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Targeting the quorum sensing mechanism in gram-positive strains using therapeutic peptides: A promising quorum quenching approach

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Abstract: The rising incidence of antibiotic resistance in bacterial pathogens, notably *methicillin-resistant Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (*S. epidermidis*), poses a considerable challenge to global public health. Conventional antibiotic treatments are increasingly undermined by the swift emergence of resistance. In light of this, anti-virulence strategies have gained attention as a viable alternative, concentrating on inhibiting the production of virulence factors instead of directly eliminating bacteria. The current study investigated the anti-virulence properties of the free QQ-5 peptide in relation to both *S. epidermidis* and MRSA. *In vitro* experiments, including a hemolysis assay, revealed a reduction in hemolytic activity for both bacterial strains upon the introduction of the QQ-5 peptide. Additionally, the MTT assay conducted with endothelial cells validated the safety profile of QQ-5, as no significant cytotoxic effects were detected. Moreover, kinase activity assays indicated that QQ-5 effectively and inhibits the phosphorylation of *AgrC*, highlighting its mechanism of action. The results of these studies suggest that QQ-5 specifically inhibits *AgrC* kinase in the agr system, representing a mechanistically distinct approach compared to conventional quorum-quenching strategies. QQ-5 exhibits robust anti-virulence activity and holds significant promise as a therapeutic agent for the treatment of MRSA and *S. epidermidis* infections.

Keywords: Anti-virulence; *Methicillin-resistant Staphylococcus aureus;* QQ-5 peptide; Resistance; *Staphylococcus epidermidis*

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INTRODUCTION

The escalating prevalence of antibiotic resistance among bacterial pathogens, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), and *Staphylococcus epidermidis* (S. *epidermidis*) represents a significant threat to global public. Traditional antibiotic therapies, which rely on bactericidal or bacteriostatic mechanisms, are increasingly compromised by the rapid evolution of resistance. In response, anti-virulence strategies have emerged as a promising alternative, focusing on the disruption of virulence factor production rather than direct bacterial killing (D'Aquila *et al*., 2024). In MRSA, the expression of virulence genes is predominantly regulated by the accessory gene regulator (*Agr*) quorum sensing (QS) system. The *agr* system, in particular, plays a pivotal role in modulating the production of virulence factors in gram positive bacteria. The *agr* signaling cascade is initiated by the binding of an autoinducing peptide (AIP), a thiolactone-containing signaling molecule, to the membrane-embedded sensor histidine kinase, *AgrC.* This interaction triggers a two-component signaling pathway, culminating in the activation of virulence gene expression (Tan *et al*., 2022).

Four distinct *agr* subgroups have been identified in MRSA, each characterized by the production of a unique AIP and its cognate *AgrC* receptor. Notably, while each subgroup's AIP activates its corresponding *AgrC*, heterologous AIPs often exhibit inhibitory effects, a phenomenon known as *agr* interference. This cross-inhibition has spurred considerable interest in leveraging native AIPs for the development of quorum quenchers (QQs)—therapeutic agents designed to disrupt QS and attenuate bacterial virulence (Pereira *et al*., 2022).

Therapeutic peptides face two major inherent limitations: membrane impermeability and poor in vivo stability, which significantly hinder their development as effective drugs. (Lau & Dunn, 2018). Recent advancements in peptide discovery technologies, such as the Random Non-standard Peptide Integrated Discovery (RaPID) system, have facilitated the identification of novel macrocyclic peptides with potent *agr* quorum quenching activity (Wiedmann *et al*., 2020). These peptides competitively inhibit *AgrC* by binding to its sensor domain, thereby blocking the activation of the *agr* signaling cascade and reducing the production of virulence factors. This approach represents a paradigm shift in antimicrobial therapy, as it targets the upstream regulatory mechanisms of bacterial physiology, thereby suppressing a broad spectrum of virulence factors. In contrast, conventional antibiotics typically target specific bacterial proteins, which often leads to the rapid emergence of resistance (Mayville *et al*., 2020). The therapeutic potential of QQs has been further explored through the development of nanoparticle-based delivery

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systems. For instance, QQ-3 peptide-loaded nanoparticles have demonstrated efficacy against MRSA strains, highlighting the feasibility of this delivery approach (Alenazi *et al*., 2024). Using delivery system is a logical solution to overcome the stability/permeability challenges observed with the peptide.

Building on these findings, this study has investigated the anti-virulence efficacy of the free QQ-5 peptide against both *S. epidermidis* and MRSA. In vitro assays, including hemolysis test that showed inhibition in hemolysis activity for both *S. epidermidis* and MRSA when adding the QQ-5 peptide. In addition, the MTT assay using endothelial cells, have confirmed the safety profile of QQ-5, with no significant cytotoxicity observed. Furthermore, kinase activity assays have demonstrated that QQ-5 effectively inhibits *AgrC* phosphorylation, underscoring its mechanism of action.

