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The role of tetK-siRNA in inhibition the biofilm formation as a first line of antibiotic resistance by regulation the TetK drug efflux pump in staphylococcus aureus

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***Abstract*---**The Staphylococcus aureus (S. aureus) is a common cause of different infections in the community. To determine the effect of siRNA on the mRNA of the tetK gene in S. aureus, 21-23 bp small interfering RNA (siRNA) duplexes were constructed against the mRNA of the tetK gene. The effect of siRNA on tetK mRNA expression was determined using reverse transcription PCR (RT-PCR). To assess changes in biofilm formation in response to siRNA activity, the usual tube technique was adopted. In vitro, tetK-siRNAs inhibited S. aureus mRNA expression and activity. The efficacy of siRNA was determined by comparing the biofilm formation in S. aureus before and after tetK-siRNA was introduced into the bacteria. In this investigation, both modified tetK-siRNA sequence and unmodified tetK-siRNA sequence, were employed. RT-qPCR revealed that both modified tetK-siRNA sequence and unmodified tetK-siRNA sequence significantly suppressed the expression of tetK-mRNA, P = (0.04, and 0.03) respectively at ($P < 0.05$) comparison with control. Regarding the biofilm formation results after treatment with modified tetK-siRNA sequence were 3 / 4 (75 %) negative biofilm formation and 1 / 4 (25 %) positive biofilm formation, while within unmodified tetK-siRNA sequence, 2 / 4 (50 %) negative formation and 2 / 4 (50 %) positive biofilm formation compared with control. According to the findings, siRNA targeting of tetK looks to be a novel strategy for treating S. aureus illnesses.

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Introduction

S. aureus is the major and most common pathogen, distinguished by its ability to coagulate blood plasma, Coagulase positive staphylococci (CoPS), from other species of *Staphylococcus* that are disease-causing human diseases (Crosby *et al.*, 2016 and González-Martín *et al.*, 2020). *S. aureus* is a widespread pathogen linked to severe community and hospital-acquired infections, and it has long been identified as a major public health concern. Since it possesses a wide variety of virulence factors, this Gram-positive bacterium can overcome all host defense barriers. *S. aureus* is also one of the most common pathogens in biofilm-related infections of indwelling medical devices, which cost developing countries billions of dollars per year in healthcare costs (Samrot *et al.*, 2021). Multidrug-resistant (MDR) *S. aureus* is a superbug pathogen that causes severe infections, consequently serious diseases. The recalcitrant nature of *S. aureus* biofilms is one of the key reasons for antibiotic therapy's lack of success against infections caused by this resistant pathogen, resulting in an increasingly serious situation wide world. As a result, the discovery of novel biofilm inhibitors is critical to controlling the pathogen's biofilm formation (El-Hamid *et al.*, 2020).

A main feature of *S. aureus* infections' recalcitrance, like that of many other species, is their resistance and invasion, which is due to their innate ability to form biofilms on natural and abiotic surfaces (Laverty *et al.*, 2013). Indeed, lethal infections caused by staphylococcal biofilms are especially difficult to remove, and their treatment has become increasingly difficult and expensive. These biofilms protect cells not only from host immune defenses, but also from chemotherapeutic agents' effects (Paharik & Horswill, 2016 and Van Acker *et al.*, 2014). As a result, *S. aureus* biofilms become increasingly resistant to numerous antimicrobials (McCarthy *et al.*, 2015). This has caused a lot of concern because it could put a huge strain on healthcare systems (Römling & Balsalobre, 2012).

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 to 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 *tetK* gene in *S. aureus* and explore the effects of silencing *tetK* on biofilm formation, which is considered a factor of bacterial virulence. Since *tetK* encodes the TetK efflux pump protein, a member of the major felicitated superfamily MFS transporter, and because of the efflux pump role in the formation of biofilm in bacteria, silencing the *tetK* gene has a significant role in inhibiting the biofilm formation.

Materials and Methods

Isolation and Culture Conditions of Bacteria

S. aureus isolates used in this study were isolated in 2021 from patients with injuries in the emergency ward of Azadi Hospital Government, Iraq, and a swab was taken from patients with burns of different ages ranging from 4 - 60 years of both genders with the help of doctors and specialists residing in the emergency ward. Initially, they were identified after a number of media and tests that were applied for 64 isolates. In most cases, bacterial isolates were identified after 24-48 hours of incubation with Mannitol Salt Agar (MSA), and blood agar plates at 37 ˚C to differentiate Gram-positive and negative bacteria. Tests such as coagulase and catalase were performed to differentiate *S. aureus* from other Staphylococci. Finally, we confirmed *S. aureus* identification by VITEK2 Compact System. There were 21 confirmed isolates of *S. aureus,* which were cultured on brain heart infusion broth (BHI) provided by (Haibo, Qingdao, China) and incubated at 37 ˚C.

