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Isolation, Characterization, and efflux pump-mediated antibiotic resistance in biofilm forming Staphylococcus Saprophyticus

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ABSTRACT

Staphylococcus saprophyticus, a coagulase-negative is getting more attention for its role in infections and hospital-acquired persistence. This study examines at the biofilm-forming abilities, antibiotic resistance profiles, and efflux pump activity of environmental S. saprophyticus isolates. Biofilm development showed increased biomass at 45°C and alkaline pH, indicating adaptive virulence. Antibiotic susceptibility testing revealed multidrug resistance, specifically to erythromycin, rifampicin, and tetracycline. Efflux pump activity was assessed using the ethidium bromide cartwheel method, with fluorescence intensity inversely proportional to efflux efficiency. Isolates that showed substantial biofilm development also had active efflux systems, indicating a synergistic resistance mechanism. These findings highlight S. saprophyticus clinical importance as a resilient pathogen, as well as the necessity for integrated therapy strategies that address both biofilm and efflux-mediated resistance. The study recommends increased environmental surveillance and the potential use of efflux pump inhibitors to restore antibiotic efficacy.

Keywords: *Biofilm, Efflux pump, Rifampicin, Staphylococcus saprophyticus, Antibiotic susceptibility*.

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1. INTRODUCTION

Staphylococcus saprophyticus is a Gram-positive, coagulase-negative staphylococcus that distinguishes itself as both a harmless commensal and an opportunistic pathogen(Ali, Naser et al. 2024). Its ability to build biofilms, which are structured microbial communities enclosed in an extracellular polymeric substance (EPS) including polysaccharides, proteins, lipids, and extracellular DNA, is critical to its transformation from commensal to pathogen (Karygianni, Ren et al. 2020). Biofilms allow bacteria to cling securely to both biotic and abiotic surfaces, such as uroepithelial cells and urinary catheters. More crucially, efflux pump activity is a major mechanism of antimicrobial resistance in *S. saprophyticus* (Lila, Rajab et al. 2023). Efflux pumps are membrane proteins that actively evacuate harmful chemicals, decreasing intracellular drug concentrations and allowing bacteria to survive under antibiotic treatment (Huang, Wu et al. 2022). Transporters such as NorA and NorB are well-studied in *Staphylococcus aureus*, but their roles in *S. saprophyticus* are less well understood, despite evidence that they contribute significantly to multidrug resistance (Stephen, Salam et al. 2023). Importantly, biofilm formation and efflux activity are mutually reinforcing processes. Efflux pumps control quorum-sensing signals and may influence biofilm-associated phenotypes, and biofilm-associated stress conditions frequently induce efflux pump expression (Subhadra, Kim et al. 2018). The limited therapy options make therapeutic tasks even more difficult. *S. saprophyticus* has innate resistance to novobiocin, which is important for diagnostic purposes but can be troublesome in terms of reducing therapy options (Syiemlieh 2020). Despite its clinical importance, there are significant gaps in knowing how biofilm capacity, efflux pump activity, and resistance phenotypes interact in *S. saprophyticus* (Martins, Ferreira et al. 2019). Unlike *S. aureus* or *S. epidermidis*, investigations relating efflux activity to biofilm formation and multidrug resistance in this species are limited. Closing this gap is critical, as recurring infections caused by resistant *S. saprophyticus* contribute to antibiotic overuse, and findings about efflux pump roles may pave the way for therapeutics utilising efflux inhibitors or anti-biofilm methods. With this in mind, the current work aims to identify and characterise *S. saprophyticus* from clinical samples, assess its potential to form biofilms, quantify its antibiotic resistance, and analyse efflux pump activity using the ethidium bromide cartwheel method (Favour and Isaac 2020). By linking these factors, the researchers hope to gain a better understanding of how biofilm formation and efflux pump activity combine to produce resistance and persistence in this pathogen (Alav, Sutton et al. 2018). These findings will help us understand *S. saprophyticus* pathogenic

methods, develop more effective therapeutics for recurrent UTIs, and highlight the potential of combining conventional antibiotics with efflux pump inhibitors or anti-biofilm medicines (Mancuso, Trinchera et al. 2024). Such techniques are critical for improving outcomes in urinary tract infections and addressing growing resistance trends caused by biofilm-forming *S. saprophyticus* (Hashemzadeh, Dezfuli et al. 2021).

