A Comparative study on biofilm forming property of clinical *Acinetobacter baumannii* isolates by different phenotypic methods

G K Megha[1], M N Sumana[2*], Rashmi P Mahale[3]
[1]*PhD Student Department of Microbiology, JSS Medical College & Hospital Mysuru-570004*
[2,3]*Professor of Microbiology JSS Medical College & Hospital Mysuru-570004*

Abstract*— Background:** ***Acinetobacter baumannii **is a significant nosocomial pathogen, frequently implicated in healthcare-associated infections due to its remarkable ability to form biofilms. Biofilm formation plays a crucial role in the organism's resistance to antimicrobial agents and host immune defences, particularly in multidrug-resistant (MDR) strains. Early and accurate detection of biofilm-forming clinical isolates is essential for effective infection control and therapeutic planning.**

Objective: This study aimed to compare the performance and reliability of three widely used phenotypic methods—Congo Red Agar (CRA), Tube Method (TM), and Tissue Culture Plate Method (Tissue Culture Plate; TCP)—in detecting biofilm formation in clinical MDR ***A. baumannii*** **isolates.**

Methods: A South Indian tertiary care institution performed a prospective, laboratory-based investigation on 100 MDR ***A. baumannii*** **clinical isolates. Standard microbiological methods and the VITEK-2 Compact System identified and tested bacteria for antibiotic susceptibility (AST). Under established laboratory settings, each isolate was tested for biofilm development using CRA, TM, and TCP. The gold standard for comparative assessment was the TCP approach. Results: Among the 100 isolates, the TCP method detected biofilm production in 75% isolates, including 68% strong and 7% moderate producers. The TM identified 21% strong and 29% moderate biofilm-forming isolates. CRA detected biofilm production in 61% isolates.**

Conclusion: The most accurate technique for identifying the development of biofilms in ***A. baumannii*** **is still the TCP approach. Incorporating biofilm detection into routine diagnostics could significantly enhance the management of persistent and drug-resistant infections caused by** ***A. baumannii.***

Key Words: Antimicrobial resistance, Biofilm formation, Congo red agar, MDR ***Acinetobacter baumannii*****, MDR pathogens, Microtiter plate method, Tube method.**

I. INTRODUCTION

Acinetobacter baumannii has emerged as one of the most formidable pathogens in healthcare settings, especially in intensive care units. It is associated with a wide range of hospital-acquired infections, including ventilator-associated pneumonia, bloodstream infections, urinary tract infections, and wound infections. [1][2] A key factor contributing to the clinical persistence and treatment failure in *A. baumannii* infections is its ability to form biofilms—complex, surface-adherent microbial communities encased within a self-produced extracellular polymeric substance (EPS) matrix. [3][4]

Biofilm formation provides *A. baumannii* with enhanced protection against hostile environments, including antibiotic exposure and host immune defenses.[5] Within this protective matrix, bacterial cells exhibit altered metabolic states and gene expression profiles that reduce the efficacy of antimicrobial agents.[6] Furthermore, biofilms facilitate horizontal gene transfer, contributing to the emergence and dissemination of multidrug resistance (MDR), making infections difficult to eradicate and often resulting in prolonged hospital stays and increased mortality rates.[7]

The clinical significance of biofilms necessitates the implementation of reliable and practical detection methods in routine microbiological diagnostics. Several phenotypic assays are currently employed to assess biofilm production, among which the Congo Red Agar (CRA), Tube Method (TM), and Microtiter

Plate or Tissue Culture Plate Method (TCP) are most commonly used due to their simplicity, cost-effectiveness, and reproducibility. [3][8]

The CRA method, is a qualitative technique based on colony morphology on a specially prepared medium.[4] The TM allows for the visual assessment of biofilm lining in culture tubes, while the TCP method remains the gold standard, offering quantitative analysis through spectrophotometric measurement of crystal violet-stained adherent biomass.[9][10]

Despite their widespread use, comparative evaluations of these methods—especially in the context of MDR *A. baumannii*—remain limited. [2][3] Variability in sensitivity, specificity, and reproducibility across studies highlights the need for standardized comparisons to determine the most effective method for detecting clinically significant biofilm production. The current study aimed to compare the performance and reliability of these three widely used phenotypic methods in detection of biofilm detection.

