

Correlation between Biofilm Formation and Multi-Drug Resistance among Clinical Isolates

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ABSTRACT

Introduction: Biofilms are often found in communities of microorganisms in chronic and persistent infections, exhibiting high resistance against antimicrobial agents. Biofilm serves as a barrier, impeding the penetration of drugs and constraining their effectiveness. Multiple methods, such as the Tissue Culture Plate method, Congo Red Agar method, Tube method, bioluminescent assay, and fluorescent microscopic examination, can be used to evaluate biofilm production. **Methods:** The study included a total of 300 clinical isolates representing a range of bacterial species, including *Acinetobacter baumannii* (n=9), Coagulase Negative Staphylococcus (n=7), *Enterobacter aerogenes* (n=7), *Enterococcus faecalis* (n=15), *Escherichia coli* (n=137), *Klebsiella pneumoniae* (n=23), *Proteus mirabilis* (n=4), *Pseudomonas aeruginosa* (n=16), *Salmonella typhi* (n=11), and *Staphylococcus aureus* (n=68). Associations among isolates capable and incapable of biofilm formation and their multidrug resistance phenotypes were evaluated. **Results:** Among the 300 clinical isolates tested, 289 isolates (96.3%) exhibited biofilm formation. The most prevalent biofilm-forming organisms were *A. baumannii* (n=9), *Citrobacter koseri* (n=1), Coagulase Negative Staphylococcus (CONS) (n=7), *E. aerogenes* (n=7), *E. faecalis* (n=15), *E. coli* (n=137), *Klebsiella oxytoca* (n=1), *K. pneumoniae* (n=23), *P. mirabilis* (n=4), *P. aeruginosa* (n=16), *S. typhi* (n=11), *S. aureus* (n=68), and *Streptococcus pneumoniae* (n=1). The biofilm-forming isolates demonstrated increased resistance compared to isolates that did not form biofilms. **Conclusion:** Antimicrobial resistance represents a critical characteristic of infections involving biofilms. The study identified biofilm production in 92.7% of the isolates tested via TCP and in 72.3% of the isolates using the CRA. Furthermore, it was observed that pathogens with multidrug resistance (MDR) exhibited a higher biofilm production tendency than non-producing pathogens.

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INTRODUCTION

Microbial communities known as biofilms are encased within their own self-produced extracellular polymeric matrix [1]. Bacteria can adopt two distinct growth modes, the first being planktonic cells, while the second involves sessile aggregates known as biofilms [2]. Biofilms are microbial communities in which microbes generate extracellular polymeric substances (EPS), including proteins (less than 1-2%, which may include enzymes), DNA (less than 1%), polysaccharides (comprising 1-2% of the total), and RNA (less than 1%). In addition to these components, water accounts for up to 90%, playing a

crucial role in the nutrient flow within the biofilm matrix [3]. Biofilm architecture comprises two primary elements: a network of water channels for nutrient transport and dense cell clusters lacking prominent pores. Microbial cells within biofilms exhibit distinctive arrangements and display pronounced variations in physiology and physical properties [4]. Pathogenic microorganisms exploit biofilm formation to adhere to implants and prosthetic devices. [5]. Microorganisms adhere to surfaces and secrete polysaccharides, forming biofilms that create an optimal environment for genetic material exchange, which, in turn, contributes to the emergence of drug-resistant

