 100 µl of gold nanoparticle suspension,100 µl of diluted *P. aeruginosa*, and 20 µl of Resazurin dye (Sigma, USA) was added and mixed thoroughly. Each well has a different concentration of gold nanoparticles. The plates were then incubated overnight aerobically at 37 °C. The antibacterial properties of gold nanoparticles were assessed using visual inspection, wherein the vitality of bacteria was examined. The evaluation was conducted by measuring the conversion of resazurin to resorufin, which occurs through a reduction reaction that is, dependent on the metabolic activity of the bacterial cells. In the event that the Resazurin dye retains its blue coloration, this signifies that the nanoparticles have effectively killed the bacteria. Conversely, if the dye assumes a pink color, this shows that the bacteria remains viable and possesses the ability to convert the Resazurin dye into Resorufin.

Loading of siRNA on gold nano partials (AuNPs)

First of all, prepare the mixture solution for silencing gene by Mixing 3 mL of (100ppm)AuNPs with 1 mL of (3M) NaCl, incubated 5 min at room temperature, then Prepare different concentration of NaCl (2, 1.5, 1, and 0.5 M) to be add gradually for the silencing solution. siRNA designed, arrives from the manufacturer in a tube with the required sequence written on it, then we dissolve it with deionized water.

In order to facilitate the binding and loading of the siRNA on to the surface AuNPs, 20 µl of (100 pmol/l) siRNA that previously prepared was combined with 4 mL of a mixed solution, incubated for 4 h at room temperature The conjugation is then facilitated by electrostatic interactions between the negatively charged phosphate groups of siRNA and the positively charged or neutral functional groups on the AuNPs surface. Next, add 200 µl of other concentrations of NaCl after every 20 min starting from the slowest concentration to the highest to the silencing solution.[27]

Confirmation of the association between AuNPs and siRNA was performed by gel electrophoresis, Safe red dye was used instead of ethidium bromide that because it's binds to single-stranded DNA. However, the binding is not as strong as its binding to double-stranded DNA resulting in fewer distortions, which reduces the effectiveness of detection, When using single-stranded DNA, it requires around 10 times more nucleic acid for equivalent detection.[28]

Transfection of siRNA -AuNPs complexes into bacterial cells

Once the AuNPs have been loaded with siRNA, then must optimize the transfection conditions to achieve efficient uptake of the loaded AuNPs with siRNA into bacterial cells.

Overnight cultures of *P. aeruginosa* were cultured with siRNA loaded AuNPs, and then incubated at 37°C in shaker incubator for 4h. Several methods can be used to transfect siRNA molecules into bacteria, and the efficiency of transfection conditions may vary depending on various factors, such as bacterial strain, nanoparticle size, nanoparticle concentration, and incubation time.[29]

Extraction of RNA and RT-qPCR

RNA was extracted using RNA extraction kit *TransZol* Up Plus RNA purification Kit (TransGen, China) in order to determine the expression of *MexB* gene of different *P. aeruginosa* isolated strains following the manufacturer recommendations using quantitative Real Time PCR (qRT-PCR).[30]

Determination of RNA expression levels of efflux pump genes (*MexB*) using quantitative real time PCR (qRT-PCR)

The GoTaq 1-Step RT-qPCR System (Promega, USA) enables detection of RNA expression levels using a one-step RT-qPCR method, combining GoScript Reverse Transcriptase and GoTaq qPCR Master Mix in a single step real-time amplification reaction.

Prepare the reaction mix by combining the 10 µl GoTaq qPCR Master Mix, 0.4µl GoScript RT Mix, 0.4µl forward primers, 0.4µl Reverse primer, and 7.8µl Nuclease free water. primers listed in Table 2. ADd the appropriate volume of reaction mix to each PCR tube or well of an optical-grade PCR plate (*Use the instrument optical settings established for SYBR Green I assay with GoTaq qPCR Master Mix)*), then Add 1µl RNA template for total volume of reaction was 20 µl. the program for real-time PCR was set up with thermo-cycling protocol listed in Table 3.

Gene recA is a housekeeping gene that be used in this study as reference gene. The gene expression was measured by using the ΔΔCT method. The sample's normalized target amount was equal to $2^{-\Delta\Delta CT}$, and this value can be used to compare the samples' expression levels.[31]

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before conducting the study. The study protocol and the subject information and consent form were reviewed and approved by a committee on publication ethics at the Institute of Genetic Engineering and Biotechnology/Baghdad/ Iraq, under the reference No. 359 on 12 Jul 2022.

Results

Determination of efflux pump activity by ethidium bromide (EtBr) cartwheel test

Bacterial cultures were swabbed as described above onto TSA agar plates containing a 4mg/L concentration of EtBr. A fluorescent bacterial mass was detected after incubation, depending on their capacity to efflux EtBr.

