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The Role of siRNA in Inhibition the Biofilm Formation as a First Line of Antibiotic Resistance by Regulation the MsrA Drug Efflux Pump in Staphylococcus Saprophyticus

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***Abstract*---**The bacterium *Staphylococcus* saprophyticus (S. saprophyticus) is a common cause of urinary tract infections (UTI) in the community. To determine the effect of siRNA on the mRNA of the msrA gene in S. *saprophyticus*, 21-23 bp small interfering RNA (siRNA) duplexes were constructed against the mRNA of the *msrA* gene. The effect of siRNA on *msrA* mRNA expression was determined using reverse transcription PCR (RT-PCR). To assess changes in biofilm formation (BF) in response to siRNA activity, the usual tube technique was adopted. In vitro, *msrA*-siRNAs inhibited S. saprophyticus mRNA expression and activity. The efficacy of siRNA was determined by comparing the BF in S. saprophyticus before and after *msrA*-siRNA was introduced into the bacteria. In this investigation, two msrA-siRNA sequences, siRNA1 and siRNA2, were employed. qRT-PCR revealed that two msrA-siRNA sequences significantly suppressed the expression of *msrA*-mRNA, P = (0.010 and 0.002) respectively at ($P<$ 0.05) comparison with control. Regarding the BF results after treatment with two siRNA sequences, they were 5/6 (83.5%) and 4/6 (67%) negative formation and 1/6 (16.5%) and 2/6 (33%) positive formation, respectively compared with control.

***Keywords*---**biofilm formation, drug efflux, msrA gene, small interfering RNA (siRNA), staphylococcus saprophyticus.

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Introduction

Staphylococcus saprophyticus (*S. saprophyticus*) is a Gram-positive bacterium that is commonly isolated from the cattle sector as well as environmental sources such as soil, air, and water (Mohamed *et al.,* 2019). Furthermore, *S. saprophyticus* is widely found in the food-processing sector, such as on the surfaces of working areas such as floors, drains, and processing machinery, and owing to biofilm development, it may resist normal cleaning and disinfection (Harakeh *et al.,* 2006). *S. saprophyticus*, as coagulase-negative staphylococci (CoNS), contains many enterotoxin genes and may create a variety of heat-enterotoxins, causing food poisoning and human disease (Casaes Nunes *et al.,* 2016). Multi-antibiotic resistance was found in over 90% of *S. saprophyticus* isolates from ready-to-eat food, suggesting a serious hazard to public health (Wang *et al.,* 2019). Furthermore, *S. saprophyticus* is a component of the genitourinary tract microbiota and can cause urinary tract infections (particularly in young women) (Martins *et al.*, 2019).

Biofilms are microbe aggregation that are adhering to each other and/or to surfaces and are surrounded by self-produced extracellular polymeric substances (EPSs), which allow bacteria survive in adverse conditions (Tremblay *et al.*, 2013). A biofilm is made up of microbial cells that are adhering to one another and live in an organic polymer matrix that they manufacture, as well as a static (living or nonliving) surface (Shirtliff *et al.,* 2000; Goldberg *et al.,* 2002) . The biofilm matrix in staphylococci species is mostly constituted of polysaccharide intercellular adhesin (PIA). Nowadays, these bacteria pose a significant threat to human health due to the development of anti-antibiotic resistance tactics such as biofilm formation, efflux pump, and enzyme secretion (Wassenaar *et al.,* 2015). Various physical and chemical techniques, such as UV, flushing, and sanitizers, have been used to manage and remove bacterial biofilms, but with little success (Von Eiff *et al.,* 2002). As a result, innovative techniques capable of inhibiting or dispersing biofilm development are desperately needed.

A newly emerging molecular approach is a revolutionary method used to inhibit gene expression. This approach is known as RNA interference (RNAi), which was found as an antiviral mechanism in plants and other species and has now been identified as an evolutionarily conserved technique for the particular inhibition of gene expression (Yanagihara *et al.,* 2006). One of RNAi types is small interfering RNA (siRNA), which is 19-23 base pairs (bp) in length and have been shown to be effective as exogenous agents for manipulating gene expression in cultured cell- and animal-based systems. Furthermore, siRNAs are extremely stable and have a minimal toxicity (Fire *et al.,* 1998; Takeuchi *et al.*, 2009). While siRNA-based antibacterial treatments are theoretically intriguing, relatively few investigations have been published too far. One research indicated that siRNAs might efficiently alter virulence, drug resistance, and pathopoiesis by successfully inhibiting the *Staphylococcus aureus* staphylocoagulase gene (Yanagihara *et al.,* 2006), and decreasing the biofilm formation (Thompson *et al.,* 2021).