pathogens [6, 7]. Within the biofilm matrix, bacteria communicate through the production of chemotactic particles or pheromones, known as quorum sensing [8]. The availability of essential nutrients, chemotactic response towards the substrate, bacterial motility, surface adhesion mechanisms, and surfactant presence are among the factors influencing biofilm formation [8]. Microorganisms within biofilms exhibit inherent resistance to antimicrobial agents compared to their planktonic counterparts [9]. Significantly higher antimicrobial concentrations are required to effectively inhibit the growth of biofilm-associated organisms due to the potential for antibiotic resistance to increase by up to 1,000-fold [9]. During biofilm growth, adaptive mechanisms of resistance, as opposed to genetic alterations, contribute to the development of antibiotic resistance. This allows drug-resistant pathogens to evade host defenses and antibiotic treatments, resulting in persistent infections [10]. Drug-resistant biofilm-producing isolates pose a significant challenge in human diseases [11]. Biofilm formation is commonly associated with infections affecting human surfaces, including teeth, skin, and the urinary tract [12]. Biofilm formation is related to various medical conditions, such as upper respiratory tract infections, endocarditis, thrombophlebitis, and urinary tract infections (UTIs). It is particularly prevalent in the presence of medical devices [13]. Numerous bacterial species can form biofilms, including *P. aeruginosa*, *S. epidermis*, *E. coli*, *S. aureus*, *E. cloacae*, and *K. pneumoniae* [14].

Biofilms have significant implications for public health as microorganisms within biofilms display reduced susceptibility to antimicrobial agents. Susceptibility to antimicrobial agents can arise due to biofilm growth or can be acquired, resulting from the transfer of extrachromosomal elements to susceptible organisms within the biofilm. Antibiotic-resistant pathogens primarily originate in healthcare settings, resulting in extended hospitalizations, increased healthcare expenses, and elevated mortality rates. Multidrug-resistant (MDR) organisms have the potential to spread to additional healthcare facilities and within the community [15, 16, 17]. Biofilms formed by bacteria exhibit infectious properties, which can lead to nosocomial infections. Hospital-acquired infections contribute to a substantial burden of morbidity and mortality. Hospital environments are susceptible to contamination with bacterial pathogens that can grow as biofilms on surfaces. Frequently encountered nosocomial pathogens such as *P. aeruginosa* synthesize exopolysaccharides, forming intricate biofilm structures that facilitate adherence to abiotic surfaces, thus protecting the action of antibiotics [18, 19, 20, 21]. Several methods are available for detecting biofilm production, including the Tissue Culture Plate (TCP), Tube method (TM) assay, Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensor technology, and fluorescent microscopy examination [22, 23, 24, 25, 26, 27].

This study includes biofilm formation and comparing antibiotic sensitivity patterns between biofilm producers and non-producers.

MATERIAL AND METHODS

A cross-sectional study conducted in a laboratory setting aimed to investigate the comparative antibiotic susceptibility among biofilm-producing and non-producing strains. The study was conducted at Star Hospital, with sample processing performed at the Microbiology Department, Modern Technical College, Pokhara University, Kathmandu, Nepal, between October and December 2018. The convenience sampling method was employed, and only specimens with positive cultures were included, while samples exhibiting mixed growth were excluded. A total of 300 bacterial isolates were obtained. The bacterial isolates were from different clinical specimens, with a prevalence rate of 61.4%, as reported by Sanchez *et al.* [12, 32]. Ethical approval was obtained from the Institutional Review Board (IRB) of the Nepal Health Research Council (NHRC), Kathmandu, Nepal (Ref. no.: 821). Written informed consent was obtained from the patients receiving care at Star Hospital.

Three hundred bacterial isolates were selected randomly from various clinical specimens obtained from Star Hospital and transferred to the Microbiology Laboratory at Modern Technical College. For subsequent analysis, the isolates were cultured on MacConkey Agar and Blood Agar plates. Biofilm detection was conducted using the tissue culture plate method and the Congo red agar technique. For the tissue culture plate method, bacterial isolates were obtained from freshly prepared agar plates and then inoculated into 10 mL Trypticase Soy Broth supplemented with 1% glucose. Subsequently, the broths were then incubated at 37 °C for 24 h. Next, the cultures were diluted at a dilution ratio 1:100 using fresh medium. Later, individual wells of sterile 96-well polystyrene plates with a flat-bottom surface treated for tissue culture were filled with a volume of 200 µL. A control organism was similarly incubated, diluted, suspended, and introduced into the corresponding wells of the tissue culture plate. For negative control, wells were filled with sterile broth inoculated with the control organism. Subsequently, the plates were incubated at 37 °C for 24 h, followed by gentle tapping to remove the contents of each well. Then, the wells were washed four times using 0.2 mL of phosphate-buffered saline (pH 7.2) to remove free-floating bacteria. The biofilm formed by bacteria that adhered to the wells was subsequently fixed using a 2% sodium acetate solution and then stained with a 0.1% crystal violet solution. The excess stain was

