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Biofilm Production Among Clinical Staphylococcus Isolates: A Comparison of Three Laboratory Detection Methods

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ABSTRACT

Background: Biofilm production is an important virulence factor of Staphylococcus species, contributing to persistent infections, antimicrobial resistance, and reduced susceptibility to conventional antimicrobial treatment. Accurate detection of biofilm production is therefore important in clinical microbiology laboratories. However, the performance of different phenotypic methods may vary.

Objective: To estimate the proportion of biofilm production among clinical Staphylococcus isolates and detection by Congo red agar, the Christensen tube method, and the crystal violet microtiter plate assay.

Methods: This hospital-based cross-sectional study included 110 consecutive eligible Staphylococcus isolates collected at Government Medical College, Thiruvananthapuram, from 20 March 2025 to 19 March 2026. Isolates were identified using conventional laboratory methods, with MALDI-TOF mass spectrometry used for species identification or confirmation. Biofilm production was assessed by three methods.

Results: Staphylococcus aureus was the predominant species among the 110 clinical Staphylococcus isolates, accounting for 78 isolates (70.9%). Biofilm production was detected using three phenotypic methods, with varying positivity rates. The Crystal Violet Microtiter Plate Assay identified the highest number of biofilm-producing isolates, with 62/110 isolates (56.4%) testing positive. In comparison, the Crystal Violet Tube Method detected biofilm production in 35/110 isolates (31.8%), while the Congo Red Agar (CRA) method identified 23/110 isolates (20.9%). Using the Crystal Violet Microtiter Plate Assay as the comparator, the count-based detection estimates for the tube method and CRA were 56.5% and 37.1%, respectively. These findings indicate that the microtiter plate assay detected a greater proportion of biofilm-producing isolates than either of the other two methods.

Conclusion: The crystal violet microtiter plate assay detected the highest proportion of biofilm-positive isolates in this collection.

Keywords: Staphylococcus; biofilm; Congo red agar; tube method; crystal violet assay; clinical isolates

1. INTRODUCTION

Staphylococcus species are Gram-positive bacteria that often live on the skin and mucous membranes of humans but can also lead to infections in the skin and soft tissues, bloodstream, heart valve infections, and infections linked to medical devices [1,2]. Staphylococcus aureus is a key pathogen because of its wide range of virulence factors, while coagulase-negative staphylococci, especially S.epidermidis, are major causes of infections in healthcare settings and those related to medical devices [3,4].The ability of these bacteria to form biofilms helps them stick to both body tissues and artificial surfaces, which leads to long-lasting infections [5,6].Biofilms are made up of groups of microorganisms trapped in a matrix that includes polysaccharides, proteins, and extracellular DNA. The process of biofilm development involves the bacteria attaching to a

surface, accumulating, maturing, and then breaking away, which is controlled by surface proteins, matrix parts, and environmental conditions [5,7,8].

Biofilm-related infections are difficult to treat because the bacteria within the biofilm are more resistant to antibiotics and less responsive to the body's immune system. This resistance is due to changes in how the bacteria use nutrients, differences in nutrient levels, and the presence of persister cells [5,9,10].Even though biofilm formation and antibiotic resistance are different processes, they can happen together in bacteria found in clinical samples, which shows

how important it is to detect biofilms [4,11].Common ways to detect biofilm formation include the Congo Red Agar (CRA) test, the Christensen Tube Method, and the Crystal Violet Microtiter Plate Assay.The CRA test looks at the shape of the colonies, the tube method checks how well bacteria stick to surfaces, and the microtiter plate test measures the amount of biofilm that has formed [11–13].Research has shown that these methods can give different results, which highlights the need to assess their effectiveness and limitations [11,12,14].As a result, this study aimed to detect biofilm production in clinical Staphylococcus isolates and compare the outcomes from the three detection methods.

2. MATERIALS AND METHODS

Study design and setting

A hospital-based cross-sectional study was conducted in the Clinical Microbiology Laboratory, Department of Microbiology, and the Postgraduate Microbiology Laboratory, Department of Medical Laboratory Technology, Government Medical College, Thiruvananthapuram, Kerala, India. The study period was 12 months, from 20 March 2025 to 19 March 2026, following Institutional Ethics Committee approval.

Study population

The study included 110 consecutive eligible Staphylococcus isolates recovered from clinical specimens received at the Central Microbiology Laboratory. Specimen types included pus, wound and respiratory specimens, blood, body fluids, catheter tips, tissue, and urine, as applicable. Mixed or contaminated cultures were excluded.

Identification of isolates

Clinical isolates were assessed by Gram staining and culture characteristics. Preliminary identification included catalase and coagulase testing and other biochemical procedures. MALDI-TOF mass spectrometry was used for species identification or confirmation.

Laboratory procedures

Biofilm production was assessed using Congo red agar (CRA), the Christensen tube method, and the crystal violet microtiter plate assay.