Before the *MexB* gene was silenced in this investigation, the findings of an ethidium bromide (EtBr) cartwheel test revealed that all seven of the isolated bacteria had active efflux pumps. According to the ability of this efflux pump to expel EtBr out of the bacteria, no fluorescent appeared.

The results of the *MexB* silencing gene experiment demonstrated that six isolates [66, 67, 49, 44,5 and PDR5 (5p)] had a decrease in the phenotypic activity of the efflux

Table 2: Primers that can be used in qRT-PCR

Target genes	Primer sequence (5'–3')	Annealing temperature °C	Product (size bp)	Reference for this study
Mex B	F:TGTTCCTGGTGATGTACCTGTT R:GAACATGGTCAGGGTGTTGAT	58	137	
recA	F:GCGGTGAAAGAAGGTGATGA R:GTAGATGCCCTTGCCGTAAA	58	120	

Table 3: Real-time PCR program

Steps	Cycles	Temperature	Time
Reverse transcription	1	37°C	15 min
Reverse transcriptase inactivation and GoTaq DNA polymerase activation		95°C	10 min
Denaturation	40	95°C	10 s
Annealing and data collection		60°C	30 s
Extension		72°C	30 s

pump, resulting in a decrease in the outflow of ethidium bromide from the bacteria, and isolate No. 18 was the only one that continued active (positive) efflux pump activity even after being treated with siRNA molecules loaded on AuNPs, as shown in Figure 1.

Detection of the efflux pump genes by conventional PCR

The DNA extracted from *P. aeruginosa* isolates have been used to detect the presence of *MexB* gene encoding efflux pumps and investigation of this gene in bacterial isolates by PCR technique for each DNA. The amplified product were 634bp have been confirmed by analysis of the band on gel electrophoresis. In this study the results revealed that *MexB* was detected in all seven *P. aeruginosa* isolates isolates, as shown in Figure 2.

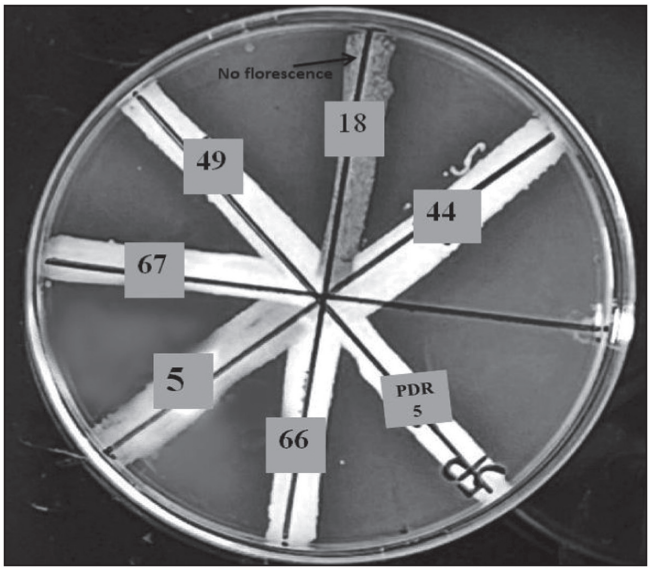

Figure 1: Determination of efflux pump activity by ethidium bromide–cartwheel method, *Pseudomonas aeruginosa* isolates treated with nanoparticles loaded with siRNA molecules were swabbed in TSA agar plates containing a 4mg/L of EtBr after overnight incubation at 37°C for 16h to determine efflux pump activity following *MexB* silencing gene experiment. Fluorescence was detected under UV light, isolates No.(66,67,49,44,5 and PDR5 (5p)) showed no efflux pump activity (presence of fluorescence)

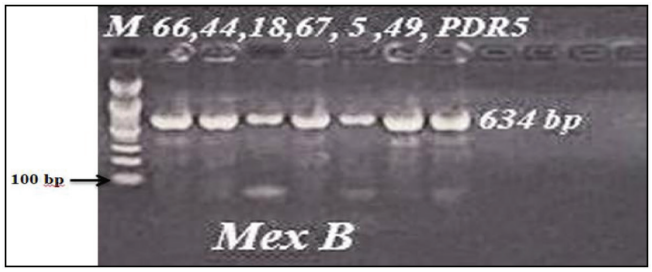

Figure 2: Detection of Efflux pump gene *MexB* of *Pseudomonas aeruginosa* isolates by 1% agarose gel electrophoresis of PCR amplified product in momoplex pattern at 70V for 1h. M represented marker (DNA ladder 100bp). *Pseudomonas aeruginosa* Isolate numbers of (66,44,18,67,5,49,PDR5) indicate the presence of *MexB* gene (634bp)

Detection the antibacterial effect of gold nanoparticles against *Pseudomonas aeruginosa* by using Rezazurin dye

Choose the appropriate concentration of AuNPLs that will use to deliver the siRNA in to bacteria was performed by Rezazurin dye which determine the antibacterial effect of AuNPLs.

our results showed that AuNPLs do not have any antibacterial activity as the evidence the color of Rezazurin dye changed to pink in all concentration of AuNPLs, as shown in Figure 3.