2. MATERIAL AND METHODS

Bacterial isolation

A total of 150 samples were taken at random from various hospital facilities in Raipur District, Chhattisgarh, India, including patient beds, dustbins, doors, and washroom basins. Each sample was first enriched by being inoculated into Luria Bertani (LB) broth. The enrichment cultures were incubated at 37°C overnight to allow bacteria in the samples to grow (Tessema 2015). After enrichment, a loopful of each overnight culture was aseptically streaked onto LB agar plates, which were made by supplementing LB broth with 1.5% agar and autoclaved to extract distinct bacterial colonies. The agar plates were then incubated overnight at 37°C under aerobic conditions.

Crystal violet assay

A qualitative microtiter plate-based biofilm analysis was used to validate isolate biofilm development. Each isolate was incubated in sterile LB broth at 37°C overnight. All pathogenic isolates were grown overnight in an LB agar slant at 37°C. Following incubation, a saline suspension was made by combining the bacterial growth with sterile saline, and the optical density (OD) was adjusted to 0.5 at 600nm (Sabaeifard, Abdi-Ali et al. 2014). The well was filled with a 20µl saline suspension of the isolate and 180µl of sterile LB broth. The microtiter plate was covered with parafilm and incubated at 37°C overnight. After the overnight incubation, the media was withdrawn from the wells, and the microtiter plate wells were washed five times with sterile distilled water to eliminate any loosely associated bacterial cells. Plates were air dried for 45 minutes before each well was stained with 200 ul of 0.1% crystal violet solution in water for 30 minutes. After staining, the plates were gently rinsed with sterile distilled water five times. To destain the wells, 200 ul of 33% acetic acid was added (Duraipandiyan, Sasi et al. 2010). At this stage, biofilm growth is visible as a blue ring on the sidewall or at the bottom of each well. The isolates that caused the ring development were identified as biofilm-forming bacteria. These isolates were chosen for further investigation. The ability of these isolates to produce biofilms was also validated using the Congo red assay.

Antibiotic Susceptibility test

The identified isolates of *Staphylococcus saprophyticus* were tested for antibiotic sensitivity using the agar well diffusion method. Seven antibiotics from various classes were chosen, and concentrations were generated in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations. To test, lawn cultures of the isolates were created by equally spreading bacterial suspension across the surface of Luria Bertani agar plates. Wells of uniform size were aseptically made on the agar with a sterile pipette tip or borer (Kaur 2019). Each well was filled with a particular volume of antibiotic solution using a pipette. The plates were then incubated at 37°C overnight to allow bacterial growth and diffusion of the antibiotics into the medium. After incubation, the zones of inhibition surrounding the wells were measured in millimeters using a scale. The results were interpreted based on CLSI standards to determine susceptibility or resistance. Isolates showing resistance to two or more antibiotic classes were classified as multidrug-resistant (MDR) strains. This method provides a reliable evaluation of the antimicrobial susceptibility profile of *S. saprophyticus* isolates.

The efflux pump activity was determined using the ethidium bromide cartwheel test.

The efflux pump activity of *S. saprophytics* isolates was evaluated using the ethidium bromide (EtBr) cartwheel test, as described by (Favour and Isaac 2020), with modest modifications. On the same day of the experiment, LB agar plates were made with different concentrations of EtBr (0 mg/L, 0.5 mg/L, 1 mg/L, 1.5 mg/L, and 2 mg/L). Each S. saprophyticus isolate (about 106 cells/mL) was streaked in a cartwheel pattern on EtBr plates. Plates were wrapped in aluminium foil to protect against light and incubated overnight at 37°C.