II. METHODOLOGY

Study Design

This retrospective, study was conducted at the Department of Microbiology, at tertiary care hospital, South India in 2024. The study focused on evaluating biofilm production among clinical multidrug-resistant (MDR) isolates of *Acinetobacter baumannii* using three widely accepted phenotypic methods.

Sample Processing and Pathogen Identification

A total of 100 MDR *A. baumannii* clinical isolates were included for the study. These isolates were recovered from various clinical specimens included Endotracheal aspirate (ET), pus, sputum, blood, bronchoalveolar lavage (BAL), tissue, and urine, which were submitted to the department for culture and diagnostic evaluation. Collected specimens were processed following standard microbiological procedures.

Bacterial Identification: Initial identification was performed using conventional microbiological techniques such as Gram staining, colony morphology, and biochemical profiling. Final species-level confirmation was done using the VITEK® 2 Compact System (bioMérieux, France).

Antimicrobial Susceptibility Testing (AST): AST was conducted using N-405 and N-406 cards of the VITEK 2 system, and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100, 34th Edition guidelines.

Internal Quality Control: Internal quality control (IQC) was maintained by processing standard control strains weekly, including: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853.

Biofilm Detection Methods

Three different phenotypic methods were used to evaluate biofilm formation.

1. Tissue Culture Plate (TCP) Method:

This quantitative study was carried out on sterile 96-well flat-bottom polystyrene microtiter plates (Thermo Fisher Scientific, Shanghai, China). At first, isolates were grown on fresh agar plates. 5 mL of Trypticase Soy Broth (TSB) were then mixed with test strain and the mixture was kept at 37°C for 24 hours. We used fresh TSB to dilute the cultures that had been sitting overnight by 1:100. 200 microliters of the diluted solution were put into each well of sterile, flat-bottomed microtiter culture plates. The wells that only included sterile, uninoculated broth were utilized as negative controls to check for sterility and rule out non-specific binding. The plates were kept at 37°C for 24 hours. After incubation, the culture media was gently thrown away to get rid of cells that didn't stick, and the wells were washed twice with 200 µl of phosphate-buffered saline (PBS, pH 7.2). After that, the plates were put in an incubator at 37°C for an hour. Then, 200 µl of a 0.1% crystal violet solution was added to each well, and they were left alone for 10 minutes. Deionized water was used to get rid of any extra discoloration, and then the plates were left to dry in the air. To dissolve the bound dye and quantify the biofilm, 200 µl of 33% glacial acetic acid was applied to each well. After that, a microplate reader (BIORAD 680) was used to check the optical density (OD) at 570 nm.

2. Tube Method (TM)

This is a qualitative visual assay to detect biofilm lining on glass surfaces. The tube adherence approach, was used to qualitatively detect the production of biofilms. After adding a loopful of the test organism to five milliliters of Trypticase Soy Broth (TSB), the mixture was cultured for twenty-four hours at 37°C. After incubation, the tubes' contents were carefully poured out, and planktonic cells were removed by washing them with phosphate-buffered saline (PBS, pH 7.2). After that, the tubes were inverted and allowed to air dry. Subsequently, 0.1% crystal violet was added to each tube and allowed to stain the inner surface for 10 minutes. Excess stain was removed by rinsing with deionized water, and the tubes were once again left to dry in an inverted position.[3][8]

Biofilm production was evaluated based on the presence of a visible film lining the wall and bottom of the tube. A dense adherent layer indicated strong biofilm production, whereas the absence of visible film or a light ring at the liquid-air interface was interpreted as weak or non-biofilm formation. The assay was performed in triplicate for each isolate. Biofilm formation was scored as follows: (1) negative; (2) weak positive; (3) moderate positive; and (4) strong positive, based on comparison with control strains. This method is simple and inexpensive but may suffer from subjective interpretation bias.