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removed using deionized water. Later, an ethanol and acetone solution in a ratio of 80:20 was applied to each well to achieve a uniform biofilm layer. The optical density of the stained biofilm adhered to the surface was measured using a microplate reader at a 550 nm wavelength. Each experiment was performed in triplicate and repeated three times. Interpretation of biofilm production was based on the criteria outlined by Stepanovic *et al.* [15, 28]. For the Congo red agar method, the CRA medium was prepared with 37 g/L of brain heart infusion broth powder, 50 g/L of sucrose powder, 10 g/L of agar No.1 powder, and 8 g/L

of Congo red indicator powder. Initially, the Congo red agar was prepared as a concentrated solution in water and subjected to autoclaving at 121 °C for 15 min, independently from the other components. Subsequently, the answer was added to the autoclaved brain heart infusion agar containing sucrose at 55 °C. Test organisms were inoculated onto the CRA plates and incubated under aerobic conditions at 37 °C for 24 h. Biofilm production was indicated by black colonies with a dry crystalline consistency. The Congo red agar method was performed in triplicate [17].

Table 1. Interpretation of biofilm production

| Average OD value | Biofilm production |
|--------------------------|--------------------|
| ≤ ODc / ODc < ~ ≤ 2x ODc | Non/ weak |
| 2x ODc < ~ ≤ 4x ODc | Moderate |
| > 4x ODc | Strong |

Optical density cut-off value (ODc) = average OD of negative control + 3× of Standard deviation (SD) of negative control.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [28]. The inoculum was directly applied to Muller-Hinton Agar (MHA). The following antibiotics were used: Amoxicillin, Cefotaxime, Nitrofurantoin, Norfloxacin, Ofloxacin, Gentamycin, Cefixime, Cotrimoxazole, Cephalexin, Amikacin, Meropenem, Piperacillin-tazobactam, Cefoxitin, and Vancomycin. Interpretation of the results was performed according to the criteria established by CLSI.

Statistical analysis: Data entry was performed using Microsoft Excel 2013, followed by analysis using SPSS version 16.

RESULTS

A total of 300 clinical isolates were obtained from various specimens. Of 300 isolates, 173 were *E. coli*, followed by *S. aureus* (n=68), *K. pneumoniae* (n=23), *P. aeruginosa* (n=16), *E. faecalis* (n=15), *S. typhi* (n=11), *A. baumannii* (n=9), Coagulase Negative Staphylococcus (CONS) (n=7), *E. aerogenes* (n=7), *P. mirabilis* (n=4), *S. pneumoniae* (n=1), *K. oxytoca* (n=1), and *C. koseri* (n=1).

The clinical specimens included urine (n=174) followed by pus (n=66), sputum (n=21), blood (n=10), tracheal aspirate (n=8), fluid (n=6), endotracheal tube (ET) (n=2), central venous pressure (CVP) (n=1) and tissue (n=1), as shown in Table 2.

