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Genotyping and siRNA-Mediated Quorum Sensing Inhibition in *Staphylococcus aureus*: Exploring New Approaches for Treating Persistent Infections

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Abstract

Emersion of persistent *Staphylococcus aureus* requires original research and developmental planning. Repression of virulence genes might be a committed therapeutic approach in this regard. Gene-quenching with short interfering RNA (siRNA) is considered an adequate factor strategy. The study demonstrates the potential of siRNA to effectively silence key genes involved in MRSA virulence and antibiotic resistance. As stated, the microbiological routine is approved to confirm bacterial isolates. TD tests were optimized for persistency affirmation. We devised 21-bp siRNA duplexes against staphylococcal protease. RT-PCR was employed to measure levels of agrII and MecA mRNA before and after siRNA treatment. The efficacy of siRNA was determined by using phenotypic, genotypic, and statistical protocols to confirm the results of the designed laboratory experiment for QS factor quenching. **Results** showed that siRNA targeting *agrII* significantly reduced the mRNA levels compared to controls. This reduction in *agrII* expression correlated with a decrease in quorum sensing activity, *agrII* silencing led to a marked decrease in the production of staphyloprotease, as appeared on the modified culture media for protease testing. This indicates that *agrII* is crucial for regulating virulence factors, its diminished virulence, making the infection less severe and easier to manage by the immune system.

In conclusion, outcomes revealed that siRNA can effectively silence key genes involved in *S. aureus* virulence and antibiotic resistance. Targeting *agrII* disrupts quorum sensing and reduces staphyloprotease production, potentially mitigating the pathogenicity of this bacterium. Targeting protease by siRNA presents an incoming delineation for curing persistent methicillin-resistant *Staphylococcus aureus* infections.

Key words; *Staphylococcus aureus,* Antibiotic screening test, persistent, MRSA, siRNA, RT-PCR.

Introduction

Staphylococcus aureus known as Gram-positive, catalase-positive cocci pertinence to a genus of Staphylococcus family Staphylococcaceae, order Bacillales. *S. aureus* uniquely tolerates adverse conditions such as high salt concentration and osmotic stress [1,2]. Among the Coagulase-Positive Staphylococci isolated from clinical materials collected from humans, *S.aureus* is the most important one [3]. Within humans, *S. aureus* correlates with several diseases, from less serious skin problems to very serious infections such as bacteremia and pneumonia. Infections are prevalent in both community-acquired and hospital-acquired settings. However, these bacteria can cause a range of potentially dangerous infections if

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allowed to enter the bloodstream or internal tissues [4].

Bacteriophages, plasmids, pathogenic islands, transposons, and chromosomal cassettes are examples of mobile genetic elements found in the accessory genome, which accounts for around 25% of the *S. aureus* genome and is obtained by horizontal transfer between strains. This region is responsible for antibiotic resistance, virulence, and immunological evasion [5].

Biofilms serve as a survival mechanism for bacteria, making them very challenging to treat because of their innate immune response and antibiotic resistance. S. aureus is a common pathogen associated with biofilm infections. Biofilm formation protects bacteria against immune system and antibiotic attacks, allowing them to persevere in the face of external threats [6-8].

Persister is a subpopulation of bacterial cells that are antibiotic-tolerant but do not modify the minimum inhibitory concentration (MIC) values in the entire population, and it is one of the most critical causes of antimicrobial therapy failure [9]. Additionally, because antimicrobial agents may be dependent on their activity at a certain stage of cell growth, this phenomenon restricts their complete development and use. To develop knowledge-based methods for the eradication of persisters produced from cells subjected to various stressful situations, it is also essential to identify their molecular and cellular characteristics [10].

For the identification and classification of *S. aureus*, the polymerase chain reaction (PCR) is a reliable standard method. Furthermore, it views the growing resistance to numerous antibiotics as a useful tool for elucidating the interrelated outcomes [11]. The application of real-time PCR tests to produce quantitative data in various bacteriological diagnostic fields is growing. This assay showed exceptional specificity and sensitivity for identifying *S. aureus* bacteria in various locations, such as nasal carriers, among others [12].

Silencing the genes of small interfering RNA (siRNA) is a useful therapeutic approach 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 *S. aureus* [13].

The current study investigates the ability of siRNA to target and silence critical genes involved in the antibiotic resistance and virulence of methicillin-resistant *Staphylococcus aureus* (MRSA). Specifically focusing on *agrII*, a component of the quorum sensing system that regulates the production of staphyloprotease, an enzyme crucial for the bacterium's pathogenicity. By disrupting *agrII,* we aim to reduce the bacterium's ability to communicate and coordinate the expression of virulence factors, thereby mitigating its pathogenic potential.

Materials and Methods

Sample collection

From July 2023 to January 2024, samples for the study were collected from patients (n=130) of varying ages suffering from various infections, as well as visiting and lying patients in several major hospitals and outpatients from various private medical laboratories in Baghdad, Iraq. Patients' socio-demographic information, including gender, age, marital status, and hospital visits with an infection profile, were recorded.

Isolation and identification of *Staphylococcus aureus*

Plates containing blood, nutrients, and mannitol salt agar were used to collect each sample, which was then incubated at 37 degrees Celsius overnight. *S. aureus* was characterized using ordinal microbiological protocol examinations and biochemical characterization processes for

representative colonies that formed and were taken before [14].

The *S.aureus* biofilm formation isolates were detected by flat bottom, untreated, polystyrene, 96-well microtiter plates using the crystal violet dye. The quantitation of *S. aureus* biofilm was utilized by the spectrophotometric technique as entirely explained [15]. All applied experiments were proceeded in triplicate.

Antimicrobial susceptibility test:

Antibiotic sensitivity testing of *S. aureus* isolates was undertaken based on CLSI standards [16], using the Kirby-Bauer disk diffusion technique on commercially available discs (Liofilchem® S.r.l. Via Scozia, Italy) that contained 17 antimicrobial agents belonging to 14 different classes. The antibiotic panel includes Rifampin (RA: 5 µg/disk), Oxacillin (OX: 5 µg/disk), Ciprofloxacin (CIP: 5 µg/disk), Moxifloxacin (MFX: 5 µg/disk), Gentamicin (Gen: 10 µg/disk), Ceftaroline (CPT: 30 µg/disk), Trimethoprim-sulphamethoxazole (TMP/SMX: 1.25/23.75 µg/disk), FusiQuality control was ensured using *Staphylococcus aureus* ATCC 25923. Results of the test and antibiogram analysis were done and interpreted as stated by the Clinical and Laboratory Standards Institute (CLSI 2022) [16].