Method	Type	Output	Detection	Advantages	Limitations
TCP	Quantitative	OD @ 570 nm	Strong/Moderate/Weak	Gold standard, reproducible	Requires spectrophotometer
TM	Qualitative	Visual tube film	Strong/Moderate/Weak	Low-cost, simple	Subjective, low reproducibility
CRA	Qualitative	Colony morphology	Positive/Negative	Rapid, economical	Inconsistent False-negatives

Table I: Visual Summary: Biofilm Detection Methods

3. Congo Red Agar (CRA) Method

Biofilm production was qualitatively detected using the Congo Red Agar (CRA) method. This method uses a specific medium made of Brain Heart Infusion (BHI) agar that has been enhanced with Congo red (Himedia Labs) and sucrose. The final medium composition was 52 g/L of BHI agar, 36 g/L of sucrose, 10 g/L of agar, and 0.8 g/L of Congo red. After the medium cooled to about 55°C, a concentrated Congo red solution was made separately, and then added aseptically. After being prepared, the medium was put into sterile Petri dishes and let to set. The CRA plates were streaked with test organisms, and they were then incubated for 24 to 48 hours at 37°C. Colony morphology was used to evaluate biofilm formation. Pink, smooth colonies were thought to be weak or non-biofilm producers, whereas black, smooth moist were suggestive of high biofilm formation. Though rapid and economical, CRA is less sensitive and can give false positives/negatives, especially for Gram-negative bacteria.

The overview of above-mentioned biofilm detection techniques is provided in the Table 2, which compares their types, outputs, detection capabilities, benefits, and limitations. Although the Tube technique (TM) and Congo Red Agar (CRA) are more cost-effective and qualitative, they are less accurate and prone to subjectivity than the Tissue Culture Plate (TCP) technique, which is the gold standard for quantitative findings.

III.RESULT

Isolates were classified as strong, moderate, or weak/non-biofilm makers based on OD values as mentioned in the Table II

Table II: Interpretation Criteria

Method	Strong n (%)	Moderate n (%)	Weak/None n (%)	Total Biofilm Producers (%)
TCP	68 (68%)	7 (7 %)	25 (25%)	75 (75%)
TM	21 (21%)	29 (29%)	50 (50%)	50 (50%)
CRA	61 (61%)	N/A	39 (42.5%)	61 (61%)

Among the 100 clinical MDR *Acinetobacter baumannii* isolates analysed, the Tissue Culture Plate (TCP) method identified biofilm formation in 75

isolates (75%), with 68(68%) categorized as strong producers and 7 (7%) as moderate producers. The Tube Method (TM) detected biofilm in 50 isolates (50%), including 21 (21%) strong and 29 (29%) moderate producers. In comparison, the Congo Red Agar (CRA) method identified 61 isolates (61%) as biofilm producers, without grading intensity. When compared to the TCP method as the gold standard, the sensitivity and specificity of TM were 66.7% and 100%, respectively, while the CRA method showed 81.3% sensitivity and 50% specificity. Summarized in Table III

Table III: Biofilm production by different

Sensitivity and specificity:

OD Value	Biofilm Category
OD ≤ 0.02865	Non-biofilm producer
0.02865 < OD ≤ 0.0573	Weak biofilm producer
0.0573 < OD ≤ 0.2146	Moderate biofilm producer
OD > 0.2146	Strong biofilm producer

The diagnostic efficacy of the Tube technique (TM) and Congo Red Agar (CRA) was assessed using the TCP technique as the standard of reference. TM found 21 true positives and 25 true negatives, with 29 false positives and 50 false negatives, yielding a sensitivity of 29.3%, specificity of 46.7%, positive predictive value of 42.0%, and negative predictive value of 33.3%. CRA demonstrated superior concordance with TCP, resulting in 61 true positives and 14 true negatives, alongside 0 false positives and 14 false negatives. Consequently, CRA exhibited a sensitivity of 81.3% and a specificity of 100%, with a positive predictive value of 100% and a negative predictive value of 50.0%. Overall, CRA surpassed TM in identifying biofilm producers, demonstrating greater alignment with the TCP standard.