After incubation, the plates were inspected using UV light. The minimal EtBr concentration for fluorescence of bacterial colonies was determined.

3. RESULT

Isolation and confirmation of the *S. saprophyticus*

Biochemical characterization showed the isolate to be gram positive cocci. It tested negative for urease and citrate utilization, while exhibiting a positive coagulase reaction, consistent with the biochemical profile of *S. saprophyticus* these phenotypic and genetic data collectively confirm the identity of the isolate as *S. saprophyticus*. Fifteen morphologically diverse bacterial strains were identified and cultured on nutrient agar, revealing a wide range of colony features. Five isolates had a mucoid colony texture, four produced smooth and glossy colonies, four had an adherent, smooth morphology, and two had a very glossy appearance. These features were consistently observed when the strains were sub-cultured on

Luria Bertani agar, with mucoid, smooth, and very glossy colonies appearing across media. Gram staining helped to distinguish the collection, revealing 10 Gram-positive and five Gram-negative bacteria. Six isolates developed dark black colonies on Congo red agar, indicating exopolysaccharide synthesis and increased biofilm-forming capacity. To assess biofilm formation, all isolates were first screened using the glass tube adhesion test and the conventional microtiter plate assay. Six strains showed considerable biofilm formation across both techniques (Figure 1 & 2). Among them, one bacterial strain consistently displayed high biofilm development, as proven by significant attachment in both glass tube and polystyrene plate forms, and was so chosen for future study. The chosen strain, Staphylococcus saprophyticus, was identified by 16S rDNA sequencing and was originally isolated from hospital grounds through nanopore sequencing an the sequencing data were submitted to NCBI database and accession number has been granted (GenBank# PV91903. Phylogenetic analysis based on the sequencing results further supported the identification, clustering the isolates within the *S. saprophyticus* clade shown in (Figure 3). It is worth noting that bacteria from such hospital environments frequently exhibit broader and more robust resistance to antimicrobial agents than their planktonic counterparts, which is consistent with previous observations that biofilm-forming bacteria have improved defence mechanisms and adaptability in clinical settings. This discovery emphasises the therapeutic significance of environmental biofilm producers, specifically *S. saprophyticus*, as a reservoir for possible multidrug-resistant bacteria in healthcare facilities.