Persistency test:

Time-killing experiments for consecutive *S. aureus* isolates approved the tolerance detection (TD) test. The test's principle is to resuscitate the subset of persistent or tolerant bacterial cells after a disk diffusion test with glucose [17].

DNA extraction:

To extract DNA, young pure colonies were placed in a test tube that contained 1 ml of distilled water boiled for 10 minutes in a water bath, and then centrifuged at 12,000 rpm for 5 minutes. The DNA-containing supernatant was collected and kept for later use at -20 °C [18].

PCR amplification and gene detection:

The detection o*f MecA, Pvl, CCrc, ccRA2B, IS1272, MecA-IS431, Cna, SdrD, Fur, agr1, agrII, agrIII*, and *agrIV* genes was performed by a conventional polymerase chain reaction test [19]. PCR amplification was performed in a 25 µl reaction mixture including 12.5 µl of green master mix, 1 µl of forward and reverse primers, 4.5 µl of template DNA, and 6 µl of distilled water. The thermal cycler authorized the PCR cycling various gradient conditions, which were carried out with the following reaction conditions: 30 consecutive cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes are performed after 5 minutes at 95°C [20]. Products Electrophoresis was done using 1.5% agarose gel, and the bands were visible under UV light. The target genes were genes that encode surface adhesion protein (*Cna*) (55c): accession no.M81736, ferric uptake regulator (*SdrD*) (55c): accession no. AJ005646.1, regulating iron availability (*Fur*) (55c): accession no. NC_007795.1.*agr* (*I, II, III, IV*) (55c) and *SCCmec* (55c) systems gene, *Pvl* (53c) and *MecA* (53c) were approved according to NCBI references. To produce a specific primer sequence, the sequence of requested genes was retrieved from NCBI and aligned using several alignments on Geneious Prime.

Small interfering RNA (siRNA) designation:

The AgrA genes of the identified *S. aureus* isolates served as templates for the designing 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 homology genes Table (1).

Table 1. Designed nucleotide sequences of small interfering RNAs (siRNAs) used in this study.

siRNA (polarity)	Sequence (5' →3')
AgrA sense	GGUGCUUGAGCAAGCUAAATT
AgrA antisense	UUUAGCUUGCUCAAGCACCTT

The study's experimental design and stages for quenching staphyloprotease production in vitro by using the siRNA gene-silencing technique;

The experiment was conducted on persistent-methicillin-resistant *S. aureus* bacteria resulting from the susceptibility screening test, which were selected as gentamicin, ciprofloxacin, and oxacillin-resistant-persistent antibiotic disk rounded isolated colonies. The persistence of these isolates was determined by the TD test protocol as previously described.

Bacterial suspension preparation:

The designed experiment was proceeded first by the inoculation of the bacteria in sterile nutrient broth tubes containing gradient concentrations of gentamicin and ciprofloxacin antibiotic solutions at subMIC concentrations within an incubation time for 24 hours at 37°C.

After overnight bacterial growth at subMIC concentrations, the work's steps were as follows:

siRNA preparation and conversion from single-stranded RNA to double-stranded RNA:

The process started with solving the designed ribose primers (AgrA siRNA sense/antisense) by using water-free nuclease with a ratio of 300 ul to the vial of primer. 40 ul of each sense/antisense was taken and gently mixed in PCR Eppendorf. Then, Eppendorf sets in the thermal cycler for a one-step approving stage with frequent temperatures ranging from 42 to 60°C and for 1 minute for each temperature degree. Furthermore, the mixture was cooled to refrigerator cooling temperature.

On the other hand, 40 ul of each sense/antisense was taken and gently mixed in PCR Eppendorf as a second step. Moreover, Eppendorf was placed in a constant state for 30 minutes. After, 50 ul of each of the siRNA primers previously prepared from the PCR machine and those prepared at stationary conditions are mixed and placed at refrigerator temperature.

Accommodation and transfection of persistent bacteria and genetic targeting using siRNA by heat shock and self-recovery:

Bacterial content was grown in a nutrient medium containing the antibiotics were separated by centrifugation at 4000 rpm for three cycles of 3 minutes each to obtain a precipitated pellet. The filtrate is discarded, and then the pellet is washed with cold distilled water three times. 50 ul of cold distilled water is added to the pellet after washing it, and 100 ul of the cold previously prepared siRNA mixture is added to the mixture of pellet and distilled water, the prepared Eppendorf mixture is placed on a crushed ice pack for 30 minutes. As well as Eppendorf was also transferred to the thermal cycler at one step temperature of (42°C) for 1 minute, and Eppendorf was directly placed in the crushed ice pack again for 2 minutes. Finally, The Eppendorf containing mixture is transferred from ice to the incubator at 37°C for one hour for the recovery process.

Phenotypic and genotypic quenching susceptibility screening and detection within enzymatic production capability on modified milk media:

The selected *S. aureus* isolates were cultured before and after the siRNA targeting experiment on a milk medium containing potassium tellurite. The spreading plate method was confirmed. While using serial dilutions, the second dilution was determined. Spreading was done by sterile cotton swabs on a modified milk medium and incubated at 37°C for 48 hours to detect the protease production efficiency before and after targeting the responsible system gene for its production as a significant and substantial virulence factor for *S. aureus*. The results were confirmed using biochemical methods as previously described, and a genetic method using the Real-time PCR tool.

Real time-PCR Screening test

Three of *S. aureus* isolates were selected for the siRNA experiment as (Sa3, Sa10, Sa28). Analysis was applied before and after treating isolates within the quenching design experiment, in addition to the 16s housekeeping gene. Implemented *S. aureus* 3 isolates with siRNA (treated (T) and untreated control (C)) were preserved in Triazole™ Reagent (Thermo Scientific, USA).

RNA extraction was performed using Promega Kit (GoTaq® 1-Step RT-qPCR System, Quantifluor RNA System. USA) according to the manufacturer's instructions. A one-step real-time PCR procedure was determined (Promega, USA). Set up done with Mic qPCR tubes containing 10µl of (qPCR Master Mix, RT mix, MgCl2, Forward primer, Reverse primer, Nuclease Free Water, RNA). The reaction tubes were subjected to a Mic qPCR Cycler (BioMolecular System, Australia). The procedure for amplification has been utilized with 15 min at 37 °C for RT enzyme activation within 1 cycle. Followed by 1 cycle for 5 min at 95 °C as an initial denaturation. 40 cycles started with 95 °C for 20s , 20s at (53°C *(MecA*), 55°C (*agr,II*), 59°C (16s)), 20s at 72 °C for measuring fluorescent signals Table (2).

