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Original Article

Chlorhexidine's potential in inhibiting *Pseudomonas aeruginosa* biofilm formation: A reverse docking study on quorum sensing proteins

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ABSTRACT

Ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* poses a significant clinical challenge due to its robust biofilm formation and resistance mechanisms. Chlorhexidine (CHX), an antiseptic agent, has shown potential in inhibiting biofilm formation, though its mechanism is not fully understood. To elucidate the mechanism of action and potential of chlorhexidine in mitigating biofilm formation, we conducted reverse docking analyses targeting key quorum-sensing proteins in *P. aeruginosa*. Protein structures of six quorum sensing and biofilm proteins were obtained from the Protein Data Bank. The structure of CHX was retrieved from PubChem and prepared for docking. Potential binding pockets in the protein structures were identified using Fpocket, and docking simulations were performed with SMINA. We generated 395 docking poses across all proteins. The highest binding affinity was observed at PslG. Additionally, CHX's high binding affinities with RhlR and LasR indicate its potential to interfere with quorum sensing pathways. CHX shows strong binding affinities to key quorum-sensing proteins, particularly PslG, RhlR, and LasR. This suggests CHX could disrupt biofilm formation and quorum sensing in *P. aeruginosa*.

Keywords: Ventilator-associated pneumonia, Pseudomonas aeruginosa, Chlorhexidine, Quorum sensing, Biofilm

Introduction

Multi-drug-resistant (MDR) bacteria pose a major medical threat, particularly *Pseudomonas aeruginosa*, which frequently develops resistance to antimicrobial agents and causes ventilator-associated pneumonia (VAP) [1, 2]. Quorum sensing (QS) is a key regulator of virulence in *P. aeruginosa*, making it an ideal target for anti-virulence strategies that minimize the risk of resistant clones [3].

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QS is an intercellular signaling system crucial for biofilm formation and maintenance in *P. aeruginosa*, regulating gene expression through molecules like N-acyl homoserine lactones (AHLs) [4]. The las system produces the signaling molecule 3-oxo-C12-HSL via LasI, which binds to LasR, while the rhl system produces C4-HSL via RhlI, binding to RhlR to regulate genes essential for biofilm and virulence, such as pel [3].

The stability of *P. aeruginosa* biofilm structure relies on polysaccharides such as alginate, pel, and psl, which serve as primary scaffolds during initial biofilm development. The synthesis of these polysaccharides is regulated by c-di-GMP, with higher concentrations promoting the production of alginate and pel, while lower concentrations enhance bacterial motility [5]. PslG is crucial for Psl biosynthesis, potentially processing the growing polysaccharide or removing aberrant polymers; however, its precise role remains unclear. Overexpression of PslG decreases Psl production, suggesting its hydrolase activity is tightly regulated by other Psl proteins to ensure proper biofilm formation [6].

A systematic review and meta-analysis found that the oral application of CHX significantly reduced the incidence of ventilator-associated pneumonia (VAP) in mechanically ventilated ICU patients (RR, 0.73 [95% CI, 0.55, 0.97]). However, the study did not find a significant effect on all-cause mortality (RR, 1.13 [95% CI, 0.96, 1.32]), indicating that while CHX is effective in preventing VAP, its impact on mortality rates requires further investigation due to the low quality of evidence [7].

To better understand how CHX works, especially in relation to quorum sensing and biofilm proteins, a detailed molecular docking study is essential [8]. This study aims to pinpoint the specific proteins that CHX might interact with within the quorum sensing and biofilm formation pathways of *P. aeruginosa*. By identifying potential protein targets, the research seeks to optimize the clinical application of CHX, enhancing its effectiveness in preventing and treating VAP.

Materials and Methods

Data preparation

The three-dimensional structures for six quorum sensing proteins from Pseudomonas aeruginosa—specifically LasR (PDB ID: 2UV0), LasL (1RO5), Psl (5BXA), PelA (5TCB), PelB (5WFT), and RhlR (8DQ0)—were sourced from the Protein Data Bank. Only the dimer forms of LasR and RhlR were used, acknowledging their propensity to dimerize [9, 10]. The proteins underwent sanitization with LePro, which included adding hydrogen atoms while accurately considering the protonation states of histidine residues. During this process, all crystal waters, ions, small ligands, and cofactors were removed to preserve the proteins' structural integrity. Chlorhexidine (CID: 9552079) was chosen as the ligand, with its isomeric SMILES representations retrieved from PubChem to generate 3D structures using Pybel. Each structure was then optimized through 500 steps of the MMFF94s force field.

Pocket detection, molecular docking, and fingerprint analysis

To identify potential binding pockets, protein structures were first cleaned and then analyzed using fpocket, focusing on pockets with a volume of at least 250 Å³ [11]. The selected pockets were visualized in PyMOL, where the getbox function was used to determine their centers and dimensions, extending the selection by 4.0 Å. Ligand docking into these pockets was performed using Smina software, with parameters set for pocket center coordinates, dimensions, exhaustiveness of 8, and up to 5 binding modes [12]. Docking results were saved in SDF format for further analysis [13]. Py3Dmol was utilized to visualize the 3D interactions between proteins and ligands [14]. The SDF files were parsed to extract essential information such as binding modes and affinities, which were then compiled into a data frame for detailed examination.