The purpose of this study was to create a siRNA against the *msrA* gene in *S. saprophyticus* and explore the effects of silencing *msrA* on biofilm formation, which is considered a factor of bacterial virulence. Since *msrA* encodes the MsrA

efflux pump protein, a member of the ATP-Binding Cassette (ABC) transporter superfamily, and because of the efflux pump role in the formation of biofilm in bacteria, silencing the *msrA* gene has a significant role in inhibiting the biofilm formation.

Materials and Method

Isolation and culture conditions of bacteria

S. saprophyticus isolates used in this study were isolated in 2020 from UTIs patients (catheter patients) in The General Hospital/ Babylon Government, Iraq. Initially, they were identified after a number of media and tests that were applied for 42 isolates. In most cases, bacterial isolates were identified after 24-48 hours of incubation with MacConkey agar, Mannitol Salt Agar, and blood agar plates at 37 °C to differentiate Gram-positive and negative bacteria. Tests such as coagulase, catalase, and urease were performed to differentiate *S. saprophyticus* from other Staphylococci. Finally, we confirmed *S. saprophyticus* identification by VITEK2 Compact System. There were 16 confirmed isolates of *S. saprophyticus,* which were cultured on brain heart infusion broth (BHI) provided by (Haibo, Qingdao, China) and incubated at 37◦C.

msrA Gene Detection by Conventional PCR

PCR investigation utilizing primers *msrA* gene (Tabel. 2) indicated the existence of the *msrA* gene responsible for erythromycin resistance. The presence of the *msrA* gene (75%) in 12 out of 16 *S. saprophyticus* isolates was discovered in this investigation. In a 2 percent agarose gel, positive strains for the *msrA* gene displayed a band of 100bp (Figure 1).

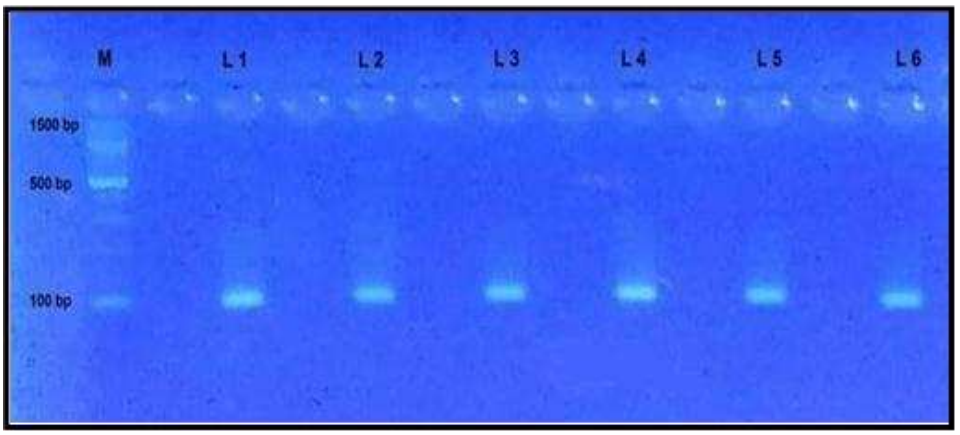

Figure 1. (2%) of agarose gel, electrophoresis shows the amplification product of msrA (band at100 bp)

Antimicrobial susceptibility testing

The resistance of the *S. saprophyticus* isolates to erythromycin (macrolides) antibiotics was investigated in Mueller-Hinton agar (Merck, Germany), by the disc

diffusion method according to CLSI (Patel *et al.*, 2017). The antibiotic disc was 15 µg provided by Bioanalyse®, Ankara, Turkey. Only 11 isolates were resistant to erythromycin.