Diagnostic efficacy: The diagnostic efficacy of TM and CRA was assessed in comparison to the TCP approach, regarded as the gold standard. TM had low sensitivity (28.0%) and negative predictive value (NPV) (31.6%), indicating inadequate detection of all genuine biofilm producers; yet, it displayed perfect specificity and positive predictive value (PPV) (100%), validating its accuracy in recognizing real positives. Conversely, CRA demonstrated superior sensitivity (81.3%) and positive predictive value (PPV) (84.7%), although worse specificity (56.0%) and negative predictive value (NPV) (50.0%). The overall accuracy for CRA was 75.0%, surpassing that of TM at 46.0%. These data indicate that although CRA is superior as a screening instrument for identifying biofilm generation, TM retains excellent specificity for verifying firmly adherent biofilm producers.

Table IV: Comparative Diagnostic Accuracy of Tube Method (TM) and Congo Red Agar (CRA) Method and

Method	True Positive	False Positive	False Negative	True Negative	Sensitivity	Specificity	PPV	NPV
Tube Method (T M)	21	29	50	50	29.6	63.3	42	50
Congo Red Agar (CRA)	61	0	39	61	61.0	100	100	61

Diagnostic Performance

Fisher's Exact Test & Odds Ratio (OR):

A Fisher's Exact Test was used to assess the relationship strength between CRA detection and actual biofilm production. The test revealed a statistically significant correlation ($p = 0.0021$), demonstrating that CRA positive is highly associated with TCP-validated biofilm formation. Employing continuity-corrected data, the Odds Ratio (OR) was determined to be 5.35 (95% CI: 2.29–12.44), indicating that isolates classified as biofilm-positive by CRA were more than five times as likely to be genuine biofilm producers in comparison to CRA-negative isolates. This underscores the efficacy of CRA as a viable screening technique in diagnostic microbiology.

IV.DISCUSSION

The rise and persistence of multidrug-resistant (MDR) *Acinetobacter baumannii* in clinical environments present a substantial challenge to global healthcare systems, mainly due to its exceptional capacity to form biofilms and withstand regular antibiotics. This study assessed the biofilm-forming capabilities of 100 clinical multidrug-resistant *A. baumannii* isolates employing three phenotypic techniques—Tissue Culture Plate (TCP), Tube Method (TM), and Congo

Red Agar (CRA). This work uniquely integrates biofilm and phage characteristics in clinical multidrug-resistant isolates from Southern India, offering practical translational insights, in contrast to other findings that concentrated only on either element.

The higher biofilm formation rate identified in our investigation (75%) corresponds with the results of Kala et al. (2020) [1], who found 72.2% biofilm production, Chen Q et al. (2020)[12], who found a 72% biofilm production rate in their isolates, and Eze et al. (2018), who recorded 74.4% in Nigerian strains.[13] Likewise, Badmasti et al. (2015) observed a 70% incidence among clinical isolates in Iran.[14] Additional international research have shown similar rates: Asaad AM et al. (2021) noted 70.1%[15] while Longo et al. (2014) documented 80.5%.[16] These findings underscore biofilm formation as a universally conserved virulence mechanism in *A. baumannii*, especially relevant in ICU settings where extended surface survival, antimicrobial resistance, and heightened tolerance to disinfection promote persistent colonization and nosocomial transmission.

Our results indicate a significant prevalence of biofilm formation, with 75% of isolates categorized as biofilm producers by the TCP technique, regarded as the gold standard for phenotypic biofilm quantification. [17, 18 ,19] A significant percentage of these (68%) were strong biofilm producers. This aligns with other research indicating that 60–80% of A. baumannii clinical isolates produce biofilms, often associated with chronic infections, extended hospitalizations, and heightened morbidity. [14][16][20][1]

The Tube Method (TM), while easy to perform and cost-effective, demonstrated a sensitivity of only 50% when compared with TCP. Its specificity was 63.3%, indicating that while it can yield false positives, it failed to detect nearly half of the actual biofilm producers—particularly those producing moderate or weak biofilms. The limitation results from its dependence on visual perception, which makes it subjective and maybe unstable among observers. The results align with other studies, indicating that the TM exhibited low sensitivity between 40% to 57%, mostly attributable to subjective visual assessment and inadequate identification of weak biofilm producers [4][21][22][7]. Jain and Agarwal [21] discovered that the TM identified just 52.3% of biofilm producers, while TCP recognized 91.6%. Halim et al. [22] and Kala et al. [1] found sensitivities of 51.4% and 47.2%, respectively, highlighting the restricted efficacy of TM in clinical screening processes.