Detection of the *tetK* Gene Using Conventional PCR

PCR investigation utilizing primers *tetK* gene (Tab. 2) indicated the existence of the *tetK* gene responsible for tetracycline resistance. In a 2 % agarose gel, positive strains for the *tetK* gene displayed a band of 127 bp, such as (Fig. 1).

Antimicrobial susceptibility testing

The resistance of the *S. aureus* isolates to tetracycline 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 30 µg provided by Bioanalysis®, Ankara, Turkey. Only 14 isolates were resistant to tetracycline.

Biofilm Formation Assay

We assessed the biofilm formation capacity in *S. aureus,* which appeared resistant to tetracycline, using the standard tube method (Christensen *et al.,* 1982). The biofilm formation of 14 *S. aureus* isolates was performed using the tube method to detect which isolates have the ability to form biofilm before and after treatment by *tetK*-siRNA sequences.

SiRNAs

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

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

Genes	siRNA Sequences		Notes
tetK	Antisense siRNA 21nt guide (5'→3')	AGUAUAAGUAGGUAAGACCAA	Without modification
	Sense siRNA 21nt passenger (5'→3')	GGUCUUACCUACUUAUACUUC	
tetK	Antisense siRNA 21nt guide (5'→3')	AAUAUUUCUAGCUACAACCAC	With 5'Fluorescein, 6-FAM modification
	Sense siRNA 21nt passenger (5'→3')	GGUUGUAGCUAGAAAUAUUAC	
Universal Negative Control (Scrambled Sequence)			

Transformation using Heat-Shock Technique

The first step in the transformation of *S. aureus* 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 h 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 RT-qPCR

TRIzol® reagent kit (Sigma-Aldrich Company, Germany), was used to extract RNA from *S. aureus*. 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. *tetK*-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. aureus' tetK* and *16S rRNA* genes, which acted as housekeeping genes (Tab. 2). The cDNAs that were used as a template for RT-qPCR were diluted to the equivalent of 1 ng RNA. As an internal reference, the transcriptional level of *tetK* 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

Target gene	Primer sequences		Size	Reference
TetK	F	5'-AGGATCTGCTGCATTCCCTTC-3'	127 bp	This study
	R	5'-TGAAGGACCTAACCCTTCACC-3'		
16S Rrna	F	5'-TGAAGAGTTTGATCATGGCTCAG-3'	527 bp	(Park *et al.*, 2011)
	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

DNA Gel Electrophoresis

DNA concentration ranged between 5-8 ng\µL and purity ranging from 1.7 to 2, as well as visually by horizontal agarose gel electrophoresis in 2 %. After performance PCR run, using specific *tetK* (F & R) primers, the results were as in (Fig. 1) which displayed bands of DNA which extracted from *S. aureus* isolates. Depending on the gel there was 8/10 (80 %) of isolates showed positive *tetK* expression. Mbindyo *et al.* also discovered that *tetK* gene was present in 75 % of *S. aureus* isolates (Mbindyo *et al.*, 2021).