Detection the loading of siRNA on AuNPs

Confirmation of the association between AuNPs and siRNA was performed by gel electrophoresis, Safe red dye was used instead of ethidium bromide that because it's binds to single-stranded DNA more efficiently. Our results are shown in Figure 4.

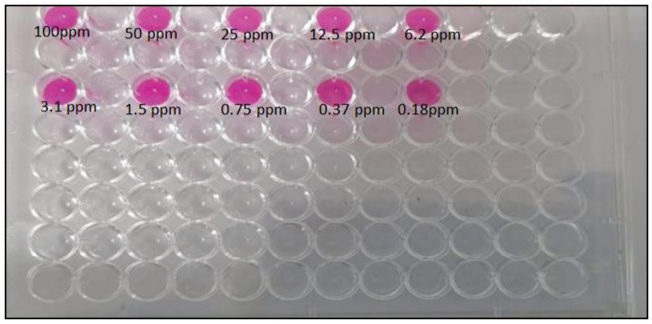

Figure 3: Determination of antibacterial effect of gold nanoparticles at various concentrations (100, 50, 25, 12.5, 6.5, 3, 1.5, 0.75, 0.38 and 0.18ppm) by using Rezazurin dye

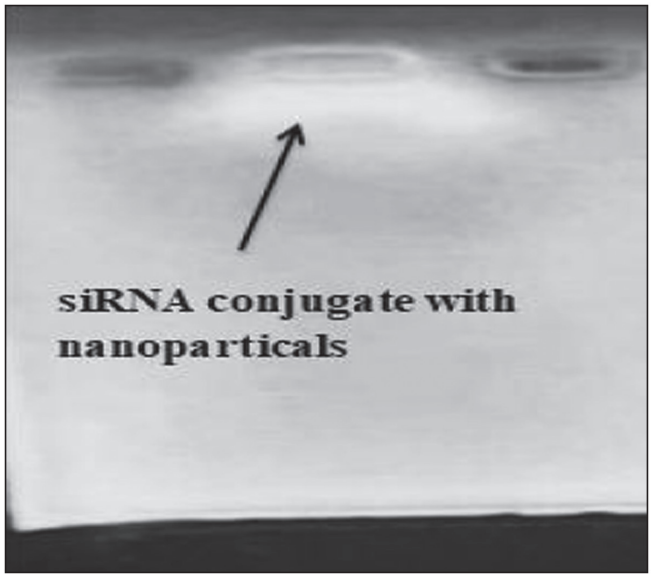

Figure 4: Agarose gel electrophoresis Confirmation of the association between AuNPs and siRNA

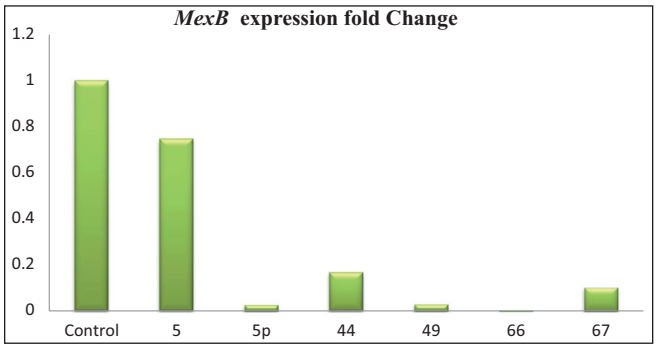

Figure 5: The expression fold change of *Mex B* in selected *Pseudomonas aeruginosa* clinical isolates numbers (5, PDR5 (5p),44,49,66,and 67) after using siRNA conjugated with gold nanoparticles for silenced the gene expression of *MexB* gene of efflux pump

Effect of siRNA loaded on AuNPs on the level of *MexB* gene mRNA expression

According to qRT-PCR results, there was a considerable decrease in *MexB* expression at the mRNA level in that expression fold change $2^{-\Delta\Delta CT}$ were (0.104, 0.031, 0.029, 0.169,0.747and 0.005) in *P. aeruginosa* clinical isolates no. (67,49, PDR5, 44,5 and 66) respectively. Treated with siRNA loaded on AuNPs *in vitro*, When compared to control, the fold changes of expression were down regulation, as shown in Figure 5.

