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Phenotypic Detection of Biofilm Formation in Clinically Significant Isolates and Its Correlation with Clinical Outcomes and Antibiotic Resistance Patterns

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ABSTRACT

Healthcare-associated infections (HAIs) are a major challenge in tertiary-care hospitals, largely driven by the ability of microorganisms to form biofilms. Biofilms are structured microbial communities embedded in an extracellular polymeric matrix that protect pathogens from host immune responses and antimicrobial agents. This study aims to detect biofilm formation in clinically significant bacterial and fungal isolates using standardized phenotypic assays and correlate these findings with antibiotic resistance patterns and patient outcomes. A cross-sectional observational study will be conducted over three years at Datta Meghe Medical College and affiliated hospitals, including approximately 700–800 unique clinical isolates from blood, urine, pus, and device-related samples. Biofilm detection will be performed using Congo Red Agar (CRA), Tube Adherence Method (TAM), and the Microtiter Plate Assay (MTP), the latter serving as the gold standard. Antibiotic susceptibility testing will follow CLSI M100 (2024) guidelines. Correlations will be analyzed between biofilm strength, antimicrobial resistance (MDR/XDR), and clinical outcomes such as hospital-stay duration, complications, and mortality. The study anticipates providing evidence for incorporating biofilm surveillance into infection-control policies and antibiotic-stewardship programs, thereby improving patient prognosis and reducing the burden of persistent HAIs.

Need for the Study

In India, and particularly in Central India, limited longitudinal data link phenotypic biofilm production with both antimicrobial resistance and clinical outcomes. Establishing a standardized, cost-effective phenotypic protocol and correlating laboratory findings with clinical variables will generate translational evidence crucial for infection-control programs. This study addresses this gap by integrating routine phenotypic assays with patient-level data to create a practical surveillance model applicable to resource-limited tertiary-care centers.

KEYWORDS: Biofilm, Antimicrobial Resistance, Phenotypic Detection, Microtiter Plate Assay, Healthcare-Associated Infection, CLSI, Central India.

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INTRODUCTION

Biofilms are highly organized microbial ecosystems enclosed in a self-produced extracellular polymeric matrix that irreversibly adheres to biological tissues or inert surfaces such as catheters and prosthetic devices. This sessile mode of growth provides remarkable survival advantages, protecting microorganisms from phagocytosis, host defenses, and antimicrobial penetration. Studies indicate that microorganisms in biofilms can resist antibiotics at concentrations up to 1,000 times higher than their planktonic counterparts. The result is persistent infection, prolonged hospitalization, and increased mortality among hospitalized and immunocompromised patients.

The clinical significance of biofilms is particularly evident in device-related infections—catheter-associated urinary tract infection (CAUTI), ventilator-associated pneumonia (VAP), and central line-associated bloodstream infection (CLABSI)—as well as in chronic wounds and otitis media. Among the key pathogens implicated are *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Candida* species. These organisms exhibit remarkable capacity for attachment and colonization of biotic and abiotic surfaces, producing a matrix rich in polysaccharides, proteins, and nucleic acids.

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Although molecular approaches such as PCR and confocal microscopy provide high sensitivity in detecting biofilm genes and structure, they are cost-intensive and impractical for routine diagnostics in developing-country laboratories. In contrast, phenotypic methods—including the Congo Red Agar (CRA) method, Tube Adherence Method (TAM), and Microtiter Plate (MTP) assay—offer inexpensive, reliable alternatives that can be readily implemented in hospital microbiology laboratories. Among these, the MTP assay provides quantitative assessment and reproducible results, making it the preferred gold-standard technique.

The increasing prevalence of multidrug-resistant (MDR) pathogens within biofilms aggravates treatment difficulty. Mechanisms include restricted antibiotic diffusion, altered metabolic activity, efflux-pump expression, and horizontal gene transfer within the biofilm community. Consequently, therapeutic failure and recurrence are common even with appropriate antibiotics. Hence, it becomes imperative to integrate phenotypic biofilm testing with antimicrobial-resistance profiling and patient outcomes.

In Central India, comprehensive data correlating phenotypic biofilm formation with antimicrobial resistance and clinical prognosis are lacking. This study therefore seeks to generate region-specific evidence by systematically detecting biofilm formation in diverse clinical isolates, correlating laboratory results with clinical outcomes, and proposing strategies for surveillance and stewardship. Such evidence can directly inform hospital infection-control committees and guide empirical antibiotic policies.