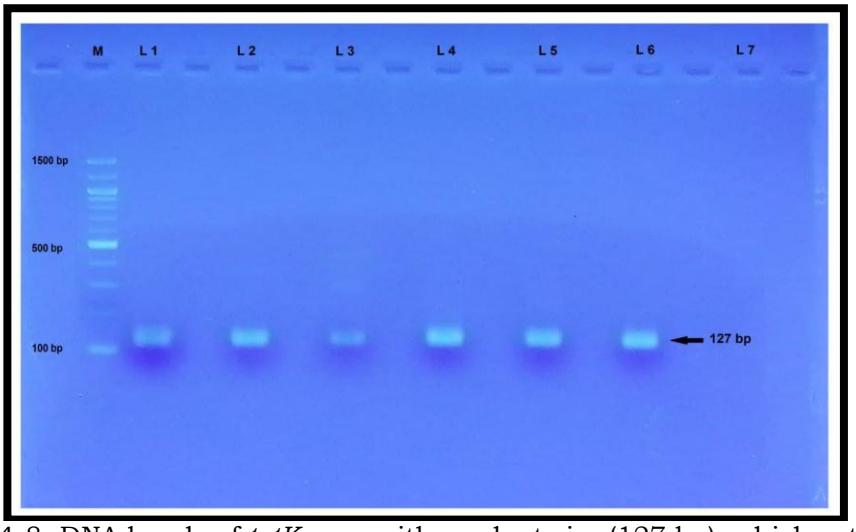


Figure 4-8. DNA bands of *tetK* gene with product size (127 bp), which extracted from resistant *S. aureus* isolates

Screening the Efficiency of Introducing Modified *tetK*-siRNA Using Fluorescent Microscope

The efficiency of heat shock following transformation with 5' Fluorescein, 6-FAM labeled siRNA was measured to confirm that the *tetK*-siRNA (modified) was transferred to the *S. aureus* isolates. Fluorescence was measured in the *S. aureus* isolates using fluorescent microscopy (Fig. 2). The efficiency of heat-shock was 4 / 8 (50 %). These findings are consistent with the findings of (Ge *et al.*, 2015), who discovered that siRNA introducing was 58 %. It is important to be mentioned that the objective of labelling (modifying) of *tetK*-siRNA related or based on efficiency of sequences in either interfering or gene expression down regulation, however, it is only was for detection and confirmation the efficiency of transformation process.

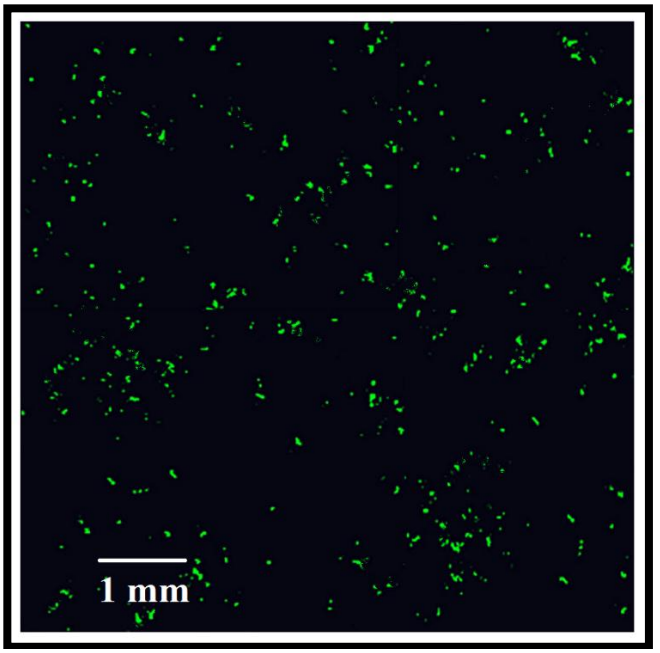


Figure. 2. Heat-shock of 5' Fluorescein, 6-FAM labeled siRNA into *S. aureus* (400×). Representative fluorescent microscopy image of *S. aureus* competent cells transformed by 5' Fluorescein, 6-FAM labeled *tetK*-siRNA sequence

Effect of siRNA in *tetK* Expression (Genotypic Effect)

According to qPCR results, there was a considerable decrease in *tetK* expression at the mRNA level in aliquots of *S. aureus* samples treated with modified *tetK*-siRNA sequence and unmodified *tetK*-siRNA sequence. When compared to control, the fold changes of expression were downregulated, P = (0.04 and 0.03) respectively, at ($P < 0.05$), such as (Fig. 3).