Table 2. Primer sequences and annealing temperatures for genes examined in gene expression.

Gene/Target	Primer Name	Sequence (5' →3')	Annealing Temperature (°C)
16S rRNA	*16S rRNA* F *16S rRNA* R	ATGCAAGTCGAGCGAAC TGTCTCAGTTCCAGTGTGGC	59°C
MecA	*MecA* F *MecA* R	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	53°C
AgrII	*AgrII* F *AgrII* R	ATGCACATGGTGCACATGC TATTACTAATTGAAAAGTGCCATAGC	55°C

Statistical analyses

Correlation was investigated and the findings were displayed using the R studio ggplot2 program. Stack charts are created using the Chart Builder tool in R studio with the ggplot2 package [21]. Analysis of Gene Expression using Livak Method.

Results

In total, out of the 130 coagulase-positive cocci, 58 isolates showed (44.62%) negative Phenotyping biofilm formation, 26 isolates showed (20.00%) weak formation percentage, moderate was shown (18.46%) for 24 isolates and strong biofilm distribution appeared in the percentage of (16.92%) for 22 Staphylococci, P-value from Chi-Square Test shows (p= 0.766), which means there is no significant association between Biofilm and Group (Figure 1).

The calculations illustrate that female patients were about (87) samples whom *Staphylococcus aureus* bacteria were isolated from, female elucidate for multidrug (43) isolates, extended resistance (14) isolates and multidrug sensitivity (30) isolates categorization within (P-value = 0.0006), for male patient calculation were about (43) samples, moreover, the state appears for multidrug (23) isolates, extended resistance (4) isolates and multidrug sensitivity (16) isolates categorization within (P-value =0.0015), as final reckoning, total P-value for patient's gender characterization were (P-value =0.5734), which means there is no significance relation between sex and antibiotics resistance.

Depending on age, the highest prevalence of antibiotic resistance was found in people aged 20 to 70 years old, which has shown a maximum numeral of about (70) isolates, while ages <20 years old presented (11) isolates, furthermore, ages >71 years old displayed minimum number for resistance within (4) isolates, total P-value were (P =0.9836), no significance relation between age and antibiotics resistance.

According to the type of sample of clinical isolates, wound swab has the majority count within (53) isolates with a significance P-value (P= 0.0075), but for the total calculation of all types of samples with P-value (P=0.0798), there is no significance relation between types of sample isolation and antibiotics resistance. The total significance *P-value* was presented in variable sources of isolates' hospitals in Baghdad, the most prevalent hospital was Al-Kindy Hospital within (50) isolates, total P-value was (P=0.0028).

The capacity of *S. aureus* isolates to generate biofilms was another aspect examined in this investigation. One of *S. aureus's* key survival strategies is the production of biofilms in human tissues and on hospital surfaces. The majority of the *S. aureus* isolates in our investigation produced biofilms. The examined statistical analysis findings indicate a relevant association between the ability to build biofilms and the ratios of multi-extended drug resistance and multi-drug sensitivity capacities (Figure 2).

The persistence or tolerance of *S. aureus* clinical isolates was examined by using a modified TD test introduced by Gefen. After antibiotic exposure, viable bacterial cells were resuscitated by glucose. Two enriched culture media, BHI agar, and chocolate agar, were utilized along with Muller-Hinton, the standard medium used in clinical laboratories for testing the susceptibility to antibiotics. Analysis of MRSA antimicrobial susceptibility revealed resistance to linezolid, fusidic acid, and rifampin, with limited resistance to moxifloxacin and gentamicin.

PCR result analysis showed a varied diverse length of the amplicon ranging from 310 bp to > 659 bp, *Cna, SdrD*, and *Fur-designed* genes emerged as inconstant amongst MRSA isolates, throughout the obtained data analysis genetic heterogeneity among MRSA strains was noticed.

PCR analysis *SCCmec* pattern identified peculiar types and their respective percentages in MRSA isolates, which were divided into four common types, along with an unknown one, as significant ratios were detected in the unknown patterns (non-typeable), while *SCCmec* type IV, I, II, and III were variably noticed.

Following glucose resuscitation, only on BHI agar the persistent/tolerant cells were shown as significant colonies. Persistent network plot and gene detection were concluded in (Figures 3,4,5).

Persister *S. aureus* isolates demonstrate variable correlation results, related to gene content and statically calculations as shown in (Figure 6).

According to Persister, *S.aureus* isolates P-value, we couldn't conclude a statistically significant relationship between the isolates and gene content (Figure 7).

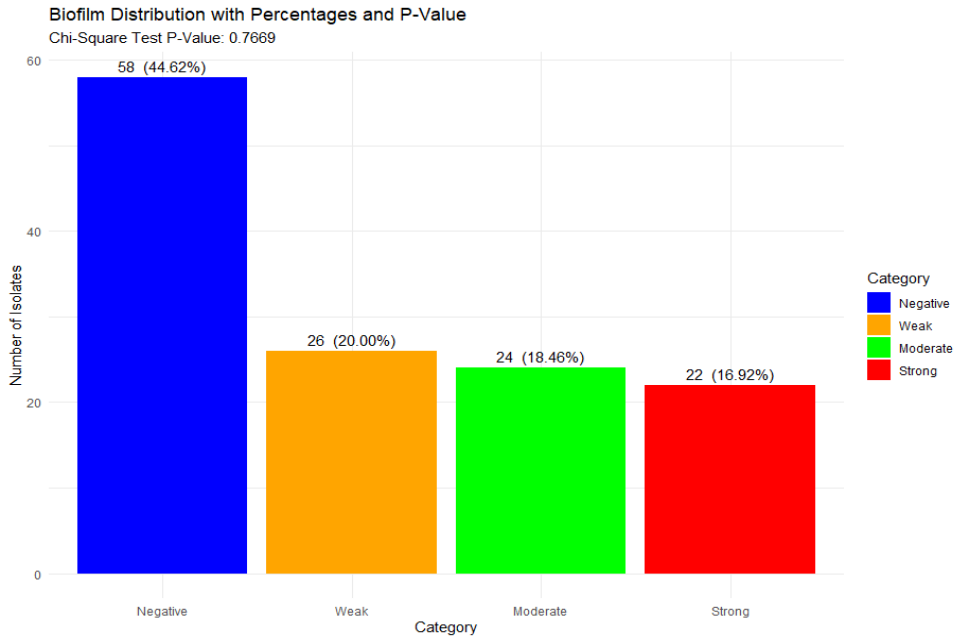

Figure 1: *S.aureus* isolates across biofilm distribution levels.