Results and Discussion

The analysis identified multiple potential binding pockets across six proteins: LasL with 11, LasR with 29, PslG with 35, PelA with 11, PelB with 7, and RhlR with 46 pockets. However, only pockets with a volume of at least 250 Å³ were considered. For each qualifying pocket, molecular docking generated 5 alternative poses, resulting in a total of 395 docking poses across all proteins [15]. This extensive docking study offers a comprehensive set of potential binding interactions for further evaluation **(Table 1)**. The pockets and ligands of each protein are visualized in **Figure 1**.

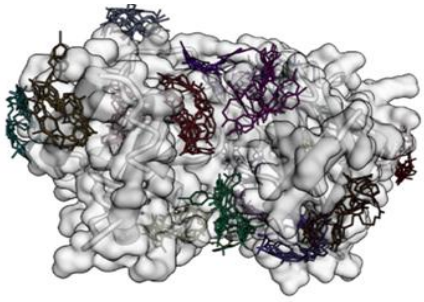
Table 1. Top 10 Binding Affinity Scores from Molecular Docking

Protein	Pocket ID	Affinity (kcal/mol)	Ligand
PslG	5BXA_pock_9	-9.30624	Chlorhexidine
RhlR	8DQ0_pock_4	-8.92511	Chlorhexidine
LasR	2UV0_pock_3	-8.85101	Chlorhexidine
PelA	5TCB_pock_1	-7.68052	Chlorhexidine
RhlR	8DQ0_pock_11	-7.59101	Chlorhexidine
RhlR	8DQ0_pock_36	-7.13941	Chlorhexidine
PslG	5BXA_pock_7	-7.12128	Chlorhexidine
RhlR	8DQ0_pock_19	-7.11689	Chlorhexidine
LasR	2UV0_pock_1	-6.99142	Chlorhexidine
PslG	5BXA_pock_36	-6.96334	Chlorhexidine

CHX exhibited the highest binding affinity to the PslG protein at pocket 9, with a binding score of -9.30624 kcal/mol. The molecular structure of CHX and its binding state in the PslG pocket 9 are visualized in **Figure 2** [16]. CHX also showed strong affinities for RhlR at pocket 4 (-8.92511 kcal/mol) and LasR at pocket 3 (-8.85101 kcal/mol). Additionally, CHX demonstrated notable binding to PelA at pocket 1 (-7.68052 kcal/mol) and multiple pockets within RhlR and PslG, with affinities ranging from -7.59101 to -6.96334 kcal/mol. These findings suggest that CHX has the potential to interact with key quorum sensing and biofilm formation proteins in *P. aeruginosa*, with PslG identified as the most promising target. However, CHX's effectiveness in disrupting biofilm-related processes and preventing VAP remains uncertain based on these results alone.

The results of this study highlight the potential of CHX as a significant inhibitor of biofilm formation in *P. aeruginosa*. Among the proteins analyzed, CHX exhibited the highest binding affinity with PslG at pocket 9, suggesting a strong interaction that may impede biofilm synthesis. The high binding affinities observed for CHX with RhlR and LasR further indicate its potential to disrupt quorum sensing pathways critical for biofilm development. Notably, CHX's interactions with multiple pockets across several proteins underscore its broad-spectrum potential, although its efficacy in vivo requires further investigation. These findings propose that PslG is CHX's main target, along with other key proteins, could be a promising strategy in preventing biofilm-

associated infections, such as VAP, though additional studies are necessary to confirm these preliminary insights and to evaluate the therapeutic viability of CHX in clinical settings. Biofilms of *P. aeruginosa* develop through a five-stage multicellular cycle, culminating in dispersal facilitated by self-generated enzymes [17]. Previous studies suggested that these enzymes, such as PslG, degrade the exopolysaccharide matrix to release biofilm seeds. PslG, an endoglycosidase targeting the Psl matrix, inhibits biofilm formation and disrupts pre-formed biofilms, sensitizing bacteria to antibiotics and macrophage attack [6]. The structural analysis of PslG revealed its significant role in biofilm disassembly, with the enzyme functioning as a monomer and containing distinct catalytic and carbohydrate-binding domains [6]. The high binding affinity of CHX to PslG pocket 9, coupled with its interactions with other biofilm-associated proteins, underscores the potential of CHX as a biofilm inhibitor. This in silico study suggests that CHX may affect biofilm formation and quorum sensing; however, the exact mechanisms and outcomes of these interactions remain unclear. Further investigation is needed to elucidate how CHX interacts with these proteins and to determine the practical implications for biofilm disruption and infection treatment [18].