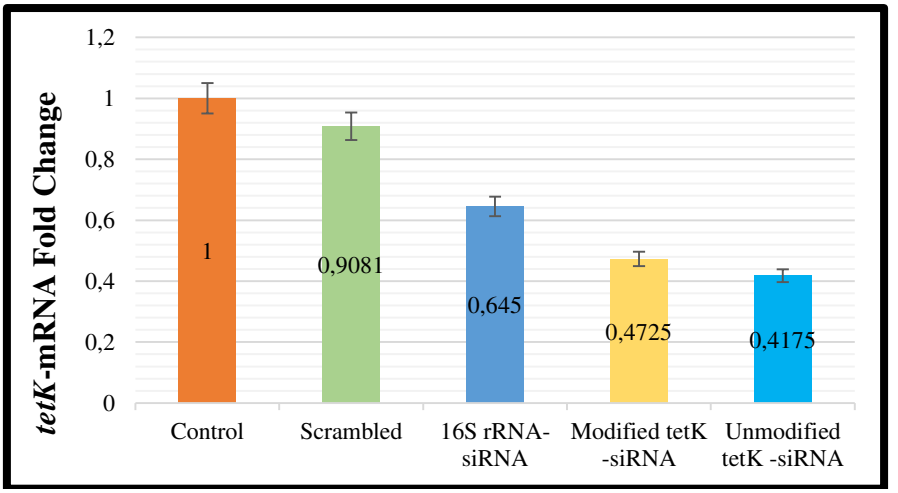
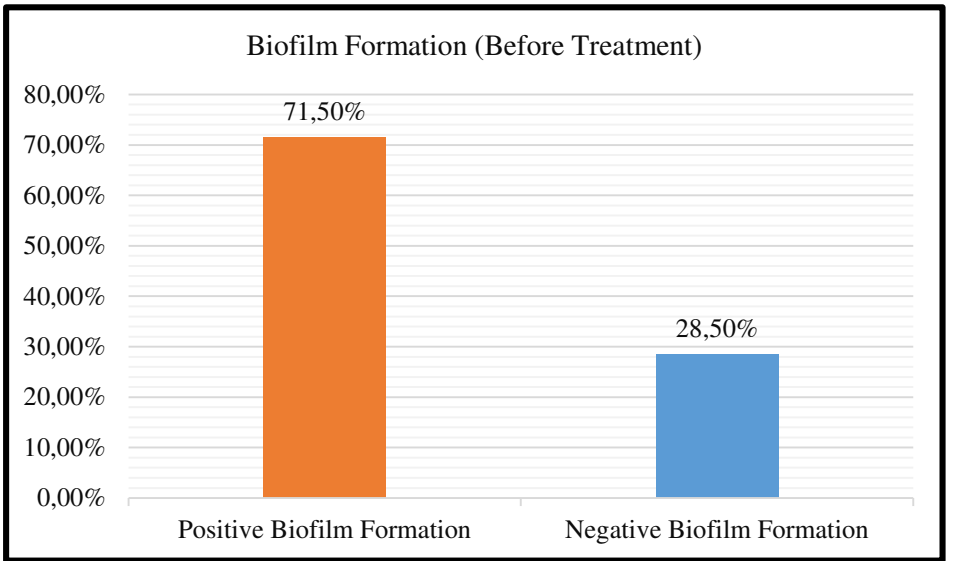


Figure 3. Effect of siRNA on *tetK*-mRNA expression

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. Ge, J. *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 (Ge, J. *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 (Phenotypic Effect)

Before treatment with siRNA sequences, biofilm formation of 14 *S. aureus* isolates was performed using the tube method. Generally, 10/14 (71.5 %) *S. aureus* isolates were biofilm positive, and among them 8/10 (80 %) isolates showed strong biofilm formation, 1/10 (10 %) isolates showed moderate biofilm formation and 1/10 (10 %) was weak biofilm producer, while 4/14 (28.5 %) could not form any detectable biofilm comparison with control (Fig. 4).



In the biofilm formation test, *S. aureus* isolates, which successfully have transformed by heat shock method, using modified *tetK*-siRNA sequence and unmodified *tetK*-siRNA sequence, were chosen for this test only. Based on the above, two aliquots (Modified and Unmodified *tetK*-siRNA sequences) from each isolate were taken. Each aliquot-separately- was tested to form a biofilm, using (the tube method). Comparison with control (aliquot from the same samples but untreated), most of the isolates did not form biofilms and the results were as follow: Out of 4 aliquots treated with modified *tetK*-siRNA sequence, 3 / 4 (75 %) were without biofilm formation, while 1 / 4 (25 %) formed short biofilms. Whereas, treated with unmodified *tetK*-siRNA sequence, 2 / 4 (50 %) were without biofilm formation, while 2 / 4 (50 %) formed short biofilms (Tab. 3), (Fig. 5), (Fig. 6) and (Fig. 7).