MATERIALS AND METHODS

QQ-5 compound was purchased from MCE (Med Chem, China). Bacterial strains wild type obtained from the library of MRSA (ATC32200) transposon from the University of Washington Genome Centre (Washington University, Washington, Seattle, USA). Endothelial cells proliferation kit I (MTT) was provided from American Type Culture Collection (ATCC). Prism software (GraphPad, Inc.) was utilized for conducting statistical analyses. The determination of statistical significance was achieved through the application of the Student's t test, one-way analysis of variance (ANOVA). A P value of less than 0.05 was regarded as indicative of statistical significance.

In-vitro kinase assay

The in vitro kinase activity was assessed using the Universal Kinase Assay Kit (abcam). Bacterial cells were lysed in ice-cold cell lysis buffer via sonication, followed by centrifugation at 10,000 × g for 20 minutes at 4°C. The resulting supernatant was collected and utilized as the substrate for the kinase assay. The kinase reaction was initiated in a 96-well plate by adding a kinase reaction mixture, which included ADP assay buffer, 100µM ATP, 10mM $MgCl_2$, 4mM $MnCl_2$ and a protease inhibitor cocktail, along with the cell lysate. The plate was incubated at room temperature for 1 hour. Subsequently, 20µL of the kinase reaction mixture was combined with 20µL of ADP sensor buffer and 10µL of ADP sensor to monitor ADP formation. The mixture was incubated in the dark for 1 hour. Finally, the fluorescence intensity of the ADP products was measured using a microplate fluorescence reader at excitation and emission wavelengths of 540 nm and 590 nm, respectively.

Cytotoxicity MTT assay

Endothelial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. For the experiments, cells were seeded at a density of 5×10^3 cells per well in 96-well plates and allowed to adhere overnight. Following adherence, the cells were treated with varying concentrations of the peptide, ranging from 3µg/mL to 30 µg/mL, and incubated for an additional 48 hours under the same conditions. Cell viability was subsequently assessed using the MTT assay (Kit I). The absorbance was measured at 570 nm using a microplate reader to quantify the results.

Hemolytic activity analysis

Bacterial strains were cultured overnight and adjusted to a 0.5 MacFarland standard. A small volume of the broth suspension was then combined with QQ-5 peptide. This mixture was subsequently spotted onto sheep blood agar and incubated overnight at both 37°C and 4°C for 24 and 18 hours, respectively (Wang *et al*., 2020). The hemolytic activity was assessed by observing the transparency surrounding the colonies on the plates.

Statistical analysis

Data analysis was conducted using Version 9 of GraphPad Prism, employing unpaired t-tests to perform statistical comparisons between the control group and the treated group.

RESULTS

In-vitro Kinase assay result

The results in the fig. 1 show that adding QQ-5 peptide, results in the inhibition of phosphorylation and kinase activity. A decrease in kinase activity is indicated by a decrease in fluorescent intensity. The QQ-5 compound, which specifically targets the histidine kinase in the *agr* system, exhibited a notable reduction in kinase activity compared to the control of untreated bacterial cells. This outcome serves as confirmation that QQ-5 functions as a competitive inhibitor for the histidine kinase within the two-component system.

In vitro cytotoxicity

Different doses range 10µg/ml to 50µg/ml were used to assess the peptide cytotoxicity against the very sensitive cell model endothelial cells. The result shown in fig. 2. The QQ-5 peptide was cytocompatible to concentration up to 50µg/ml ($p<0.01$).

Hemolytic assay

The hemolysis activity on blood agar for MRSA and *S. epidermidis* were inhibited by QQ-5 peptide as shown in figs. 3-4 with the following concentration 10 to 50 µg/ml. The *S. epidermidis* hemolysis was completely inhibited with all concentration, while, for MRSA as it is a highly resistance strain the hemolysis activity was limited with all concentration.