Table 2. Details of collected specimens and identified bacteria

| Organisms | Urine | Pus | Sputum | Blood | ET tube | Fluid | CVP Tube | Tissue | Total |
|----------------------|-------|-----|--------|-------|---------|-------|----------|--------|-------|
| <i>E. coli</i> | 109 | 24 | 1 | 0 | 1 | 1 | 0 | 1 | 137 |
| <i>S. aureus</i> | 25 | 28 | 8 | 2 | 0 | 5 | 0 | 0 | 68 |
| <i>P. aeruginosa</i> | 17 | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 23 |
| <i>K. pneumoniae</i> | 5 | 4 | 4 | 0 | 2 | 0 | 1 | 0 | 16 |
| <i>E. faecalis</i> | 5 | 8 | 1 | 0 | 1 | 0 | 0 | 0 | 15 |
| <i>S. typhi</i> | 0 | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 11 |
| <i>A. baumannii</i> | 1 | 2 | 1 | 0 | 5 | 0 | 0 | 0 | 9 |
| CONS | 5 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 7 |
| <i>E. aerogenes</i> | 5 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 7 |
| <i>P. mirabilis</i> | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| <i>K. oxytoca</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| <i>S. pneumoniae</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| <i>C. koseri</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Total | 179 | 68 | 22 | 13 | 10 | 6 | 1 | 1 | 300 |

Biofilm-producing bacteria. Of the 300 clinical isolates, 289 (96.3%) were biofilm producers, as determined by the TCP and Congo red agar methods. TCP revealed 150 isolates as strong biofilm producers, 128 as

moderate biofilm producers, and 22 as none or weak biofilm producers — meanwhile, using CRA 217 isolates were biofilm producers (Table 3).

Table 3. Frequency of biofilm production by different methods

| Method | Biofilm production | No. of isolates |
|--------------------|--------------------|-----------------|
| Biofilm production | Yes | 289 (96.3%) |
| | No | 11 (3.3%) |
| | Strong | 150 (49.5 %) |
| TCP method | Moderate | 128 (42.2%) |
| | Non / Weak | 22 (7.3%) |
| CRA method | Positive | 217 (71.6%) |
| | Negative | 83 (27.4 %) |

Among the clinical isolates obtained from the specimens, 289 were identified as the major predominant biofilm producers. All *K. pneumoniae* (n=23), *S. aureus* (n=68), *CONS* (n=7), *E. faecalis* (n=15), *P. aeruginosa* (n=16), *E. aeruginosa* (n=7), *P. mirabilis* (n=4), *K. oxytoca* (n=1), *S. pneumoniae* (n=1) and *C. koseri* (n=1) isolates were biofilm producer. Among the 137 *E. coli* isolates, 131 exhibited biofilm production. Similarly, of the 11 *S. typhi* isolates, 8 demonstrated biofilm

production, and of the 9 *A. baumannii* isolates, 7 showed biofilm production.

Most of the biofilm-producing bacteria were derived from urine (60.2%), followed by pus (22.8%), sputum (7.3%), blood (3.5%), tracheal aspirate (2.8%), fluid (2.1%), endotracheal tube (ET) (0.7%), central venous pressure (CVP) tube (0.3%), and tissue (0.3%) (Table 4).

Table 4. Biofilm formation of isolates from various clinical specimens

| Organisms | Urine | Pus | Sputum | Blood | ET tube | Fluid | CVP Tube | Tissue | Total |
|----------------------|-------|-----|--------|-------|---------|-------|----------|--------|-------|
| <i>E. coli</i> | 104 | 23 | 1 | 0 | 1 | 1 | 0 | 1 | 131 |
| <i>S. aureus</i> | 25 | 28 | 8 | 2 | 0 | 5 | 0 | 0 | 68 |
| <i>P. aeruginosa</i> | 17 | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 23 |
| <i>K. pneumoniae</i> | 5 | 4 | 4 | 0 | 2 | 0 | 1 | 0 | 16 |
| <i>E. faecalis</i> | 5 | 8 | 1 | 0 | 1 | 0 | 0 | 0 | 15 |
| <i>S. typhi</i> | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 8 |
| <i>A. baumannii</i> | 1 | 1 | 0 | 0 | 5 | 0 | 0 | 0 | 7 |
| <i>CONS</i> | 5 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 7 |
| <i>E. aerogenes</i> | 5 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 7 |
| <i>P. mirabilis</i> | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| <i>K. oxytoca</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| <i>S. pneumoniae</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| <i>C. koseri</i> | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Total | 174 | 66 | 21 | 10 | 10 | 6 | 1 | 1 | 289 |

Association of biofilm production and MDR. Compared to non-biofilm-producing organisms, biofilm-producing organisms exhibited significantly higher levels

of drug resistance to all tested antibiotics, as shown in Table 5.