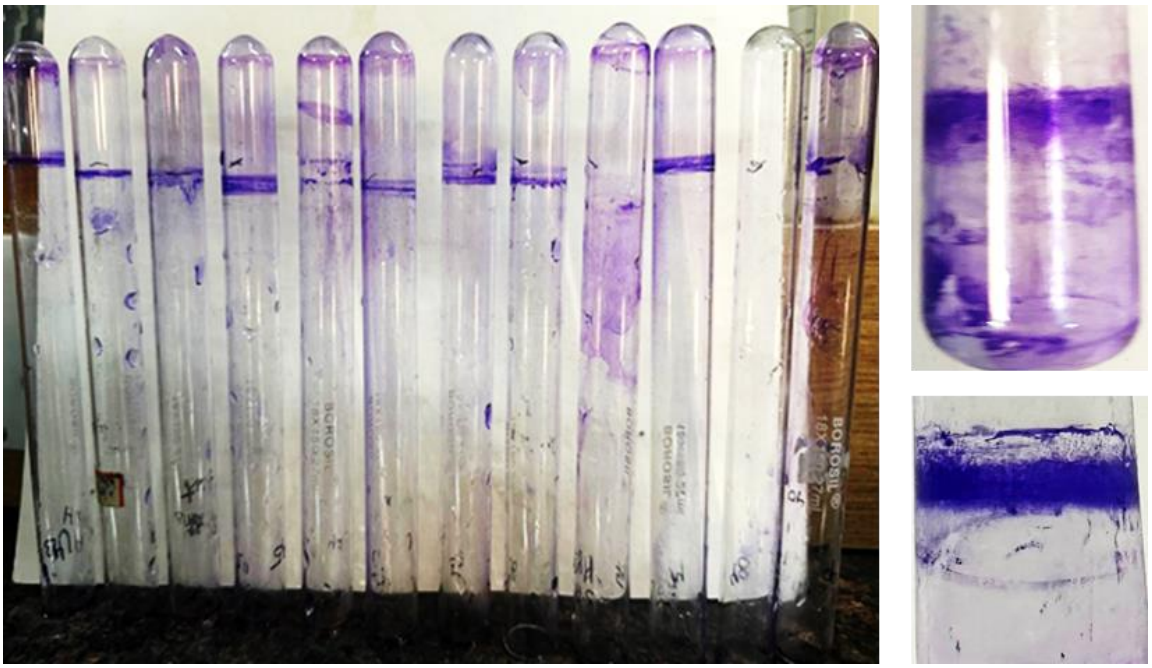

Figure 1. Stained tubes showing biofilm attachment, A. Comparative view of the tubes. B. Closed view of the selected strain