Biofilm formation assay

We assessed the biofilm formation capacity in *S. saprophyticus,* which appeared resistant to erythromycin, using the standard tube method (Christensen *et al.,* 1982). The biofilm formation of 11 *S. saprophyticus* isolates was performed using the tube method to detect which isolates have the ability to form biofilm before and after treatment by *msrA*-siRNA sequences.

SiRNAs

SiRNA sequences were synthesized against *msrA* gene in *S. saprophyticus,* (two sequences). A scrambled-siRNA sequence as negative control was synthesized as well. All siRNAs, were designed and synthesized by Qiagen Company USA (Tabel 1).

Table 1.
Sequences of siRNAs (Specific and scrambled) used in this study

Genes		Sequences of siRNA (5'→3')
msrA 1	Antisense	UUUUACCAGUACCAUUACCCC
	Sense	GGUAAUGGUACUGGUAAAACC
MsrA 2	Antisense	UGUUAAGUGAUCGUAAAUCU
	Sense	GAUUUACGAUCACUUAACAUC
Universal Negative Control (Scrambled Sequence)		

Heat shock technique

The first step in the transformation of *S. saprophyticus* was to produce competent cells with $CaCl_2$ and all reagents as well $CaCl_2$, were maintained at 4 °C or ice-cold. After preparing the competent cells of bacteria with $CaCl_2$, they were subjected to a heat shock by being placed on ice. Adding 1.5 µL (20 nanometers) of each siRNA sequence to separate tubes from the same sample. For 30 min, incubate the samples on ice. Heat shock the samples by immersing them in a 45°C water bath for exactly 60 seconds and then putting them on ice for 2 min. 1 mL of nutrient broth medium added. Shaking at 37°C, 200 rpm, for 1 hour to encourage outgrowth. After heat shock, the mixtures were put into (100 ml) nutrient agar, and cultured for 24 h at 37 °C to be prepared for the following stages (Chang *et al.,* 2017).

Extraction of RNA and qRT-PCR

TRIzol® reagent kit (Sigma-Aldrich Company, Germany), was used to extract RNA from *S. saprophyticus*. The concentration and purity of RNA were assessed by measuring the absorbance ratio at 260/280 nm and 260/230 nm using (Nabi UV/Vis Nano Spectrophotometer MicroDigital, Korea), followed by gel electrophoresis. The GoScriptTM Reverse Transcription System Kit from Promega Corporation/USA, was utilized for reverse transcription. A negative control

without reverse transcriptase was supplied to guarantee that no tainted DNA was present. *msrA*-cDNA amplifications were done in triplicate in a Real-Time PCR System (7500 Applied Biosystems, Inc., USA) using SYBR Green-based detection (Promega, Inc., USA). The compatibility of the housekeeping gene was established by the extraction of genomic DNA and RNA. The expression ratio between extracted RNA and DNA remained steady throughout the experiment. Primers were made using the sequences of *S. saprophyticus' msrA* and *16s rRNA* genes, which acted as housekeeping genes (Tabel. 2). The cDNAs that were used as a template for qRT-PCR were diluted to the equivalent of 1 ng RNA. As an internal reference, the transcriptional level of *msrA* was normalized to the transcriptional level of the *16S rRNA* gene. As previously mentioned, the relative gene expression or changes (X-fold) in transcriptional levels of induced and control samples were calculated (Pfaffl, M.W., 2001).

Table 2
Sequences of primer for target genes amplification

Gene		Primer sequences	pb size	Ref.
msrA	F	5'-GCTCTACTGAATGATTCTGATG-3'	100	**Park *et al.*, 2011**
	R	5'-TGGCATACTATCGTCAACTT-3'	bp	
16S	F	5'-TGAAGAGTTTGATCATGGCTCAG-3'	527 bp	**Park *et al.*, 2011**
rRNA	R	5'-ACCGCGGCTGCTGGCAC-3'		

Analysis of statistics

The (Statistical Package for Social Sciences- SPSS) version 23 was used to conduct the statistical analysis. The experiments were done at least three times, and the findings may be found as mean values ± standard deviations. To evaluate statistical significance, a one-way analysis of variance (ANOVA) was utilized, and the LSD was used as a Post Hoc Test. The statistical significance was determined to be ($p < 0.05$).