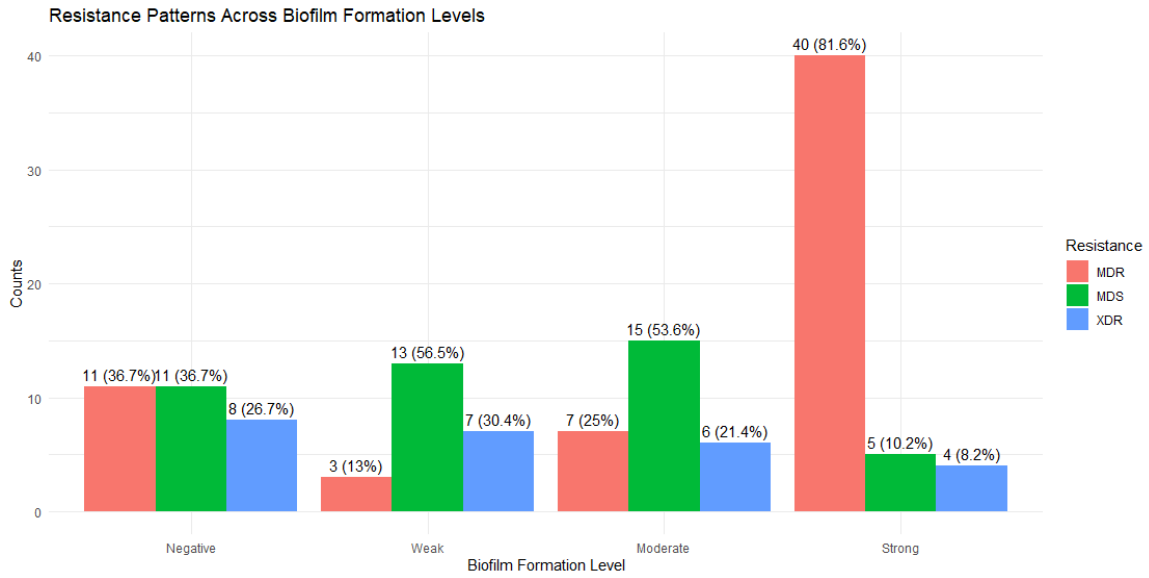

Figure 2: Resistance patterns across biofilm formation levels.

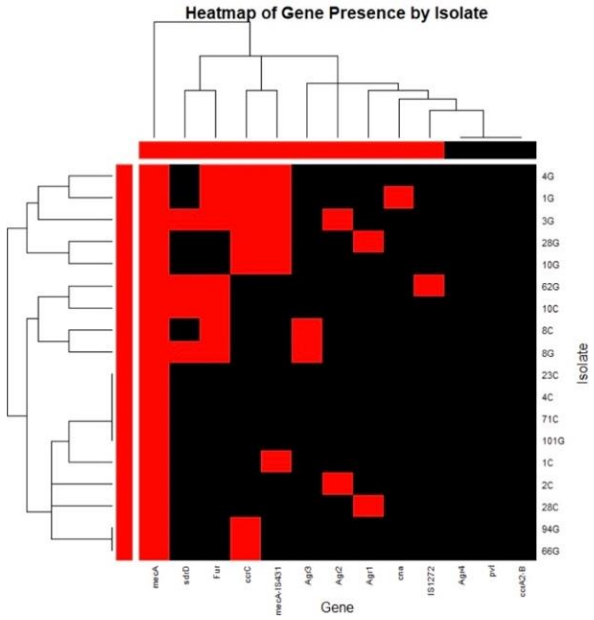

Figure 3: *Staphylococcus aureus* gentamicin and ciprofloxacin persistent isolates dendrogram with heatmap. Gene presence; red colored and absence; black colored.

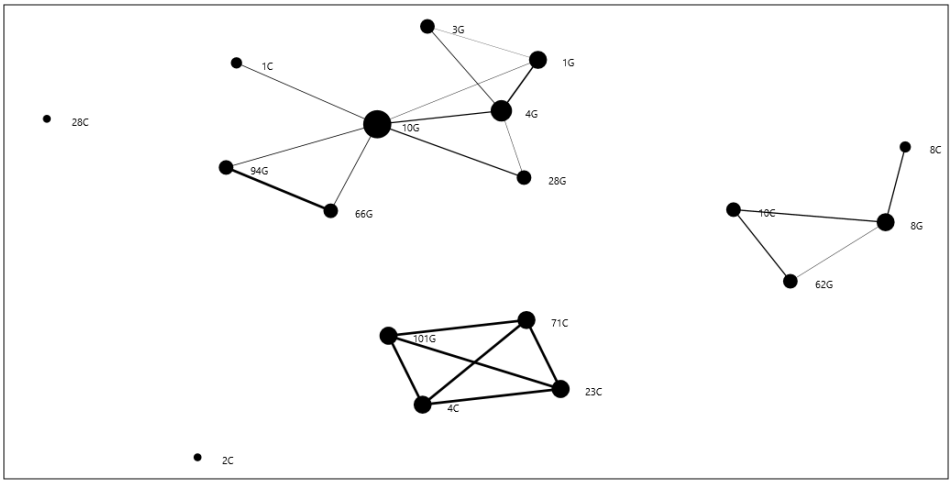

Figure 4: *Staphylococcus aureus* gentamicin and ciprofloxacin persistent isolates network plot relations.

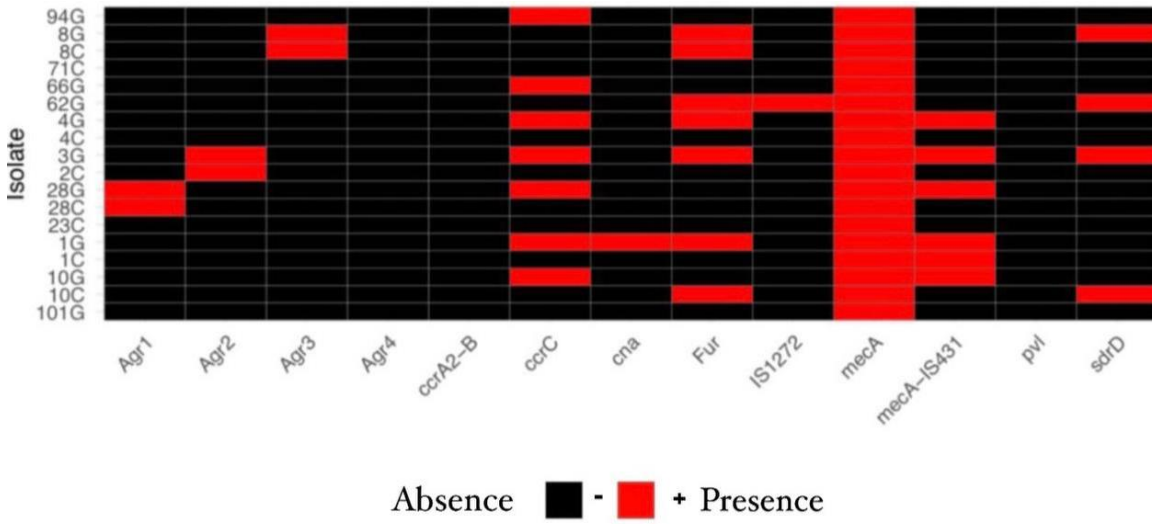

Figure 5: *Staphylococcus aureus* gentamicin and ciprofloxacin persistent isolates molecular heatmap within presence and absence genes.