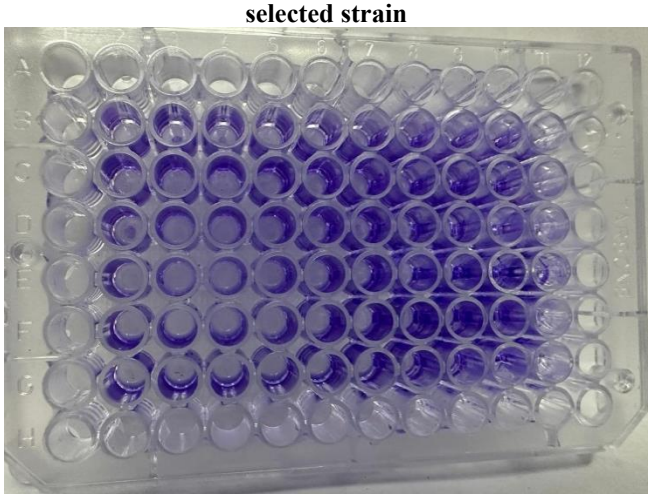

Figure 2. Biofilm screening of the isolated strains, in the microtire plate

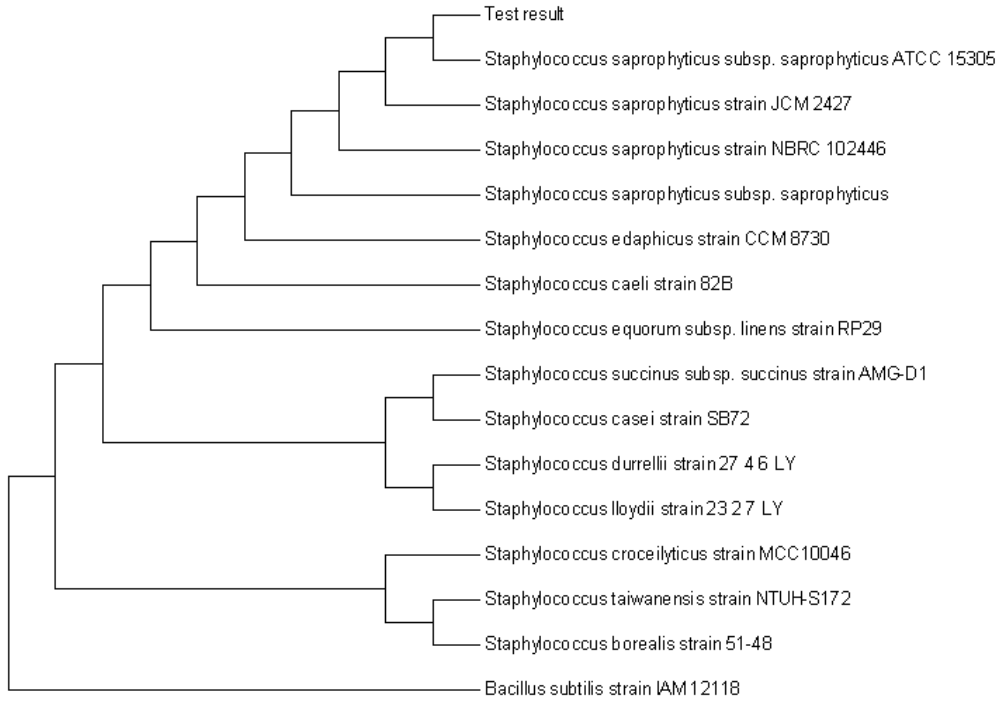

Figure 3. Phylogenetic analysis of the test strain (*Staphylococcus Saprophyticus*) based on 16s rRNA gene sequencing

Determination of antibiotic resistance pattern

Bacterial isolates showed distinct resistance patterns to antibiotics such as erythromycin, rifampicin, tetracycline, ampicillin, gentamicin, kanamycin, and streptomycin at concentrations of 100 µg, 80 µg, 50 µg, and 30 µg. CHW1 showed resistance to erythromycin, rifampicin, and streptomycin, demonstrating the presence of multidrug resistance pathways. CHW2 was resistant to erythromycin, rifampicin, and tetracycline, although CHW3 was resistant to ampicillin, rifampicin, and erythromycin. CHW4 was resistant to tetracycline, erythromycin, and rifampicin, but CHW5 was resistant solely to tetracycline as shown in Table 1. Several isolates were found to be resistant to erythromycin and rifampicin, as well as to tetracycline. The findings emphasise the importance of regular susceptibility testing and prudent antibiotic selection.

Table 1 Evaluation of bacterial resistance pattern for the different class of antibiotic

Antibiotic	**1**	**2**	**3**	**4**	**5**	**6**
Erythromycin	Resistance	Intermediate	Resistance	Resistance	Intermediate	Sensitive
Rifampin	Resistance	Resistance	Resistance	Resistance	Intermediate	Resistance
Tetracyclin	Intermediate	Resistance	intermediate	Resistance	sensitive	Resistance
Ampicillin	Resistance	Resistance	Resistance	Intermediate	sensitive	sensitive

Gentamycin	sensitive	Intermediate	Resistance	Intermediate	sensitive	sensitive
Kanamycin	sensitive	Intermediate	sensitive	Intermediate	Intermediate	sensitive
Streptomycin	Resistance	Resistance	Resistance	sensitive	Intermediate	Intermediate

Production of Biofilm at Various Concentrations, Temperatures, and pH Levels

The chosen bacterial strain was cultivated in a microtiter plate, and biofilm generation was quantitatively evaluated by measuring biomass at regular intervals. After 72 hr of incubation, biofilm development peaked at 1.62 ± 0.052 at 570 nm and thereafter decreased. This pattern is consistent with prior findings, which show that mature biofilm matrices peak at later stages of incubation before dispersal or nutrient constraint reduce biomass. Temperature and pH were the two most important environmental elements studied in terms of biofilm formation. At 45 °C, maximum biofilm formation occurred, with a corresponding absorbance of 1.543 ± 0.052 at 570 nm. This suggests that higher temperatures can improve biofilm development by boosting metabolic activity and extracellular polymeric material synthesis. Similarly, altering the pH of the medium revealed that alkaline circumstances substantially favoured biofilm accumulation for the specified strain as shown in (Figure 4). These findings are consistent with previous research, which has shown that biofilm development is generally facilitated at near-neutral to alkaline pH and optimal or slightly higher temperatures, environmental stressors such as pH shifts or suboptimal temperatures generally resulting in decreased biofilm output.