Results and Discussion

Effect of siRNA in *msrA* expression (Genotypic Effect)

According to q-PCR results, there was a considerable decrease in *msrA* expression at the mRNA level in aliquots of *S. saprophyticus* samples treated with siRNA1 and siRNA2. When compared to control, the fold changes of expression were downregulated, P= (0.010, and 0.002) respectively at (P< 0.05), and P= (0.015, and 0.028) respectively when compared to scrambled-siRNA. These findings demonstrated the efficacy of siRNA as a novel molecular inhibitor for efflux pumps, which are one of the most important sources of antibiotic resistance in bacteria. Jingping Ge *et al.* demonstrated that a load of *Lactobacillus paracasei* bacteria treated with siRNA sequence was decreased in vivo, and the current findings matched those of numerous earlier studies on the use of siRNA interfering in bacteria (Jingping Ge *et al.,* 2015). This was linked to the role of siRNA and its ability to suppress gene expression for the target gene.

Thompson *et al.* also demonstrated that *C. elegans* worms exposed to siRNA-treated *S. aureus* survived significantly longer than those exposed to untreated strains, indicating that siRNA's capacity to downregulate target gene expression might be a helpful complement to current *S. aureus* therapy options (Thompson *et al.,* 2021). In addition, another study found that employing *MexB*-siRNAs sequences, which targeted MexB efflux pumps in these bacteria, considerable reduction of MexB mRNA production in *Pseudomonas aeruginosa* in vitro (Gong *et al.,* 2014).

Effect of siRNA in biofilm formation

The findings of biofilm formation of 11 *S. saprophyticus* isolates using the tube method before treatment by *msrA*-siRNA sequences were 8/11 (73%) *S. saprophyticus* isolates were biofilm positive while 3/11 (27%) could not form any detectable biofilm compared with control (Figure 2).

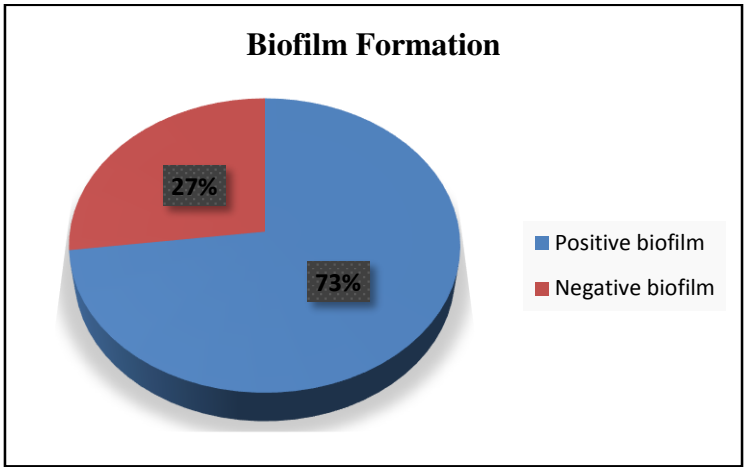

Figure 2. Biofilm Formation before Treatment

The isolates that have formed biofilm (8 isolates), were chosen to be treated by two *msrA*-siRNA sequences using heat shock method. The result of siRNA1 sequence was 5 of 8 (62.5%) isolates did not form any detectable biofilm. Regarding the aliquots treated with siRNA2 was 6 of 8 (75%) without biofilm as in (Figure 3 and 4).