Table 5. Biofilm formation and MDR

| MDR | Biofilm Producer | P-value | |
|-----|------------------|---------|----|
| | | Yes | No |
| MDR | Yes | 151 | 4 |
| | No | 138 | 7 |

Distribution of MDR among clinical isolates. Among the 289 biofilm-producing isolates, 151 (52.2%) exhibited multidrug-resistant (MDR) phenotypes among biofilm-positive and biofilm-negative organisms. Among the 11 biofilm-negative organisms, only 4 (36.4%) exhibited MDR phenotypes (Table 6).

Antibiogram of Gram-positive isolates. The predominant Gram-positive isolates were found to be MDR. Forty-seven percent of the *S. aureus* isolates were identified as MRSA. None of the MRSA isolates exhibited resistance to Vancomycin. *CONS* isolates showed higher resistance than *S. aureus* (Table 8).

Table 6. MDR in different biofilm-producing organisms

| Biofilm-producing organisms | MDR |
|-----------------------------|------------|
| <i>E. coli</i> | 61 (40.4%) |
| <i>S. aureus</i> | 32 (21.2%) |
| <i>K. pneumonia</i> | 14 (9.3%) |
| <i>E. faecalis</i> | 12 (7.9%) |
| <i>P. aeruginosa</i> | 11 (7.3%) |
| <i>A. baumannii</i> | 7 (4.6%) |
| CONS | 4 (2.6%) |
| <i>E. aeruginosa</i> | 3 (2.0%) |
| <i>P. mirabilis</i> | 2 (1.3%) |
| <i>S. typhi</i> | 2 (1.3%) |
| <i>C. koseri</i> | 1 (0.3%) |
| <i>S. pneumonia</i> | 1 (0.3%) |
| <i>K. oxytoca</i> | 1 (0.3%) |

Table 7. Susceptibility pattern rates of Gram-negative bacteria producing biofilms

| Organisms | | Antibiotic (%) | | | | | | | | | |
|----------------------|-----------|----------------|------|------|------|------|------|------|------|------|------|
| | | AK | CTX | CTR | MRP | OF | COT | PIT | NIT | NX | AMX |
| <i>E. coli</i> | Sensitive | 45.8 | 32.1 | 32.1 | 58 | 39.6 | 33.6 | 45 | 49 | 35.1 | 16 |
| | Resistant | 54.2 | 67.9 | 67.9 | 42 | 60.3 | 66.4 | 55 | 51 | 64.9 | 84 |
| <i>K. pneumoniae</i> | Sensitive | 39.1 | 26.1 | 21.7 | 73.9 | 26.1 | 47.8 | 21.7 | 13 | 21.7 | - |
| | Resistant | 60.9 | 73.9 | 78.3 | 26.1 | 73.9 | 52.2 | 78.3 | 87 | 78.3 | - |
| <i>P. aeruginosa</i> | Sensitive | 25 | - | - | 50 | 25 | - | 43.8 | 0 | 50 | - |
| | Resistant | 75 | - | - | 50 | 75 | - | 56.2 | 100 | 50 | - |
| <i>P. mirabilis</i> | Sensitive | 75 | 50 | 75 | 75 | 0 | 50 | 50 | 0 | 50 | 0 |
| | Resistant | 25 | 50 | 25 | 25 | 100 | 50 | 50 | 100 | 50 | 100 |
| <i>A. baumannii</i> | Sensitive | 0 | 0 | 0 | 14.3 | 28.6 | 0 | 0 | 0 | 0 | 0 |
| | Resistant | 100 | 100 | 100 | 85.7 | 71.4 | 100 | 100 | 100 | 100 | 100 |
| <i>E. aerogenes</i> | Sensitive | 57.1 | 57.1 | 57.1 | 71.4 | 57.1 | 57.1 | 71.4 | 42.8 | 42.9 | 28.6 |
| | Resistant | 42.9 | 42.9 | 42.9 | 28.6 | 42.9 | 42.9 | 28.6 | 57.2 | 57.1 | 71.4 |
| <i>S. typhi</i> | Sensitive | NT | 75 | 75 | 100 | 100 | 75 | 100 | - | - | 62.5 |
| | Resistant | NT | 25 | 25 | 0 | 0 | 25 | 0 | - | - | 37.5 |