Microscopic analysis of the biofilm

The presented microscopic images show the findings of the crystal violet (CV) assay, which was used to assess biofilm development by bacterial isolates. The CV test is a commonly used quantitative and qualitative method for determining the presence and density of biofilms because crystal violet binds to the extracellular polysaccharide matrix and biomass adhering to the surface. The current study reveals diverse morphological patterns of biofilm formation under the microscope, showing differences in biofilm architecture, density, and cellular organisation across the studied bacterial strains (Figure 5) of the isolates significant biofilm-forming capabilities, as seen by dense, linked networks of bacterial cells widely spread across the field. Notably, these isolates show aggregated clusters and matrix-enclosed communities, indicating strong extracellular polymeric substance (EPS) synthesis and sustained surface attachment. In contrast, less effective biofilm-formers had a sparse distribution and scattered individual cells, indicating poor adhesion and low EPS generation.

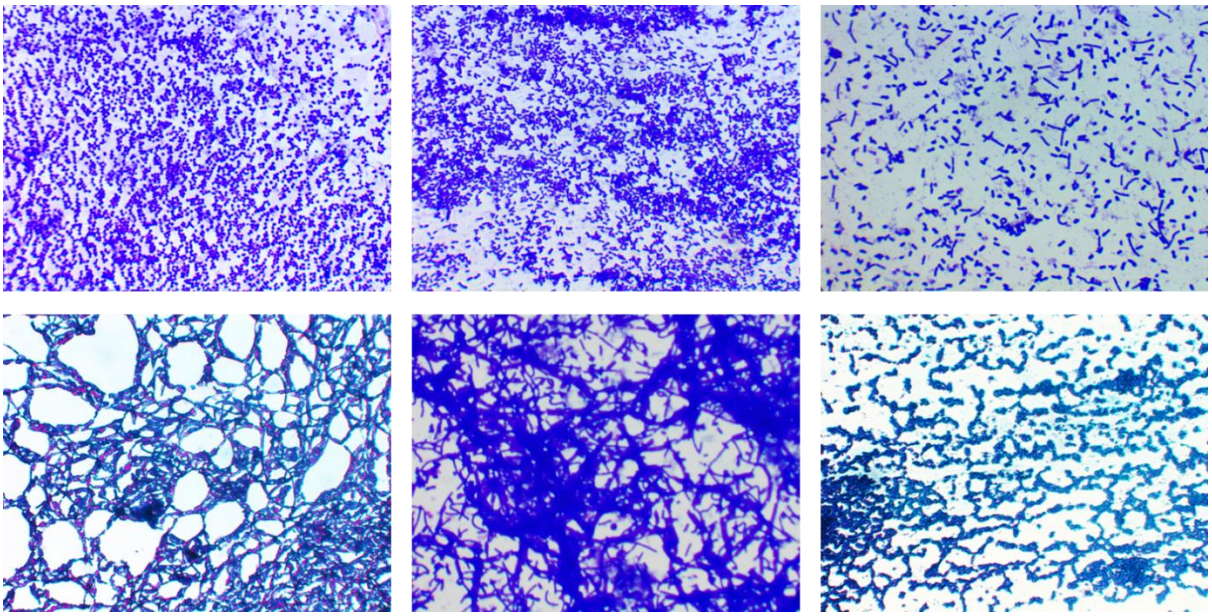

Figure 4 Microscopic analysis of the bacterial isolates using the CV assay

Etbr Cartwheel method

To assess efflux pump activity among bacterial isolates, we used the ethidium bromide (EtBr) cartwheel method on agar plates supplemented with increasing EtBr concentrations (0, 0.5, 1.0, and 1.5 mg/ml). Bacterial cultures were streaked radially in a cartwheel pattern and incubated at dark conditions. After incubation, plates were exposed to UV light to measure fluorescence intensity, which reflects intracellular EtBr accumulation and correlates inversely with efflux efficiency. At 0 mg/mL EtBr, no fluorescence was detected, demonstrating the absence of dye uptake. At 0.5 mg/mL, select isolates showed mild fluorescence, indicating limited EtBr retention due to moderate efflux activity as shown in (Figure 6). As the concentration increased to 1.0 and 1.5 mg/mL, fluorescence intensity increased in isolates with defective or low-efficiency efflux systems, but high-efflux organisms showed little fluorescence even at higher dye concentrations. This fluorescence gradient allowed for semi-quantitative separation of efflux pump activity across isolates. The approach operated efficiently for quick screening of multidrug resistance potential, particularly in finding isolates with active efflux mechanisms that contribute to antimicrobial resistance.