REVIEW OF LITERATURE

The concept of biofilm detection originated with Christensen et al. (1), who described the tube-adherence and Congo Red Agar (CRA) methods for identifying slime-producing staphylococci. These assays established simple and reproducible techniques for routine microbiology. Later, Mathur et al. (2) and Stepanović et al. (3) demonstrated that the Microtiter Plate (MTP) assay provided the most accurate quantitative assessment of biofilm formation and is now considered the phenotypic gold standard.

Biofilm-forming organisms show substantially greater antimicrobial resistance than planktonic bacteria due to the protective extracellular matrix, reduced growth rate, and active efflux mechanisms. Donlan et al. (4) emphasized that biofilm-embedded bacteria can tolerate antimicrobial concentrations up to 1,000-fold higher than free-living cells. Mah et al. (5) further explained that quorum sensing and altered gene expression underlie this tolerance. Clinical studies, such as those by Moskowitz et al. (6) and Oliveira et al. (7), confirmed the association between strong biofilm production and multidrug resistance in Pseudomonas aeruginosa and coagulase-negative staphylococci, respectively.

Hall-Stoodley et al. (8) established the clinical relevance of biofilms in chronic and device-associated infections, highlighting their role in persistence and therapeutic failure. Although global literature supports the biofilm–AMR link, data from India remain fragmented and often organism-specific. Most studies are cross-sectional, with limited integration of phenotypic assays and patient outcomes. Thus, this study will comprehensively evaluate biofilm formation in multiple organisms, correlate it with resistance patterns, and assess clinical impact, filling a major regional evidence gap.

AIM

To detect biofilm formation phenotypically in clinically significant isolates and correlate these findings with antibiotic-resistance patterns and patient clinical outcomes.

OBJECTIVES

To detect biofilm formation using standard phenotypic methods (CRA, TAM, MTP) and determine antimicrobial-resistance patterns of biofilm-forming and non-forming isolates.

To correlate biofilm formation with clinical outcomes such as duration of hospitalization, complications, and mortality and identify predominant biofilm-forming organisms in different sample types and hospital units.

Null Hypothesis

There is no significant correlation between phenotypic biofilm formation and antimicrobial-resistance patterns or clinical outcomes among clinically significant isolates.

Alternate Hypothesis

Phenotypic biofilm formation is significantly associated with higher antimicrobial resistance and poorer clinical outcomes in patients with clinically significant infections.

MATERIALS AND METHODS

Study Design and Setting

A cross-sectional observational study with prospective data collection will be conducted in the Department of Microbiology, Datta Meghe Medical College and affiliated hospitals over three years.

Inclusion Criteria

Clinically significant bacterial or fungal isolates from blood, urine, pus, wound swabs, respiratory secretions, and device tips.

Unique isolate per patient.

Exclusion Criteria

Environmental or commensal isolates.

Duplicate isolates within 14 days.

Contaminants or mixed growths not clinically significant.

Ethical Approval

The protocol will be reviewed and approved by the Institutional Ethics Committee (IEC).

METHODOLOGY

All clinical specimens including blood, urine, pus, wound swabs, respiratory secretions, and device tips will be collected aseptically from patients admitted to various wards and intensive care units of Datta Meghe Medical College and its affiliated hospitals. Each sample will be transported immediately to the microbiology laboratory and processed according to standard clinical microbiology procedures. The isolates will be obtained by inoculation on Blood agar, MacConkey agar, or Sabouraud Dextrose Agar (for fungal isolates) and incubated at 37°C for 18–24 hours. Identification of bacterial isolates will be carried out using Gram staining, colony morphology, and conventional biochemical tests. Where available, automated systems such as the VITEK 2 Compact will be used for species-level confirmation to ensure accuracy and reproducibility. Only clinically significant, non-duplicate isolates from individual patients will be included, while environmental, mixed, or commensal isolates will be excluded from analysis.

Biofilm Detection

The detection of biofilm formation will be performed using three standardized phenotypic methods: Congo Red Agar (CRA), Tube Adherence Method (TAM), and the Microtiter Plate (MTP) Assay. The CRA method will serve as a preliminary screening test. In this method, isolates will be inoculated onto CRA plates prepared with Brain Heart Infusion agar supplemented with 5% sucrose and 0.8 g/L Congo red dye and incubated at 37°C for 24–48 hours. Black, dry crystalline colonies will indicate strong biofilm production, whereas pink or red colonies will be considered non-producers. The Tube Adherence Method will be employed as a semi-quantitative technique in which isolates are inoculated into tryptic soy broth with 1% glucose, incubated at 37°C for 24 hours, and then stained with 0.1% crystal violet after washing The formation of a visible film on the inner wall of the tube will be interpreted as positive for biofilm formation.