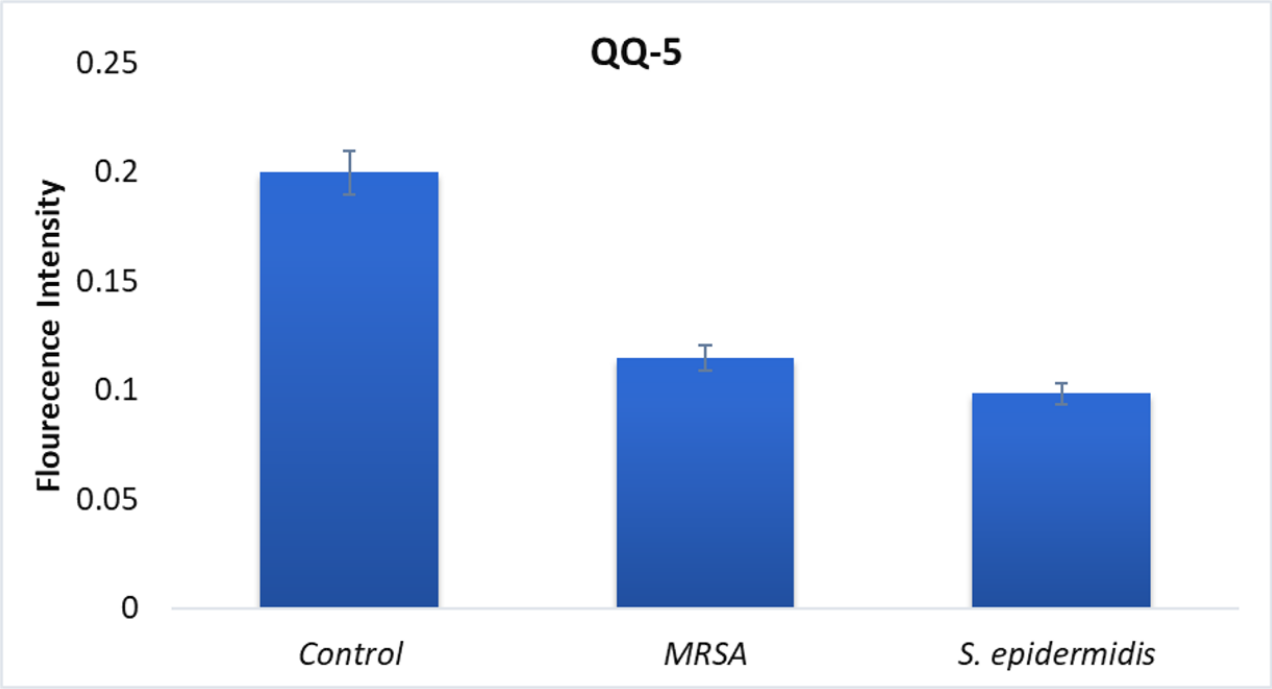

Fig. 1: The in-vitro Kinase assay, decrease in kinase activity in respect to control. Fluorescence intensity decrease indicating reduced kinase activity after QQ-5 treatment (mean ± SD, n=3) ($p < 0.05$).