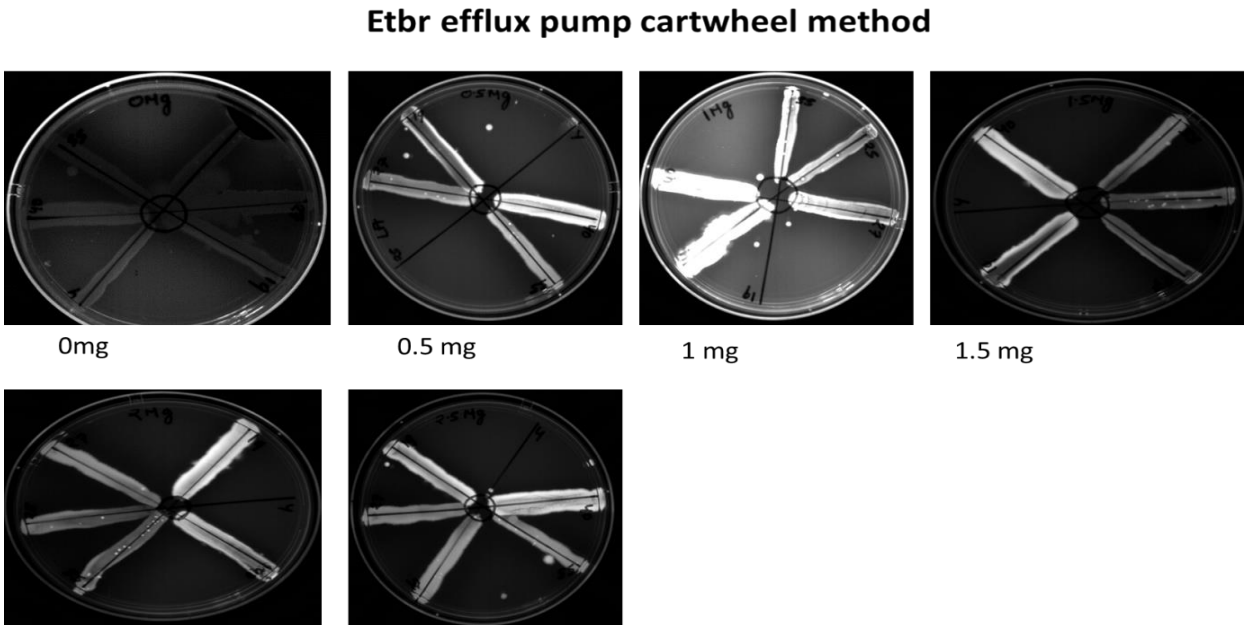

Figure 5 Ethidium Bromide-agar cartwheel method. Isolates no 1,2,3,4,5,6 removing ethidium bromide from bacteria and reducing fluorescence