Table 8. Antimicrobial susceptibility testing patterns of biofilm-producing Gram-positive bacteria as a percentage

| Organisms | | Antibiotic (%) | | | | | | | | | |
|--------------------|-----------|----------------|------|------|------|------|------|------|------|------|------|
| | | AK | CTX | CTR | CN | OF | PIT | NIT | VA | CX | COX |
| <i>S. aureus</i> | Sensitive | 64.7 | 27.9 | 42.6 | 32 | 56 | 78 | 56 | 100 | 53 | 42 |
| | Resistant | 35.3 | 72.1 | 57.4 | 68 | 44 | 22 | 44 | 0 | 47 | 58 |
| CONS | Sensitive | 100 | 42.8 | 42.8 | - | 42.8 | 42.8 | 0 | 28.6 | 57.2 | 28.6 |
| | Resistant | 0 | 57.2 | 57.2 | - | 57.2 | 57.2 | 100 | 71.4 | 42.8 | 71.4 |
| <i>E. faecalis</i> | Sensitive | 20 | 13.3 | 20 | 44.4 | 46.7 | 40 | 33.3 | 13.3 | 13.3 | 20 |
| | Resistant | 80 | 86.7 | 80 | 55.6 | 53.3 | 60 | 66.7 | 86.7 | 86.7 | 80 |

DISCUSSION

A laboratory-based cross-sectional study was conducted in the Microbiology laboratory from October to December 2018. Various clinical specimens were included, and antimicrobial susceptibility testing was performed using the Kirby-Bauer disc

diffusion method. Biofilm production was assessed using both Congo red agar and tissue culture methods. Most microorganisms (99.9%) demonstrated the ability to produce biofilm on various surfaces, including biological and inert surfaces [29, 30].

In our study, Biofilm formation was detected in 289 isolates (96.3%), while Verma *et al.* (2016) reported 66% [31]. Sanchez *et al.* (2013) found that of 205 clinical isolates, 126 (61.4%) were biofilm production [32]. Biofilm production in the present study was higher compared to those previous studies. This difference may be attributed to the inclusion of largely multidrug-resistant hospital.

Biofilm-producing organisms mainly were from urine samples (60.2%), followed by pus samples (22.8%), sputum samples (7.5%), blood samples (3.5%), tracheal aspirate samples (2.8%), fluid samples (2.1%), endotracheal tube samples (0.7%), central venous catheter (CVP) samples (0.3%), and tissue samples (0.3%). In a similar study, biofilm-producing organisms were most commonly found in urine samples (30%), followed by urinary catheter tips (25.7%), pus samples (12.8%), sputum samples (11.4%), intravenous catheter tips (10%), and naso-bronchial lavage samples (10%) [29]

In our study, of the 137 *E. coli* isolates 131 were biofilm producers, including all *S. aureus* (N=68), *K. pneumoniae* (n=23), *P. aeruginosa* (n=16), *E. faecalis* (n=15), *E. aerogenes* (n=7), *P. mirabilis* (n=4), CONS (n=7), *C. koseri* (n=1), *K. oxytoca* (n=1), and *S. pneumoniae* (n=1) isolates. Of the 11 *S. typhi* isolates, 8 were biofilm producers, and of the 9 *A. baumannii* isolates, 7 exhibited biofilm production. According to Sanchez *et al.* (2013), among 23 *S. aureus* isolates, 21 strains (91%) tested positive, and among the 54 *K. pneumoniae* isolates, 41 tested positive [32]. Similarly, 36 *P. aeruginosa* and 53 *A. baumannii* isolates, 30 and 29 were biofilm producers. Among the 39 *E. coli* isolates, only 5 were biofilm producers. The clinical isolates in our study that demonstrated biofilm production were similar to the findings of Sanchez *et al.* (2013).