Based on qRT-PCR results, it was determined that there were variations and differences in the levels of gene expression among the isolates used, particularly in isolate No. 5, which displayed a slightly lower level of gene expression after using siRNA loaded on AuNPs compared to other isolates that displayed a huge lower level.

It's critical to note that *P. aeruginosa* clinical isolate number (18) was continued exhibit positive efflux pump activity, as mention before, even after gene silencing of *MexB* gene so, they were Excluded from qRT-PCR.

Discussion

The fast emergence of medication resistance in *P. aeruginosa* has made it a particularly difficult infection to treat while also remaining a common opportunistic pathogen in hospitalized patients.[32]

Numerous other RNDs and other drug family pumps are encoded in the *P. aeruginosa* genome. MexA-MexB-OprM and MexX-MexY-OprM mediate naturally occurring and acquired drug resistance, respectively. The MexA-MexBOprM efflux pump system was the first to be identified as a naturally occurring drug-resistant mechanism for several antibiotics, and it has since grown to be the most extensively researched of the efflux pump systems. The resistance phenotype has been connected to the efflux system.[33,34]

The results of an ethidium bromide (EtBr) cartwheel test revealed that all seven isolated bacteria had functional efflux pumps, Before silenced MexB gene in this investigation. These findings are consistent with numerous investigations, including those in.[35] After employing siRNA against the MexB gene, the efflux pump's phenotype was abolished. This activity was also measured using EtBr-cartwheel techniques.

Regarding agreement between phenotypic and PCR gene detection methods Seven (100%) of *P. aeuroginosa* isolates carrying efflux pump genes had phenotypic efflux pump activity. A perfect association between phenotypic and gene presence in bacteria was shown by Khalek *et al.*,[36] supporting our findings. According to qRT-PCR results, MexB expression at the mRNA level decreased in *P. aeruginosa* clinical isolates that were treated with siRNA *in vitro*. Our results are in agreement with many other studies, including,[14] which show that siRNA treatment decreased msrA expression at the mRNA level in aliquots of *S. saprophyticus* samples, and the same results in study presented by Gong *et al.*,[37] which indicated that use of an siRNA to silence the MexB gene expression. The same outcomes were found in Yanagihara *et al.*'s study,[16] which mentions how siRNA can decrease the expression of coagulase mRNA and protein. ADditionally, the isolates employed in the silencing gene experiment varied and had different degrees of gene expression, as seen by our qRT-PCR results. Depending on a study by Yanagihara *et al.*,[16] the amount or level of siRNA that was injected into the bacteria may depend on the permeability of the bacteria that takes in siRNA.

Our research differs slightly from that of Yanagiharal *et al.*,[16] who used a siRNA duplex molecule to efficiently silence the coagulase gene to lower virulence in methicillin-resistant *Staphylococcus aureus.* SiRNAs expressed by plasmid vectors were used in a distinct study by Greenfield, *et al.*[38] In our study, we used single-strand siRNA loaded on AuNPLs for efficient delivery to *P. aeruginosa* to mute *MexB* gene expression. According to our findings, it has been proposed that utilizing a siRNA molecule to mute *MexB* gene expression could be a useful method for lowering drug resistance in *P. aeruginosa* by changing the expression and function of the MexA-MexB-OprM efflux pump. In this study, the *MexB* gene was temporarily silenced by siRNA; however, there was no permanent change in gene expression (knockdown). There is no data on the effectiveness of single-strand siRNA in bacterial silencing. On the other hand, several studies in gene silencing have successfully used siRNA duplexes delivered to bacteria using plasmid vectors to permanently alter gene expression.[39]

These findings demonstrated that Gold-conjugated Nanoparticle siRNA is a potent new molecular inhibitor of efflux pumps, one of the most significant sources of bacterial drug resistance. ADditionally, before attempting gene silencing tactics in bacteria, it is important to

carefully weigh the potential hazards and advantages, as with any genetic modification.

Conclusion

Gold nanoparticles conjugated with siRNA effectively inhibited *MexB* gene expression and efflux pump activity in *P. aeruginosa*. The use of single-strand siRNA sequences for the purpose of targeting MexB-mRNA appears to be a novel approach in the treatment of *P. aeruginosa*, with the potential to mitigate its virulence. Moreover, the results of this study underscore the demonstration of the fundamental concept supporting the use of small interfering RNAs (siRNAs) and position them as a valuable adjunct to conventional treatment strategies for *P. aeruginosa*.

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Nil.

Conflicts of interest

All the authors certify that they have no conflict of interest to disclose in relation to the subject matter or materials discussed in the present study.

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