4. DISCUSSION

The current work studies *Staphylococcus saprophyticus* isolates from hospital settings in a range of ways, with an emphasis on biofilm formation, antibiotic resistance profiles, and efflux pump function. These findings add to a growing body of evidence emphasising *S. saprophyticus* clinical relevance as a developing multidrug-resistant pathogen, notably in the context of urinary tract infections (UTIs) and nosocomial persistence. Biofilm production is an important virulence mechanism that promotes bacterial survival in unfavourable environments. Our investigation found that *S. saprophyticus* isolates formed strong biofilms, especially at 45 °C and alkaline pH conditions. These findings are consistent with recent studies by (Hashemzadeh, Dezfuli et al. 2021) and (Khalil, Alorabi et al. 2022) which found that biofilm biomass in staphylococci peaks under physiologically relevant stresses such as high temperatures and pH level changes. *S. saprophyticus* ability to modify its biofilm architecture in response to external factors suggests a complex regulatory network that favours persistence in the urinary tract and on abiotic surfaces like catheters. Microscopic study showed the presence of dense, matrix-enclosed bacterial populations, which is associated with substantial extracellular polymeric substance (EPS) production. This structural integrity not only promotes adhesion but also prevents antibiotic penetration, which contributes to therapeutic failure. The observed variations in biofilm morphology among isolates may be due to strain-specific changes in quorum sensing, EPS composition, or regulatory gene expression, as previously described by (Jeske, Arce-Rodriguez et al. 2022). Antibiotic susceptibility testing indicated concerning resistance patterns among the isolates, with numerous strains showing resistance to three or more antibiotic classes, classifying them as multidrug-resistant (MDR). Resistance to erythromycin, rifampicin, tetracycline, and ampicillin was particularly frequent, consistent with recent surveillance studies (Makarov, Ivanova et al. 2022) that show growing resistance in coagulase-negative staphylococci (CoNS), including *S. saprophyticus*. Our phenotypic data clearly indicate that efflux-mediated resistance

may play an important role in these patterns, especially in bacteria with high biofilm-forming capacity. The ethidium bromide (EtBr) cartwheel approach yielded semi-quantitative results on efflux pump activity among isolates. The intensity of fluorescence under UV light was inversely associated with efflux efficiency, allowing strains with high and low efflux to be differentiated. Isolates that showed limited fluorescence even at greater EtBr concentrations most likely had active efflux systems capable of evacuating harmful chemicals, including antibiotics. This finding is identical with the work of (Rolbiecki, Korzeniewska et al. 2022) and more recent research by (Sun 2024), who established the EtBr cartwheel test as a reliable screening technique for efflux-mediated resistance. The relationship between biofilm formation and efflux activity is especially remarkable. Biofilm-associated stress conditions, such as food constraint and oxidative stress, have been shown to upregulate efflux pump genes (e.g., norB, mepA), increasing bacterial survival under antimicrobial pressure (Sinha, Aggarwal et al. 2024). Future research should use qPCR or transcriptome analysis to validate the expression of critical resistance factors. Furthermore, assessing the efficacy of EPIs and anti-biofilm agents in vitro may provide translational significance to the findings. Another area to investigate is the effect of mobile genetic elements like plasmids and transposons in transmitting resistance characteristics. Whole-genome sequencing of high-resistance isolates may reveal novel resistance genes or regulatory networks involved in the reported traits.

5. CONCLUSION

This study highlights the multifactorial resistance mechanisms used by *Staphylococcus saprophyticus* isolates from hospital settings, with a specific emphasis on biofilm development and efflux pump function. The capacity of these isolates to produce robust biofilms under stress conditions, such as high temperature and alkaline pH, reveals their flexibility and persistence in urinary tract and nosocomial environments. Biofilm design not only promotes surface adherence, but it also serves as a physical and biochemical barrier to antimicrobial drugs, resulting in treatment failure and chronic infections. Antibiotic susceptibility profile demonstrated multidrug resistance in several isolates, particularly to erythromycin, rifampicin, and tetracycline. The EtBr cartwheel test revealed phenotypic evidence of operating efflux pump systems, which are likely responsible for the reported resistance by expelling antibiotics and other harmful chemicals. The link between substantial biofilm production and low EtBr fluorescence supports a mutually beneficial interaction between biofilm-mediated protection and efflux-driven resistance. These findings have important implications for infection prevention and treatment efforts. The discovery of MDR *S. saprophyticus* in ambient samples emphasises the need for increased surveillance and stronger hygiene standards in healthcare settings. Furthermore, the data supports the use of efflux pump inhibitors and anti-biofilm medicines as adjuncts to conventional antibiotics. Addressing these pathways through combined diagnostic and treatment approaches is critical to reducing the clinical effect of this new pathogen while maintaining antimicrobial efficacy.

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