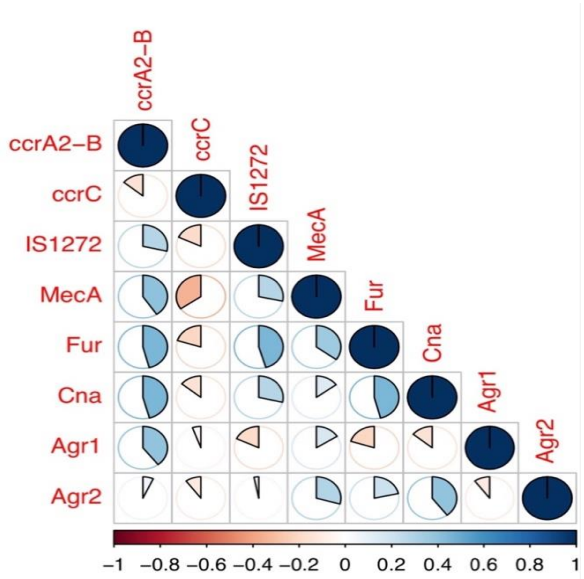

Figure 6: Statical correlation of persister *S.aureus* isolates related to gene content.

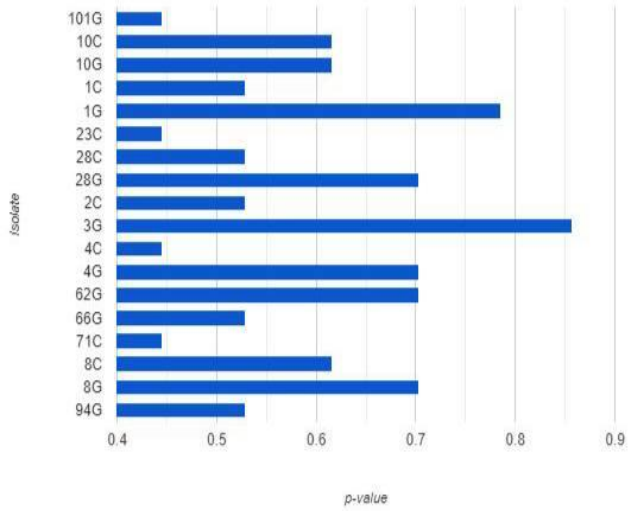

Figure 7: *Staphylococcus aureus* gentamicin and ciprofloxacin persistent isolates P-value related with genes content.

There are many differences between isolates which mainly correlate to the differences in biofilm formation abilities and antibiotic resistance ability. Depending on the implemented siRNA study designed experiment, prominence showed successful quenching for protease enzyme production of *S.aureus* persistent-methicillin resistant isolates. Moreover, selected *S.aureus* treated and untreated (Sa3, Sa10, Sa28) isolates were cultured on modified milk agar media with potassium tellurite for 48 hours, results revealed a comparison between protease production before and after the siRNA transfection experiment (Figure 8).

Real-time PCR screening outcomes for the selected *Staphylococcus aureus* persistent-methicillin resistant isolate (Sa3, Sa10, Sa28) before-after siRNA transfection experiment disclosed harmonized results with phenotypic examination test. Summarized data of gene expression for all 3 isolates provide variable ranges for each treatment within fold changes 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 quorum sensing (QS) in persistent MRSA isolates (Figure 9,10).

For isolate (T3), the calculated fold change is approximately 0.055. This indicates a substantial downregulation in gene expression for the agrII gene within the treatment compared to the control one. In isolates (T10) and (T28) fold change is present at 1.05, 1.052 indicating a minor change in gene expression between the combined treatment and control isolates. In conclusion, treatment for isolate (T10) and (T28) seems to have a minor stimulatory effect on gene expression, but the small magnitude suggests caution in interpreting the biological significance of this change (Figure 11).

Fold change for MecA gene isolate (Sa3, Sa10, Sa28) was recorded at (218.27, 2.828, 14404), which means the treated sample (T3) exhibits a significant upregulation compared to the control sample. While (T10) shows a moderate upregulation compared to the control sample. Finally, it demonstrates a substantial upregulation compared to the control sample (Figure 12).

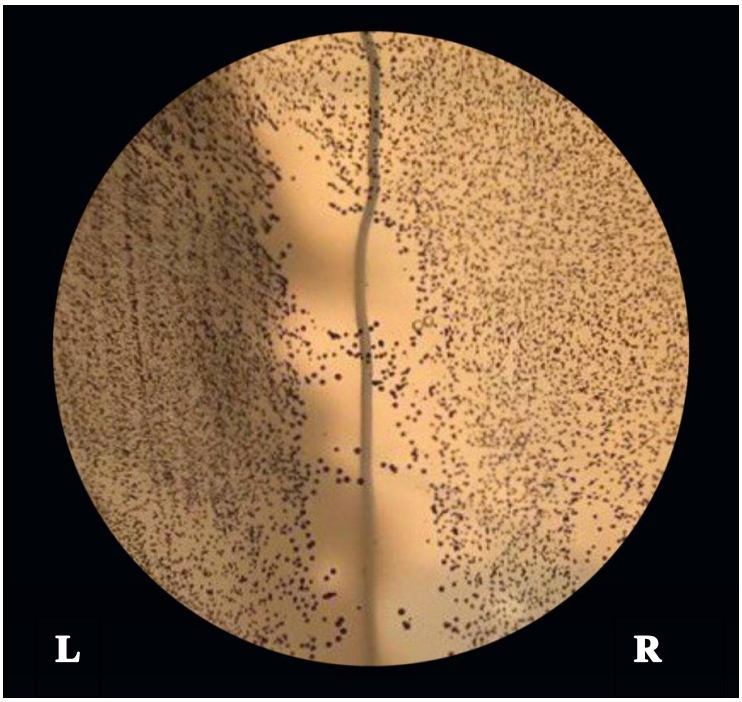

Figure 8: *Staphylococcus aureus* persistent-methicillin resistant isolate (Sa28) 48hr culture results on modified milk agar media with potassium tellurite before and after the designed siRNA quenching experiment. The figure shows colonies with black centers; the right side presented bacterial growth before siRNA transfection which appears as a halo zone as a result of protease production, while the left side has a dusky appearance indicating there is no enzyme production as the conclusion.