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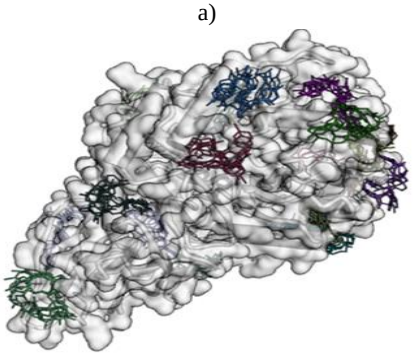

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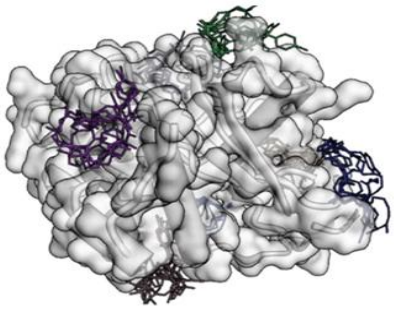

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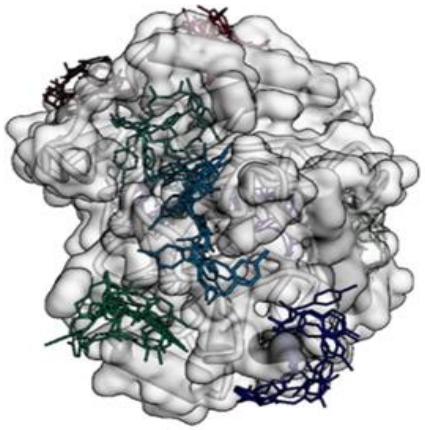

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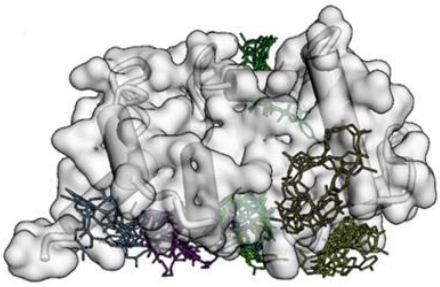

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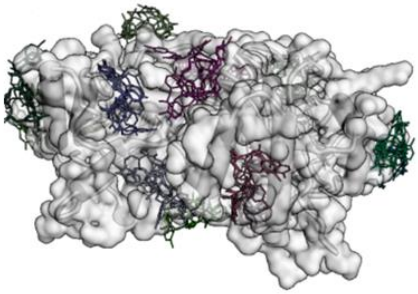

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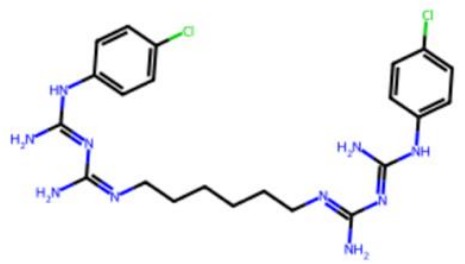
Figure 1. Molecular docking of a) LasI, b) PslG, c) LasR, d) PelA, e) PelB, and f) RhlR with chlorhexidine at identified pockets. Each pocket shows five poses of the ligand bound to the site.

P. aeruginosa often colonizes intubated patients, with 10-20% developing VAP, which has a high mortality rate of 30-40%. The QS circuit in *P. aeruginosa* regulates key virulence factors, including elastase, rhamnolipids, pyocyanin, and cyanide, which contribute to infection [19]. Targeting QS proteins may also be beneficial; this study showed that CHX could bind to numerous QS proteins with high affinity, such as RhlR and LasR. These interactions suggest that CHX could disrupt QS-regulated virulence, potentially preventing the development of VAP in colonized patients. Further research is needed to confirm these findings and explore the therapeutic potential of CHX in clinical settings.



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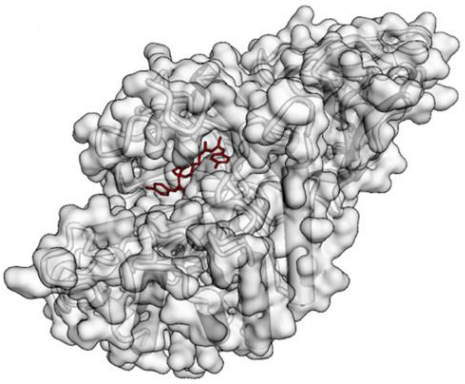

b)

Figure 2. a) Visualization of Chlorhexidine molecular structure and b) Interaction of Chlorhexidine with PslG Pocket 9 which has the highest binding affinity in this study.

Conclusion

This study highlights CHX as a potent inhibitor of biofilm formation and quorum sensing in *P. aeruginosa*. The high binding affinity of CHX to PslG and other key proteins suggests its potential to disrupt critical pathways in biofilm development and virulence. Further, in vivo studies are required to confirm these findings and evaluate the clinical viability of CHX as a therapeutic agent for preventing and treating biofilm-associated infections, such as VAP.

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