Table 3
Represent of isolates that biofilm positive with biofilm negative, after treatment with *tetK*-siRNA

	Positive biofilm degree					**Biofilm Formation**	
	***tetK*-siRNA**		Strong	Moderate	Weak	+	-
S. aureus	Modified	No.	0	0	1	1	3
		%	0 %	0 %	100 %	25 %	75 %
	Unmodified	No.	1	1	0	2	2
		%	50 %	50 %	0 %	50 %	50 %

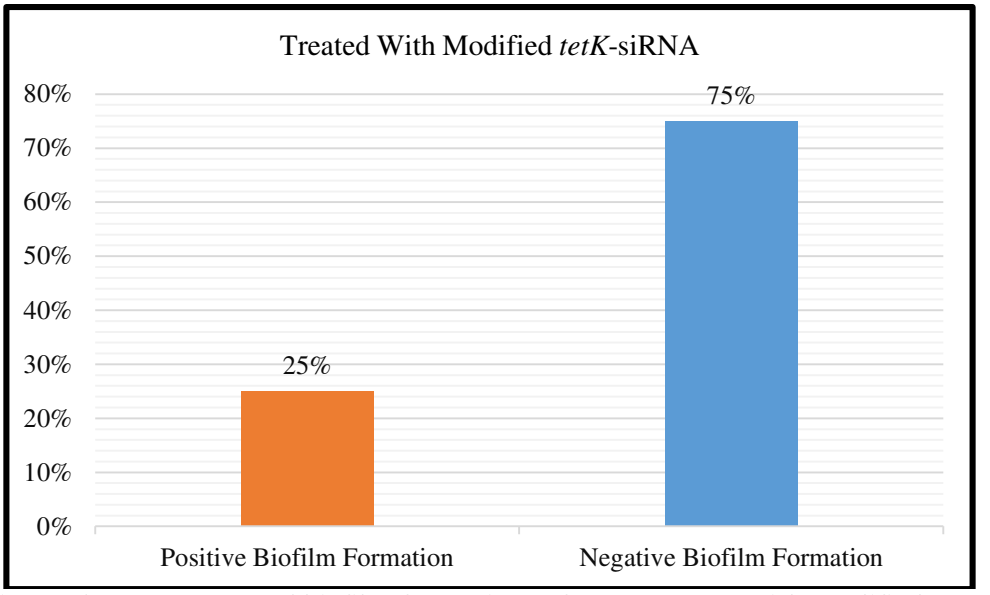


Figure 5. Percentages of biofilm formation, after treatment with Modified *tetK*-siRNA sequence

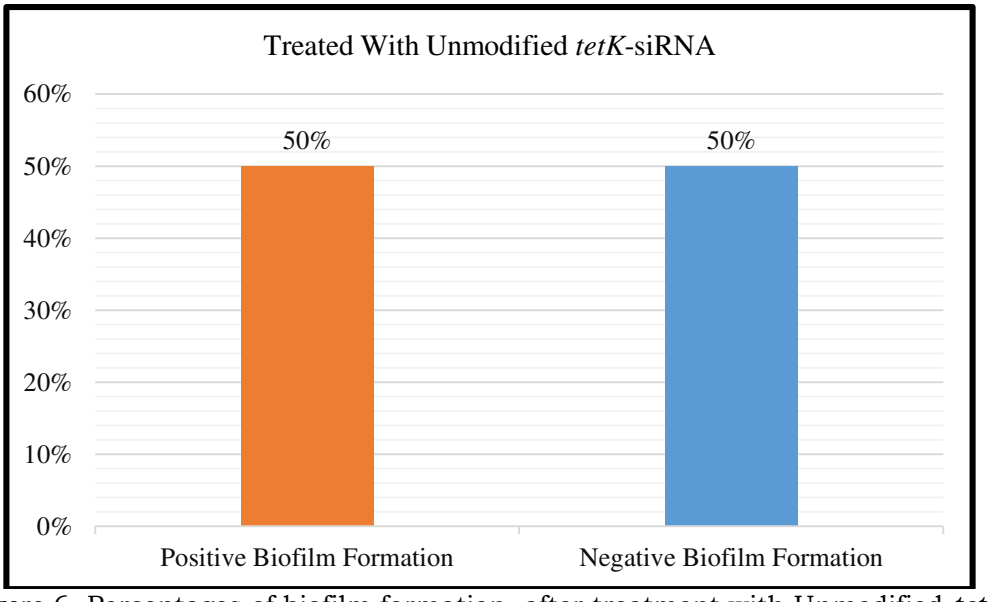


Figure 6. Percentages of biofilm formation, after treatment with Unmodified *tetK*-siRNA sequence