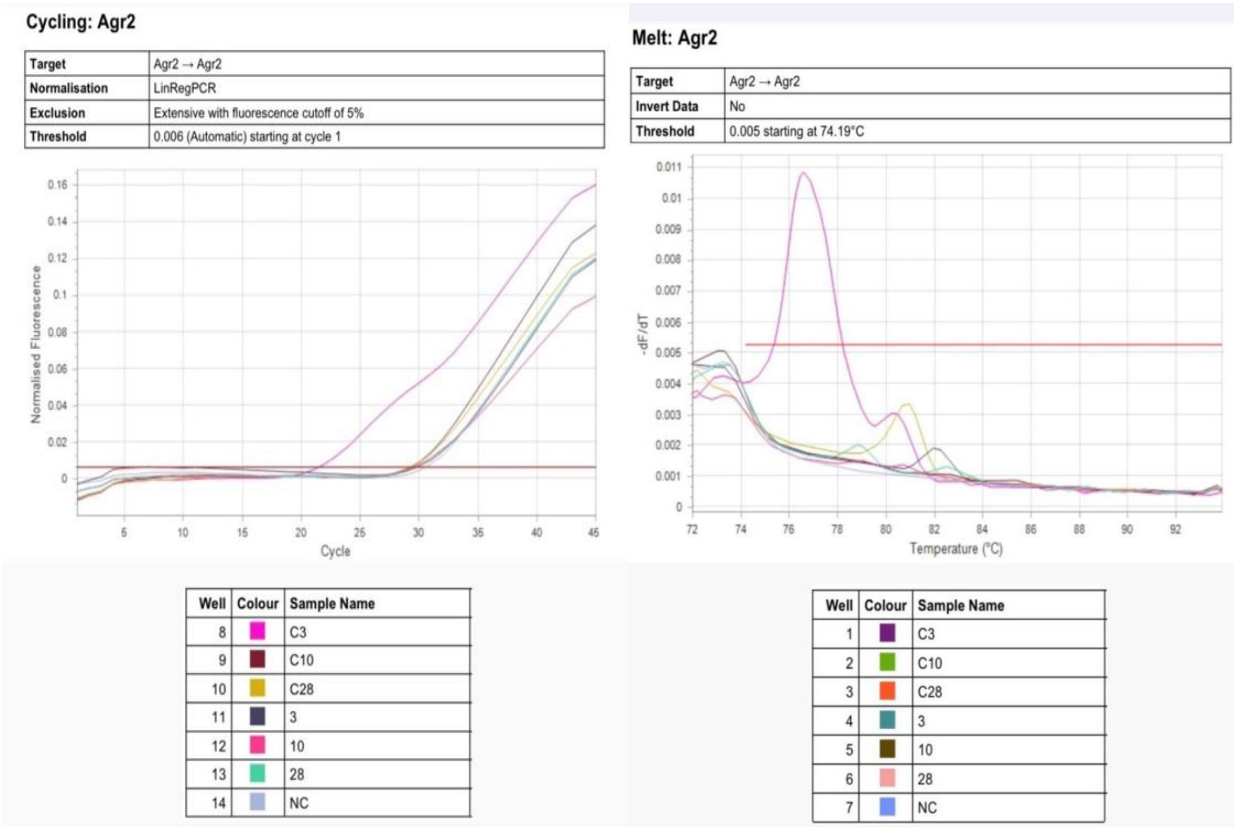

Figure 9: *AgrII* reported gene expression curves for cycling and melting steps of the selected bacterial isolates before (c) and after (t) siRNA treatment.