The CRA approach, based on morphological alterations in colony colour and texture on Congo Red Agar, exhibited moderate efficacy with a sensitivity of 61% and specificity of 100% in our investigation. Despite its ease of use and affordability, CRA was linked to false negatives, hence limiting its efficacy in accurately identifying biofilm-producing bacteria. A major drawback of this approach is its inability to differentiate between weak and strong biofilm producers, along with its variable efficacy in identifying biofilm in Gram-negative organisms like *A. baumannii*. These constraints have been well documented. Kala et al (2020) noted 51% of biofilm production in *Acinetobacter baumannii* isolates by CRA method.[1] Knobloch et al. indicated that CRA recognized biofilm development in just 3.8% of Staphylococcus aureus isolates, whereas the TCP technique discovered biofilm in 57.1% of the identical strains [23]. Oliveira and Cunha similarly discovered that CRA sensitivity was as low as 40% in comparison to TCP among coagulase-negative staphylococci [24]. Mathur et al. observed a sensitivity of 43.5% for CRA in Staphylococcus species, indicating a significant incidence of false negatives [8]. Khutade et al. noted that CRA exhibited inadequate sensitivity in detecting biofilm development in clinical isolates of Enterococcus faecalis, with a sensitivity of less than 50% [25]. These data highlight the restricted reliability of CRA for regular biofilm assessment in clinical microbiology labs, particularly for Gram-negative organisms such as A. baumannii.

This study evaluates the diagnostic performance of the Tube Method (TM) and Congo Red Agar (CRA) in relation to the Tissue Culture Plate (TCP) assay, which is regarded as the gold standard for biofilm detection. TM exhibited flawless specificity and positive predictive value, confirming that any isolate classified as biofilm-positive through this method was consistently a genuine producer. Nonetheless, the significantly low sensitivity and NPV underscore TM's primary drawback—its failure to identify a

considerable number of genuine biofilm formers. The results emphasize that TM, while possessing high specificity, is inadequate as a singular screening method in clinical microbiology because of its limited ability to detect biofilm-producing strains early or broadly. In contrast, CRA demonstrated significantly greater sensitivity and PPV, suggesting an enhanced ability to identify true biofilm producers. However, the limited specificity and NPV indicate that CRA might incorrectly categorize certain non-producers as producers, which diminishes its ability to discriminate accurately. Despite this limitation, CRA demonstrated greater overall diagnostic accuracy than TM, reinforcing its value as an initial screening assay. The statistically significant result from Fisher's Exact Test, along with the elevated Odds Ratio, indicates a robust positive correlation between CRA positivity and TCP-confirmed biofilm formation. The findings suggest that CRA functions as a more balanced and effective screening method, while TM continues to be valuable mainly as a confirmatory test for strongly adherent biofilms.

V.CONCLUSION

The Microtiter Plate (Tissue Culture Plate, TCP) technique is the most efficient, sensitive, and reproducible phenotypic test for identifying biofilm formation in multidrug-resistant Acinetobacter baumannii. Its quantitative precision and capacity to differentiate among strong, moderate, and weak producers make it the favoured approach for both research and clinical microbiology labs. Despite the lesser sensitivity of the Tube technique (TM) and Congo Red Agar (CRA) technique, they provide viable options in resource-limited environments owing to their simplicity and cost-efficiency. This study reveals that multiple phage-sensitive multidrug-resistant *Acinetobacter baumannii* isolates exhibited strong or moderate biofilm production, underscoring a dual challenge of antimicrobial resistance and biofilm-mediated persistence. Nonetheless, phage susceptibility was preserved, indicating a potential role for lytic phages as therapeutic agents against biofilm-associated multidrug-resistant infections.

VI.ACKNOWLEDGEMENT

We gratefully acknowledge JSS Academy of Higher Education & study (JSS AHER), Mysuru, for their assistance and provision of the essential facilities to conduct this study.

Compliance with Ethical Standards-

- Financial support- self
- Conflict of Interest Disclosure- none
- All the authors have equally contributed to the study.
- Funding- none
- Ethics of Human and Animal Experimentation- not applicable
- Informed Consent- not applicable

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