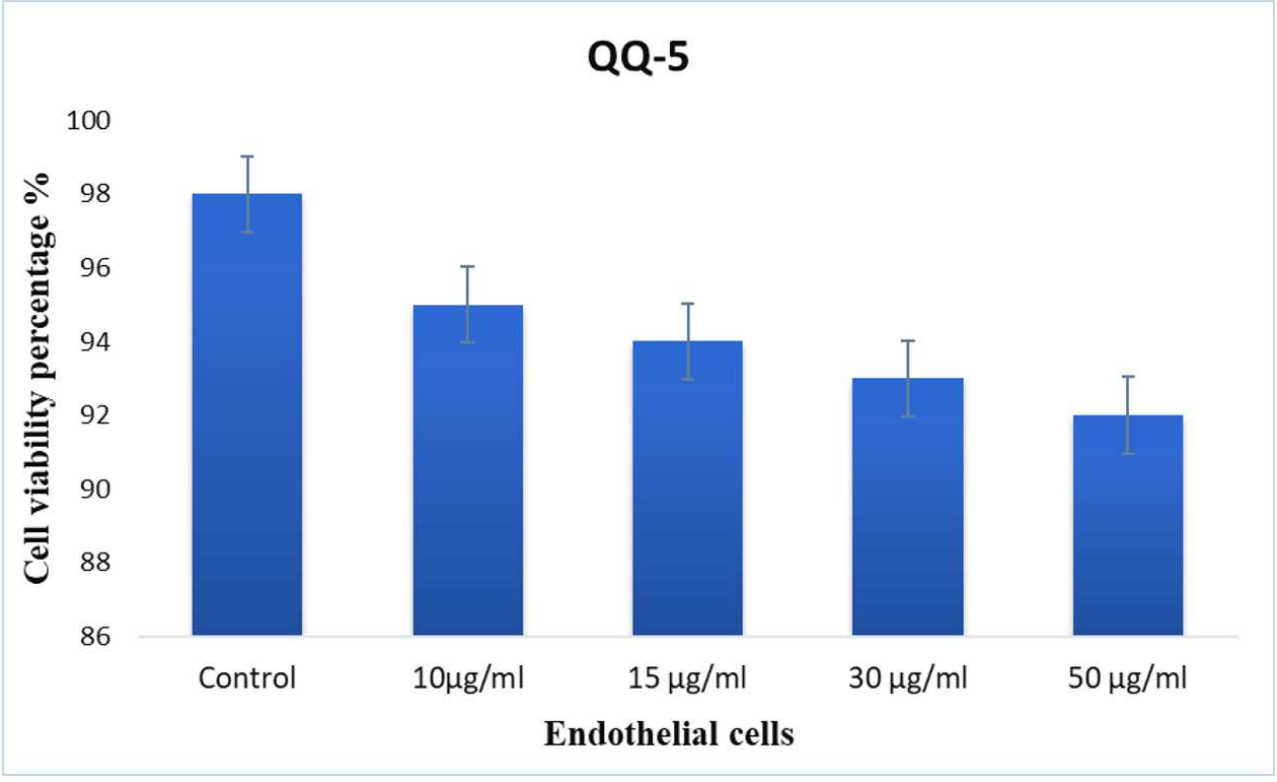

Fig. 2: MTT assay for the QQ-5 peptide, no significant decrease in viability at all concentration used. (mean ± SD, n=3) ($p<0.05$).

Fig. 3: Hemolysis activity, a) *S. epidermidis* treated with QQ-5 peptide, the hemolysis activity was inhibited with all concentration used (10- 50µg/ml)

Fig. 4: Hemolysis activity, a) MRSA treated with QQ-5 peptide, the hemolysis activity was limited with all concentration used (10- 50 µg/ml).

DISCUSSION

MRSA is a leading pathogen contributing to antimicrobial resistance (AMR) in both community and healthcare settings.

The pathogenicity of many bacterial infections is multifactorial, making it challenging to pinpoint the exact contribution of each virulence factor. Previous research has demonstrated that mutations in the *agr* locus result in the loss of virulence factor expression, highlighting the critical role of this system in bacterial pathogenicity (Sharma *et al*., 2019).

Targeting QS, particularly through histidine kinase (HK) inhibition, has emerged as a promising strategy to attenuate virulence in resistant strains. The present study aimed to assess the efficacy of the QQ-5 peptide in targeting and inhibiting the kinase within the two-component system (TCS). A kinase reaction assay was employed, with a reduction in fluorescent intensity serving as an indicator of decreased kinase activity. The assay results demonstrated that the QQ-5 peptide functions as a competitive inhibitor of the histidine kinase in the TCS, confirming its ability to disrupt kinase activity.

The present study utilized endothelial cells as a model to evaluate the cytotoxicity of the peptide. This cell line is widely regarded as a suitable and sensitive model for such investigations. The findings demonstrated that the QQ-5 peptide displayed cytocompatibility at concentrations of up to 50µg/ml, highlighting its potential for further development as a therapeutic agent.

One of the most potent virulence factors employed by MRSA is the secretion of toxins (α, β, γ, δ), which play a critical role in promoting tissue damage. Reducing these toxin can significantly diminish the severity of infections and delay biofilm formation (Mansour *et al*., 2021). The results of hemolysis assay revealed that QQ-5 effectively inhibited the hemolytic activity of *S. epidermidis* at all tested concentrations. While the impact of QQ-5 on MRSA had not been previously investigated, the study demonstrated that the hemolytic activity of MRSA was also significantly reduced upon the addition of the QQ-5 compound. These findings suggest that QQ-5 holds promise as a potential therapeutic agent for mitigating the virulence of both *S. epidermidis* and MRSA.

This study explored the antimicrobial efficacy of the histidine inhibitor peptide QQ-5 against a highly resistant strain of MRSA and wild-type *S. epidermidis*. The QQ-5 peptide was internalized by the bacterial cells and competitively bound to the sensor histidine kinase *AgrC*, thereby inhibiting the interaction between autoinducing peptide (AIP) and *AgrC*. This disruption of the *agr* quorum-sensing system led to the suppression of key virulence factor production in both *S. epidermidis* WT and MRSA. The results of these studies suggest that QQ-5 has anti-virulence efficacy and holds significant promise as a therapeutic agent for the treatment of MRSA and *S. epidermidis* infections. By combining QQs with conventional antibiotics, it may be possible to develop synergistic antimicrobial therapies that minimize the risk of resistance development. This dual approach not only enhances the efficacy of existing treatments but also aligns with the urgent need for innovative strategies to combat antibiotic-resistant pathogens.

CONCLUSION

Targeting the *agr* quorum-sensing system with quorum-quenching compounds like QQ-5 presents a promising anti-virulence strategy against *Staphylococcus aureus*. Future studies should prioritize optimizing the delivery mechanisms and pharmacokinetic profiles of these peptides, alongside rigorous evaluation in preclinical and clinical models. These advancements will be essential for translating these findings into effective therapeutic interventions against MRSA and other antimicrobial-resistant infections.

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NA, design of study, methodology, chemical analysis and writing final draft. HKA, methodology, chemical analysis and writing. Both leading authors made equal contributions.

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Conflicts of interest
The authors declare no conflict of interest.

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