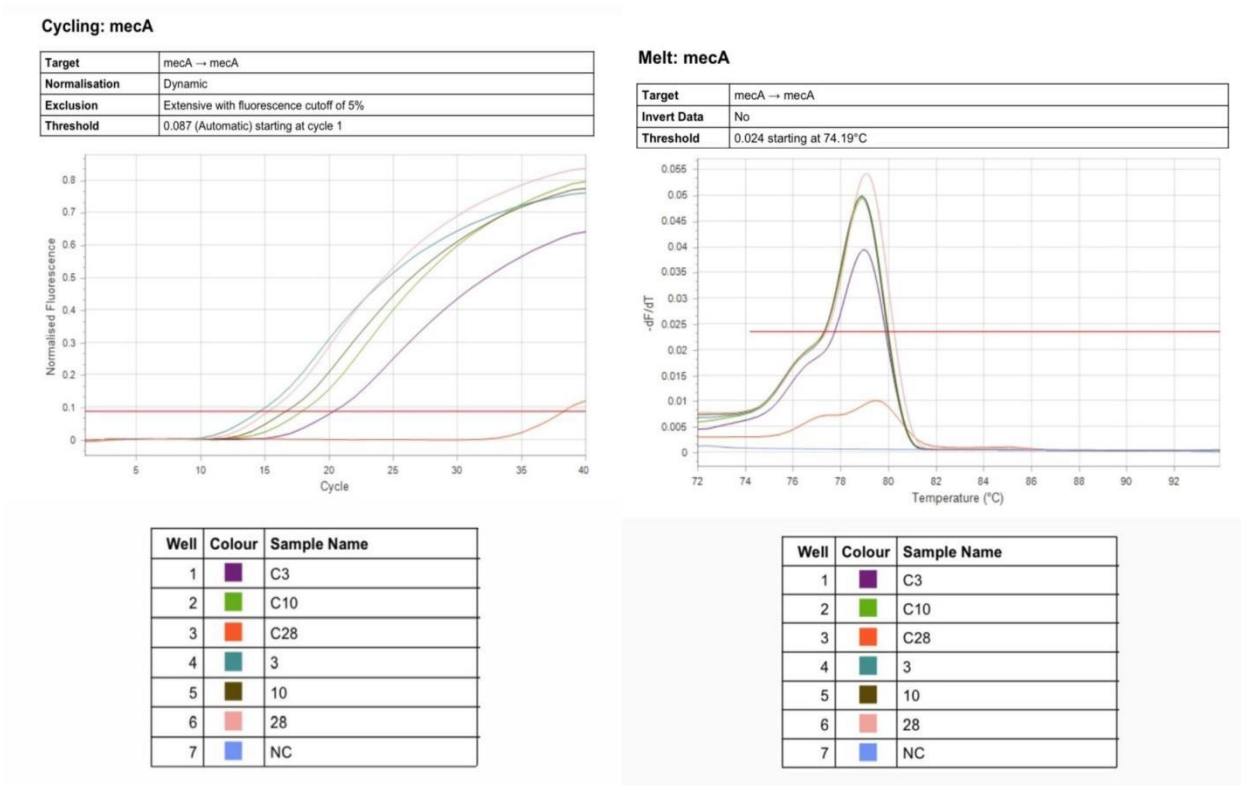

Figure 10: *MecA* reported gene expression curves for cycling and melting steps of the selected bacterial isolates before (c) and after (t) siRNA treatment.

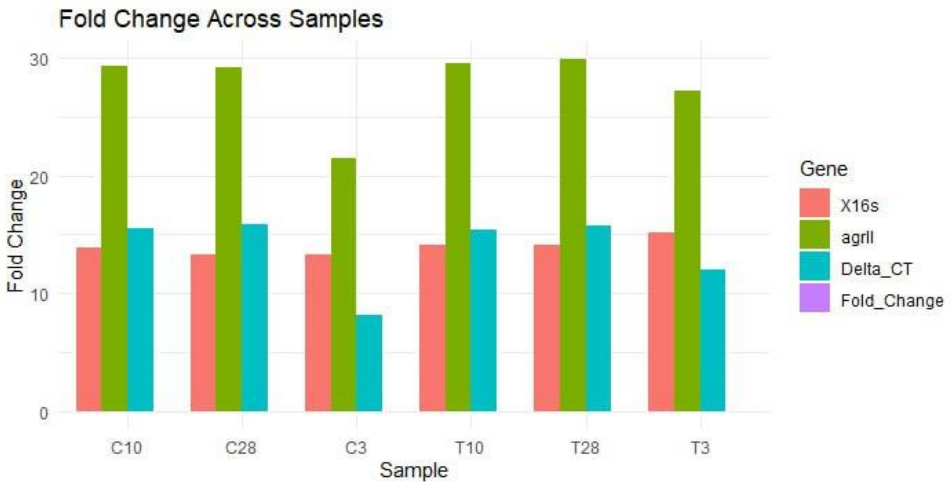

Figure 11: *AgrII* fold changes across tested samples.

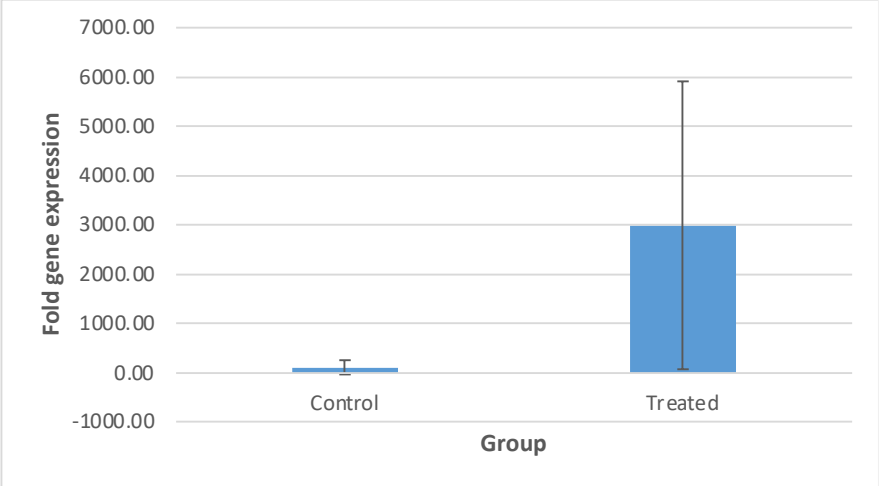

Figure 12: Total fold change and gene expression for *MecA* gene in tested samples.

Discussion

This study aimed to demonstrate three main points: First, the employing of small interfering RNAs (siRNAs) in various *S. aureus* strains could reduce the transcriptional response in genes impacted by the grand protease, *MecA,* and *AgrII* circuits. Second, perturbing the agrA and QS system gene cassette regulatory circuits with siRNAs could attenuate pathogenesis in persistent methicillin-resistant *S. aureus* isolates. Third, it examined the prevalence of *S. aureus* isolates from many major hospitals in Baghdad province.

The study findings indicated that the prevalence of *S. aureus* in these hospitals had different gene presentations in comparison to other studies, although the overall rate was lower than other hospital-acquired bacteria. This is significant because *S. aureus* has virulence genes, is intrinsically resistant to common antimicrobial agents, and can form biofilms. Variable reports of *S. aureus* prevalence in Baghdad show higher variability in isolate collection and distribution compared to a 2015 study. The isolates were obtained from urine (n=44), blood (n=9), cerebral spinal fluid (n=7), skin tissues (n=6), sputum (n=5), vaginal swabs (n=2), wound swabs (n=53), and ear swabs (n=4) [22,23].

All these isolates were approved by standard biochemical procedure. *S. aureus* was more prevalent in females than males, with 14 XDR and 43 MDR isolates in females (P-value 0.00069) compared to 4 XDR and 23 MDR isolates in males (P-value 0.00159). *S. aureus* infections are significant because they can affect gender infertility, impacting sperm activity in males and various phases of a woman's life, including reproduction, fertilization, pre-pregnancy, and pregnancy [24].

The study also found that The majority of *S. aureus* infections with multi-extended drug resistance occurred in individuals between the ages of 20 and 70. Hospitalized infants, children, and other age groups can vary in their susceptibility to *S. aureus* infections based on gender, historical profiles, and other factors [25-27].

Although vancomycin and TMP/SMX are considered to be effective treatments for S. aureus infections, recent studies have raised concerns about their long-term effectiveness.

Alternative antimicrobial agents, such as linezolid and gentamicin, have been reported for their performance against invasive *S. aureus* infections [28,29].

In this study, tigecycline (TG) and gentamicin (GEN) showed efficacy against *S. aureus*, while rifampicin (RA) was found ineffective. Due to its unavailability in Iraqi hospitals, Quinupristin-dalfopristin was not studied.