In a similar study by Verma *et al.* (2016), among 168 biofilm producer isolates, *E. coli* was the most prevalent pathogen, accounting for 56 out of 96 isolates, followed by *K. pneumoniae* with 30 out of 45 isolates. *A. baumannii* exhibited biofilm formation in all 6 isolates, while *P. aeruginosa* showed biofilm production in 5 out of 7 isolates. Similarly, *E. cloacae*, *P. mirabilis*, *C. diversus*, and *Proteus vulgaris* demonstrated biofilm formation in all 5, 5, 2, and 2 isolates, respectively [31]. Similar findings were observed in a study conducted by Hassan *et al.* (2011), which reported 25 (22.7%) isolates with strong biofilm production and 45 (41%) isolates with moderate biofilm production [33]. The rate of moderate biofilm production was consistent with our study. Only 11 isolates were identified as biofilm producers using the CRA method out of 111. The significant biofilm production observed in this study using the CRA method could be attributed to device-associated pathogens. In a regional survey in India, among 152 isolates, 53.9% of the isolates demonstrated biofilm production, while 46% did not exhibit biofilm production [34].

In a separate study conducted by Verma *et al.* (2016), out of a total of 111 strains, 66% (111 strains) demonstrated biofilm production by the TCP method, while the remaining 34% (57 strains) did not exhibit biofilm production [31]. Nevertheless, 58% of the isolates exhibited biofilm production using the CRA method, while the remaining 42% did not. In contrast, the TCP method demonstrated a sensitivity of 95% and specificity of 100%, surpassing the CRA method, which exhibited a sensitivity of 74% and specificity of 90.9%. The TCP method provides a quantitative and convenient approach for assessing biofilm formation in microorganisms. Likewise, a study by Saha *et al.* (2018) reported 89.7% of the clinical isolates from hospitalized patients as biofilm producers; at the time, in

outpatients, only 10.3% exhibited the same feature [35]. Bacterial biofilms account for 65% of nosocomial infections, emphasizing their significant contribution to healthcare-associated conditions.

Organisms that produce biofilms exhibited a higher prevalence of MDR than organisms that did not. Of the 289 organisms that produced biofilms, 151 were identified as having MDR phenotypes. However, among the 11 non-biofilm-producing organisms, only 4 organisms exhibited MDR. In this study, among the 289 biofilm-producing bacteria analyzed, the predominant MDR organism was *E. coli* (40.4%), followed by *S. aureus* (21.2%), *K. pneumoniae* (9.3%), *E. faecalis* (7.9%), *P. aeruginosa* (7.3%), *A. baumannii* (4.6%), coagulase-negative Staphylococci (2.6%), *E. aerogenes* (2.0%), *P. mirabilis* (1.3%), *S. typhi* (1.3%), *K. oxytoca* (0.7%), *C. koseri* (0.7%), and *S. pneumoniae* (0.7%).

Likewise, in a study by Summaiya *et al.* (2012), it was found that *A. baumannii* (35.1%) was the most common multidrug-resistant organism, followed by *P. aeruginosa* (18.9%), *K. pneumoniae* (18.9%), *E. coli* (13.5%), and *S. aureus* (10.8) [36]. In the present study, most biofilm-positive clinical isolates resisted cephalosporins, aminoglycosides, carbapenems, fluoroquinolones, nitrofurantoin, and co-trimoxazole. Drug resistance among the biofilm producers was similarly elevated in the study by Sanchez *et al.* (2013), mirroring our research [32].

A concerning rise in the production of biofilms and the development of drug resistance has been observed among isolates of Gram-negative and Gram-positive bacteria obtained from various clinical samples.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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