Microtiter Plate (MTP) Assay

The Microtiter Plate (MTP) assay will be considered the gold standard quantitative method. Overnight bacterial cultures adjusted to 0.5 McFarland standard will be inoculated into 96-well flat-bottom microtiter plates containing tryptic soy broth with 1% glucose and incubated at 37°C for 24 hours. Wells will then be gently washed with phosphate-buffered saline to remove planktonic cells, fixed with methanol, and stained with 0.1% crystal violet. After washing and air-drying, 200 µL of 95% ethanol will be added to solubilize the stain, and optical density (OD) will be measured at 570 nm using an ELISA reader. Biofilm production will be graded as non, weak, moderate, or strong based on the mean OD values relative to the negative control (Stepanović criteria). For internal quality control, Staphylococcus epidermidis ATCC 35984 will serve as a positive control and S. epidermidis ATCC 12228 as a negative control in each batch of tests.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing (AST) will be conducted for all isolates using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar, following the latest Clinical and Laboratory Standards Institute (CLSI) M100 guidelines. A standardized inoculum of 0.5 McFarland turbidity will be used, and antibiotic discs representing major antimicrobial classes—including β-lactams, aminoglycosides, fluoroquinolones, carbapenems, and glycopeptides—will be tested. The results will be interpreted as sensitive, intermediate, or resistant according to CLSI interpretive charts. Multidrug resistance (MDR) and extensively drug resistance (XDR) will be defined as per the CDC–ECDC joint criteria (2011). Quality control will be ensured by using E. coli ATCC 25922, S. aureus ATCC 25923, and P. aeruginosa ATCC 27853 as control strains.

Clinical Data Collection:

Clinical data corresponding to each isolate will be collected prospectively using a structured proforma. Parameters such as patient demographics, underlying diagnosis, hospital unit (ICU or ward), device usage, duration of hospital stay, complications, and outcome (discharge or death) will be recorded. This information will be used to establish correlations between biofilm formation, antibiotic resistance, and patient outcomes

Biosafety and Waste Management:

All procedures will be performed under Biosafety Level 2 (BSL-2) conditions in accordance with the Biomedical Waste Management Rules (2016). Decontamination of work surfaces with 1% sodium hypochlorite and autoclaving of all biological waste will be strictly followed. Quality assurance will include adherence to Standard Operating Procedures (SOPs), regular equipment calibration, and periodic internal and external quality audits.

STATISTICAL ANALYSIS

All data obtained from microbiological and clinical observations will be compiled using Microsoft Excel and statistically analyzed with IBM SPSS version 27.0 and R software version 4.3.2. Descriptive statistics will be applied to summarize categorical variables as frequencies and percentages, and continuous variables as mean ± standard deviation (SD) or median with interquartile range (IQR) as appropriate. The prevalence of biofilm formation among different clinical isolates will be determined, and comparisons between biofilm producers and non-producers in relation to antimicrobial resistance patterns will be assessed using the Chi-square test or Fisher's exact test for categorical data. Continuous variables such as hospital-stay duration will be analyzed using the Student's t-test or Mann–Whitney U test, depending on data normality verified through the Shapiro–Wilk test. To determine the independent association between biofilm formation, multidrug resistance, and adverse clinical outcomes (prolonged stay, complications, mortality), binary logistic regression analysis will be performed, adjusting for confounding factors such as age, comorbidities, and device use. Correlation strength will be expressed as odds ratios (OR) with 95% confidence intervals (CI). All statistical tests will be two-tailed, and a p-value < 0.05 will be considered statistically significant. Graphical representation of results, including bar charts and scatter plots, will be used for data visualization, ensuring clarity and reproducibility in the final analysis.

OUTCOMES

Establishment of biofilm prevalence among clinical isolates for the determination of association between biofilm formation and antimicrobial resistance.

Correlation between biofilm presence and clinical outcomes such as hospital stay and mortality and formulation of biofilm surveillance protocol for hospital infection-control programs.

Generation of evidence to guide empirical therapy and antimicrobial-stewardship policies...

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