Results of several antimicrobial susceptibility tests were reported from Iraq and other locations. According to a Baghdad University study, 40 (69%) of the *S. aureus* isolates were resistant to cefoxitin and gentamicin. The Vitek-2 compact system was used to test for vancomycin susceptibility, and the results showed that 57 (98%) of the S. aureus isolates were sensitive to the antibiotic, while only one isolate showed intermediate resistance to their demonstrations [30].

The study also investigated the biofilm formation ability of the isolates of *S. aureus*, finding that the majority were biofilm producers. Statistical analysis cleared a relationship between biofilm formation ability, multi-extended drug resistance, and multi-drug sensitivity. Chronic *S. aureus* infections are complicated by frequent relapses due to drug resistance and the formation of persistent cells, which are metabolically inactive and highly tolerant to antibiotics.

The high prevalence of methicillin-resistant *S. aureus* (MRSA) strains and the development of non-growing, dormant persisters subpopulations that show high levels of antibiotic tolerance and contribute to chronic or recurrent infections are considered to be major challenges in the treatment of *Staphylococcus aureus* infections, according to the results of tests used in this report [31].

The study applied a tolerance detection (TD) test for persistent *S. aureus* isolates, showing that the modified TD test can detect levels of antibiotic tolerance or bacterial persistence. Resuscitated colonies within the inhibition zone can assist in distinguishing between tolerant and persistent isolates. Standard microbiological techniques were used to identify MRSA and PCR with particular primers allowed for the rapid determination of genetic diversity. PCR results showed variability in MRSA genetic contents, possibly due to environmental factors or stress-induced mutations. *Agr* dysfunction findings were consistent with a Korean study but disagreed with another study showing higher Agr system ratios. The study found no presence of the *Pvl* gene in VRSA or MRSA isolates, aligning with *mecA* gene presence but differing from a study in Babylon province.

According to this study PCR results, MRSA shows a different variability in their genetic contents, this is due to several criteria, once it might be returned for the isolate genetic diversity according to the isolate collection environment or isolates stress pressure that may lead to make a mutation that performed to quench, minimize, or delete functions of some genes parts or systems. Our findings for Agr system components agreed with the Korean study [32], while they disagreed with studies that show a higher percentage of Agr system appearance ratio [33].

For this protocol findings, there is no presence of *Pvl* gene in VRSA or MRSA isolates, and this was agreement in presence of *mecA* genes but in disagreement with the study of Babylon province which demonstrated that 19 (79%) out of 24 isolates had a positive result for pvl toxin gene [25].

The *Pvl* gene's absence might indicate lower virulence or suggest different epidemiological origins. MRSA without *Pvl* might rely on other resistance mechanisms. This study explored using siRNA to quench quorum sensing systems, particularly *AgrII* and *MecA*, reducing virulence and making the bacteria more susceptible to immune system attacks and antibiotics. siRNA targeting of *AgrII* and *MecA* decreased transcriptional response, consistent with reduced virulence.

This study prospects a novel-convenient gene-silencing technique for quenching and downregulation for quorum sensing systems or parts within expressions as staphyloprotease production as a central virulence factor for their pathogenesis. The experiment was characterized by ease of implementation and availability of materials compared to other molecular genetic experiments. This method used to quench the virulence factor gene for enzyme secretion was based on using designed siRNA molecules as an inhibitory agent processor.

Repression of *agr* quorum sensing circuit and *MecA* could also lead up to a remaining cell within a quiescent stage while metabolic activity is designed to keep the infection surviving until cell density reaches a threshold level. In the absence of this communication, cells enter the exponential phase more quickly and divide occurs unchecked concerning cell density. Once the cells have entered the exponential phase, doubling time is quick because siRNA repression of the *agrII* and/or *MecA* regulon lowers the metabolic burden of the cells due to decreased transcriptions of virulence genes.

All of the selected strains present a reduced transcriptional reaction when treated with designed AgrA/siRNA molecules, consistent with the inhibition of a virulence-regulating circuit. According to other studies, Agr mutants have attenuated virulence [34,35]. *Agr* activity is arguably the main regulator of *S.aureus* virulence, other regulatory systems can influence its activity and that of its effector, RNAIII and many regulatory RNAs and proteins influence the transcription of RNAII and RNAIII [36, 37]. Strains exposed to siRNA targeting with both *agrII* and *MecA* previously detected positive genes showed lower gene expression in comparison to control strains that were untreated with a siRNA molecule.

In this study, the results revealed that targeting *agrII* with siRNA disrupts the signaling process by which *S. aureus* coordinates the virulence factors expression based on cell density. While quorum sensing regulates the production of staphyloprotease as a degradable-promotable factor for host tissues and infection, Disarray of *agrII* results in lower production of these enzymes, reducing the bacterium's ability to cause damage and spread. Therefore, by disrupting *agrII*, biofilm formation is inhibited (whenever quorum sensing is essential for biofilm development and maintenance) leading to making the bacteria more susceptible to immune system attack and antibiotic treatment capabilities. Although AgrA/siRNA effectively targets and silences key genes involved in quorum sensing virulence, this may not directly affect the *MecA* gene. Within upregulation of the *MecA* gene, encoding PBP2a (penicillin-binding protein 2a) and is responsible for methicillin resistance (MRSA) ability, it might be an indirect consequence of the stress imposed on the bacteria by the disruption of the Agr system. This could be a compensatory response by the bacteria to enhance its survival under adverse conditions. Furthermore, the *MecA* MRSA gene and its silencing would require specific siRNAs targeting for that gene.

This study reviewed the siRNA technique as a safe method of genetic targeting, as well as the genetic targeting was applied and captured after the bacteria manufactured their mRNA. Hitting targets via DNA may cause a genetic mutation, which leads to the possibility of producing higher virulence capacity, thus obtaining infection with more aggressive-resist bacteria. Although AgrA/siRNA effectively targets quorum-sensing virulence genes, it may not directly affect the *MecA* gene. The study suggests AgrA/siRNA as a promising therapeutic strategy but highlights the need for additional strategies to address methicillin resistance.

Conclusion

The study's characteristics and obtained findings highlight the proof of principle for siRNA use and establish it as a valuable supplement to conventional treatment options for *S. aureus* infections. Persister-caused chronic and recurrent infections will continue and probably become more widespread as a result of

the growing number of immunocompromised patients and the use of medical devices. New strategies to identify and repress *S. aureus* persistent-methicillin-resistant protocols have been proposed and recommended. With a better knowledge of how to gene-quench persister cells, reducing biofilm accumulation and other virulence factors repression, gene-silencing with siRNA procedures could be the incoming promised therapeutic dependence protocol.

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