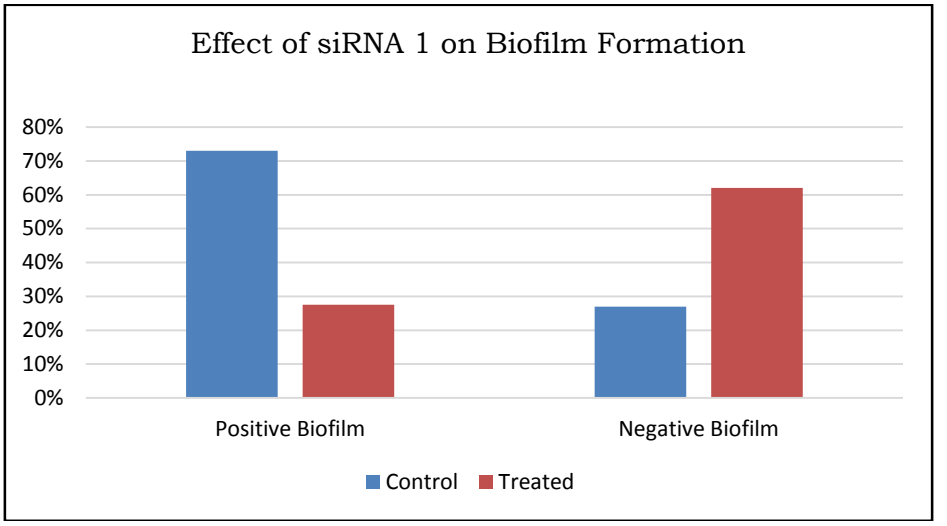

Figure 3. Effect of siRNA 1 on biofilm formation

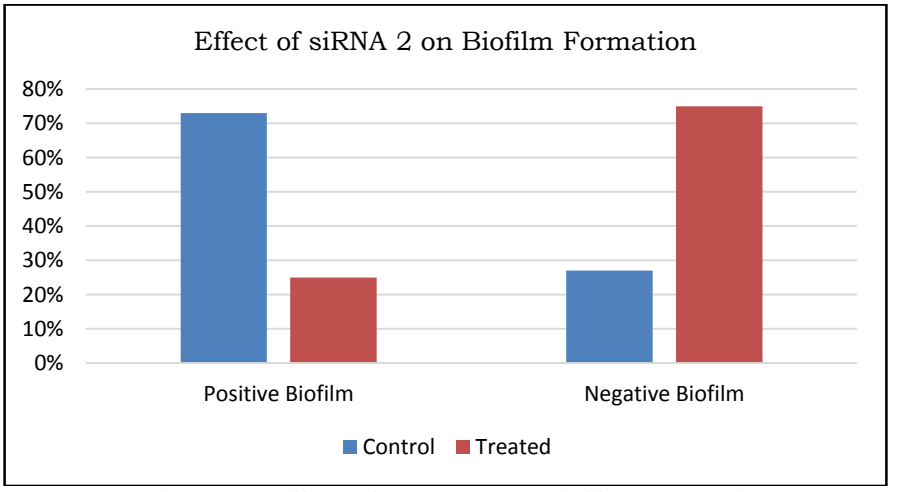

Figure 4. Effect of siRNA 2 on Biofilm Formation

Totally, the results were consistent with Wang *et al.,* who showed that baicalin (chemical material and an inhibitor to MsrA efflux pump), decreased biofilm formation, and only a few bacteria adhered to the glass (Wang *et al.,* 2019). In another recent study, Kim *et al.,* revealed the effects of *Rumex japonicus* Houtt (RJH) plant extract as an anti-biofilm, and bactericidal agent on MDR *S. aureus* isolates, which led to a large reduction in biofilm formation (81.9 %), (Kim *et al.*, 2022). Furthermore, antibiotics and RNA interference of the *rsmY* and *rsmZ* genes were demonstrated to efficiently inhibit biofilm formation in *P. aeruginosa* (Gong, et al., 2014). Thompson *et al.* had found that biofilm formation, significantly ($p < 0.05$), decreased in siRNA treated *S. aureus* strains, as a result of targeting *sarA and agrA* which they related to MsrA efflux pump (Thompson *et al.*, 2021). Explanation of this case is due to interference between the *msrA*-siRNA sequence and the mRNA of the *msrA* gene, which led to downregulate its expression and thus blocking the MsrA efflux pump, resulting in the inhibition of the quorum-

sensing system, which prevented biofilm formation in *S. saprophyticus* (Wang *et al.*, 2019).

Conclusion

In vitro, siRNAs suppressed both mRNA expression of the *msrA* gene and the function of the *S. saprophyticus* MsrA efflux pump, resulting in decreased biofilm formation compared to control. The use of siRNA sequences to target *msrA*-mRNA looks to be a new method for treating *S. saprophyticus* pathologies and reducing virulence. Furthermore, the findings of this study emphasize the proof of principle for the use of siRNAs and position it as a valuable supplement to traditional *S. saprophyticus* treatment approaches.

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