Statistical analysis

Results are presented as frequencies and percentages.

Ethics approval

Ethics approval was obtained from the Human Ethics Committee, Government Medical College, Thiruvananthapuram (reference number: 07/03/2025/MCT; approval date: 20 March 2025).

3. RESULTS

A total of 110 Staphylococcus isolates were included. Staphylococcus aureus accounted for 78 isolates (70.9%), followed by S. epidermidis (17; 15.5%), S. haemolyticus (10; 9.1%), S. hominis (3; 2.7%), and S. capitis (2; 1.8%).

3.1 Biofilm detection by method

Table 1 presents the biofilm detection results obtained using the three phenotypic methods.

Method	Biofilm positive, n (%)	Biofilm negative, n (%)
Congo red agar	23 (20.9)	87 (79.1)
Christensen tube method	35 (31.8)	75 (68.2)
Crystal violet microtiter plate assay	62 (56.4)	48 (43.6)

Table 1. Biofilm detection among clinical Staphylococcus isolates (n = 110).

The crystal violet microtiter plate assay identified the largest number of positive isolates. Based on the reported positive counts, the tube method's positive count was 56.5% of the crystal-violet-positive count (35/62), and CRA's was 37.1% (23/62).

3.2 Biofilm categories by crystal violet assay

Biofilm category	Number (%)
Non-producer	48 (43.6)
Weak producer	23 (20.9)
Moderate producer	31 (28.2)
Strong producer	8 (7.3)
Total	110 (100.0)

Table 2. Distribution of biofilm production categories by the crystal violet microtiter plate assay.

3.3 Biofilm production by species

Species	Total isolates	Biofilm producers, n	Within-species production, n (%)
Staphylococcus aureus	78	43	55.1
Staphylococcus epidermidis	17	11	64.7
Staphylococcus haemolyticus	10	5	50.0
Staphylococcus hominis	3	2	66.7
Staphylococcus capitis	2	1	50.0
Total	110	62	56.4

Table 3. Species-wise distribution of biofilm-producing isolates based on the crystal violet microtiter plate assay.

4. DISCUSSION

In the present study, Staphylococcus aureus was the predominant species among the 110 clinical Staphylococcus isolates, accounting for 70.9%. Biofilm production was detected using three phenotypic methods, with the Crystal Violet Microtiter Plate Assay showing the highest positivity rate (56.4%), followed by the Christensen Tube Method (31.8%) and Congo Red Agar (CRA) (20.9%). The higher detection rate obtained with the microtiter plate assay may be attributed to its ability to assess adherent biofilm biomass under standardized conditions. In contrast, the tube method

relies on the visual detection of stained material adhering to the tube surface, while CRA depends on characteristic colony morphology and pigmentation, which may not consistently reflect biofilm formation. The microtiter plate assay also enabled the classification of isolates into non-producers (43.6%), weak producers (20.9%), moderate producers (28.2%), and strong producers (7.3%), with moderate producers representing the largest category among the biofilm-positive isolates. These findings highlight the variability in biofilm detection according to the method employed and support the usefulness of the microtiter plate assay for comparative assessment of biofilm biomass.

Species-wise analysis revealed biofilm production in 55.1% of S. aureus, 64.7% of S. epidermidis, 50.0% of S. haemolyticus, 66.7% of S. hominis, and 50.0% of S. capitis isolates. Although S. aureus contributed the highest absolute number of biofilm-producing isolates, S. hominis and S. epidermidis showed higher within-species positivity rates. However, the small numbers of S. hominis and S. capitis isolates warrant cautious interpretation. Biofilm formation among both S. aureus and coagulase-negative staphylococci is clinically relevant because it may contribute to persistent infections and reduced susceptibility to antimicrobial treatment. The overall positive counts for the tube method and CRA represented 56.5% and 37.1%, respectively, of the positive count obtained with the microtiter plate assay; these are count-based comparisons.

Limitations

The study was conducted at a single tertiary care hospital and included 110 isolates. Molecular methods for biofilm assessment and scanning electron microscopy were not performed because of resource and funding limitations.

5. CONCLUSION

The crystal violet microtiter plate assay detected the highest proportion of biofilm-positive clinical Staphylococcus isolates among the three methods assessed.

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AUTHOR CONTRIBUTIONS

Devikrishna S contributed to the conceptualization and design of the study, data collection, laboratory investigations, data analysis, interpretation of results, and manuscript preparation. Dr. Smitha Pious Francis and Dr. Pradeep Kumar M contributed to supervision, methodological guidance, critical review, and intellectual input. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

ETHICS APPROVAL AND CONSENT

Ethics approval was obtained from the Human Ethics Committee, Government Medical College, Thiruvananthapuram (reference number: 07/03/2025/MCT; approval date: 20 March 2025). Consent details were not specified in the source manuscript.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author.

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