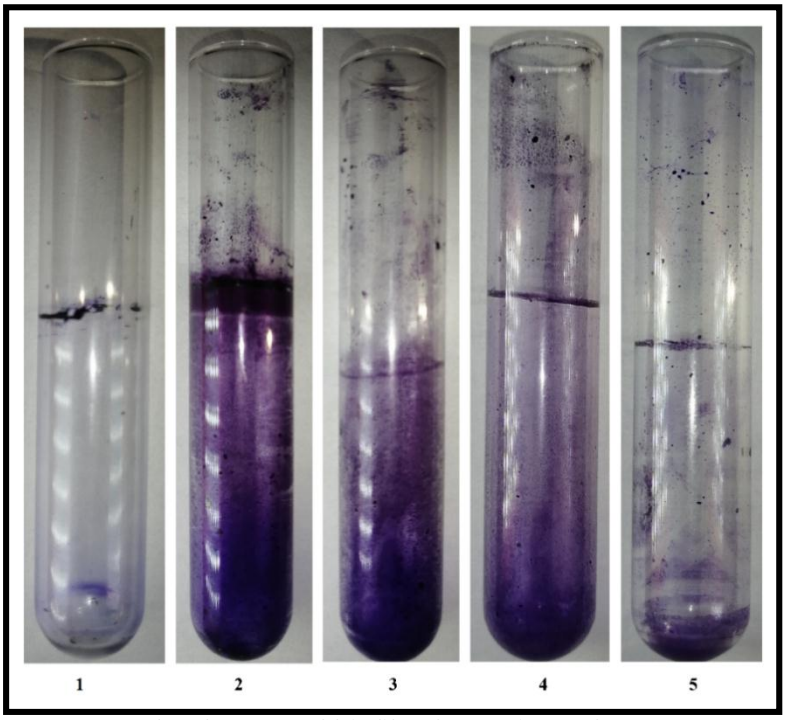


Figure 7. Represent the degrees of biofilm formation, after treatment with both *tetK*-siRNA (Modified) and *tetK*-siRNA (Unmodified) sequences, (1) without *S. aureus* and without siRNA trated, (2) *S. aureus* without siRNA treated, (3) biofilm formation strongly after treatment with siRNA, (4) biofilm formation moderately after treatment with siRNA and (5) biofilm formation weakly after treatment with siRNA

These results- totally- were consistent with those, of Gong, *et al.*, (2014) found that using antibiotics and RNA interference of the small regulatory RNA [(riboregulator Y) (*rsmY*)] and small regulatory RNA [(riboregulator Z) (*rsmZ*)] genes effectively reduced biofilm formation in *P. aeruginosa* (Gong, *et al.*, 2014). In addition, Thompson *et al.* found that biofilm formation, significantly ($p < 0.05$), decreased in siRNA treated *S. aureus* strains, as a result of targeting Staphylococcal accessory regulator (*sarA*) and Accessory gene regulator A (*agrA*) which they related to Macrolides and Streptogramins Resistance A (MsrA) efflux pump protein (Thompson *et al.*, 2021). On the other hand, Loratadine decreased biofilm development in *S. aureus*, according to (Cutrona *et al.*, 2019), whereas Zheng *et al.* discovered that loratadine analogues selected their capacity to reduce *S. aureus* biofilm development and pathogenicity (Zheng *et al.*, 2022). In addition, *S. aureus* biofilm biomass was reduced by 51 %, 49 %, and 50 % respectively, when azoles, such as clotrimazole, tioconazole, econazole, and tetracycline, were combined with tetracycline to disrupt TetK efflux pumps (MFS transporter). Successfully performed biofilms were significantly reduced when azol es and tetracycline were combined (Mahey *et al.*, 2021). Interesting, two phytochemicals, 7-hydroxycourmarin and indole-3-carbinol, were found to prevent biofilm production in *S. aureus* cells overexpressing *tetK* (Monte *et al.*,

2014). Antisense is a type of complementary sequence that interacts to its target mRNA and suppresses the expression for the same gene (Moustafa *et al.*, 2021).

Conclusions

In vitro, siRNAs suppressed both mRNA expression of the *tetK* gene and the function of the *S. aureus'* TetK efflux pump, resulting in decreased biofilm formation compared to control. The use of siRNA sequences to target *tetK*-mRNA looks to be a new method for treating *S. aureus* 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. aureus* treatment approaches.

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