

Biofilm-Forming Clinical Isolates - A Risk Factor for Multidrug Resistance

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Abstract - Biofilm formation acts as a major driver of antimicrobial resistance, allowing bacteria to withstand antibiotic concentrations 10 to 1,000 times higher than their planktonic counterparts. Biofilms represent one of the most adaptable microbial features that significantly contribute the virulence of pathogenic bacteria. The enhanced survival mechanism promotes the emergence of multi-drug resistance bacteria posing the greatest threat to human health worldwide. Therefore, the evaluation of multi-drug resistant biofilm-forming bacteria needs to be undertaken to improve treatment strategies and clinical outcomes. The present study focuses on the identification, and analysis of biofilm forming ability among clinical isolates collected from the hospitals in Namakkal district, Tamil Nadu, India. Out of all isolates tested, biofilm production varied among species, Higher biofilm formation was observed in *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Enterococcus* spp., *Vibrio* spp., and *Shigella* spp. Moderate biofilm formation was observed in *Escherichia* spp. and *Proteus* spp. In contrast no biofilm formation in *Salmonella* spp. Furthermore, the biofilm-producing bacterial isolates demonstrated higher multi-drug resistance compared to non-biofilm producers, indicating a strong association between biofilm formation and antimicrobial resistance.

Key Words: biofilm formation, multidrug resistance, clinical isolates, antimicrobial susceptibility, extracellular polymeric substance, Namakkal district.

1. INTRODUCTION

The intricate structural complexity of biofilms, composed by embedded extracellular polymeric substances (EPS) matrix, contributes a major role in microbial drug resistance (1). Biofilms are formed by single species or poly species communities, including bacteria, fungi, diatoms and protozoa, that colonize in both biotic and abiotic surfaces, exhibits enhanced resistance to removal and antimicrobial treatment (2). They cause recurrent infections in natural and artificial ecosystems. Natural ecosystems like teeth, urinary and respiratory tract, gastro intestinal systems, chronic wounds and implanted medical devices (3). Biofilm formation serves as a survival strategy that enables bacteria to withstand hostile environment leading to increased tolerance towards antibiotics, biocides, desiccation and host immune system (4).

Formation of biofilms is a dynamic multi step process include, attachment, microcolony formation, maturation and

dispersal. First step in biofilm formation is Reversible attachment, where planktonic microbes attached to the natural or artificial environment, followed by irreversible attachment of the cells. During this stage bacteria firmly anchors by secreting extracellular polymeric substances, which includes polysaccharides, proteins lipids and extracellular DNA. Bis-(3'5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is an intracellular signalling molecule that plays a vital role in early events of biofilm formation by restricting flagella mediated swimming motility and increasing biofilm matrix production (5) & (6).

Following successful adhesion to a surface, microorganisms begin to proliferate and aggregate within a self-produced extracellular polymeric substance (EPS) matrix, leading to the formation of microcolonies, often associated with elevated intracellular levels of cyclic di-GMP (c-di-GMP). Flagella-mediated motility facilitates initial interactions between microorganisms and surfaces, while type IV pili play a crucial role in cell-cell interactions and aggregation during microcolony formation (7).

Extracellular polymeric substances (EPS) play a crucial role in biofilm maturation by facilitating microbial attachment to surfaces, stabilizing the three-dimensional structure of the biofilm, and promoting cell-cell aggregation. The EPS matrix provides protection against various environmental stresses, including host immune responses, antimicrobial agents, oxidative damage, and metal ions. Additionally, it serves as a reservoir for signaling molecules involved in quorum sensing, as well as metabolic products and enzymes. Mature biofilms often develop complex architectures, such as 'mushroom' or 'tower-like' structures, in which microorganisms are spatially organized according to their oxygen tolerance and metabolic activity (7 & 8).

In the final stage, some cells detach from the mature biofilm and return to the planktonic state. This process is triggered by factors such as nutrient limitation, environmental stress, or signaling molecules. Dispersed cells can colonize new surfaces and initiate the formation of new biofilms, contributing to the spread of infection (6).

Antimicrobial resistance is a serious hazard to global public health and threatens the effectiveness of contemporary medicine. Morbidity, mortality, and healthcare expenses have gone up as a result of the growth in infections that are

extensively and multi drug-resistant (9). Multi-drug resistant (MDR) bacteria are well-recognized to be one of the most important current public health problems. The Infectious Diseases Society of America (IDSA) recognizes antimicrobial resistance as “one of the greatest threats to human health worldwide” (10).

When a bacterium is resistant to at least one antibiotic in three (or more) different antibiotic classes it is said to be multidrug-resistant. This can happen in two separate ways (11). The first way of resistance development is through resistance genes to the particular antibiotic, the second methodology is resistant strategy through efflux pumps, enzymatic degradation, target modification and altered permeability (12).

Multidrug-resistant (MDR) bacteria arise through the acquisition of multiple resistance genes, often located on mobile genetic elements like plasmids, transposons, and integrons. These genes enable bacteria to resist antibiotics through mechanisms such as drug inactivation, target modification, and enhanced efflux. Key genes and gene families involved in multidrug resistance include β -Lactam resistance genes, which produce enzymes that break down β -lactam antibiotics (penicillins, cephalosporins, carbapenems) like blaTEM, blaNDM, blaKPC, mecA etc. (13).

Aminoglycoside resistance genes modify aminoglycoside antibiotics to prevent binding to the bacterial ribosome; these include strA/strB, aac, aad and aph (14). Efflux pump genes encode transport proteins that pump antibiotics out of the bacterial cell, including acrAB/EF, mexAB/CD, norA/B, tetA and tetB (15). Quinolone resistance genes (qnrA, qnrB, qnrS) are plasmid-mediated genes that protect DNA gyrase from quinolones, while gyrA, gyrB, parC and parE chromosomal mutations are frequent in fluoroquinolone-resistant bacteria. Among vancomycin and colistin resistance genes, the vanA cluster is responsible for high-level resistance to vancomycin in Enterococcus and VRSA, and mcr-1 is responsible for plasmid-mediated resistance to colistin (polymyxin), a last-resort drug. Other resistance genes include sul1, sul2 and sul3, which confer resistance to sulfonamides; cstB3, responsible for chloramphenicol resistance; ermA and ermB, which encode methylases conferring resistance to macrolides, lincosamides and streptogramin B; and tet(M), tet(O) and tet(S), which confer tetracycline resistance through ribosome protection (16).

Hence the biofilm formation and genetic drivers play an important role in multidrug resistance. Biofilm formation significantly enhances antimicrobial resistance by limiting antibiotic penetration through the extracellular polymeric matrix, promoting the survival of persister cells, and altering bacterial metabolic activity (17 & 18). Additionally, the close proximity of cells within biofilms facilitates horizontal gene transfer, further contributing to the spread of multidrug resistance. As a result, biofilm-associated infections are often

difficult to eradicate and are associated with increased treatment failure (19).

Biofilm-associated multidrug-resistant pathogens are frequently implicated in hospital-acquired infections, particularly those associated with indwelling medical devices such as catheters, prosthetic implants, and ventilators. These infections are often chronic, recurrent, and difficult to treat, posing a significant burden on healthcare systems (20 & 21). Despite extensive studies on biofilm formation and antimicrobial resistance, limited data are available on the correlation between biofilm production and multidrug resistance among clinical isolates around the Namakkal, Tamil Nadu region.

2. MATERIALS AND METHODS

Study Area and Sample Collection

A total of 36 non-duplicate clinical bacterial isolates were obtained from tertiary care hospitals located in the Namakkal District, Tamil Nadu, India. The panel comprised diverse genera, including *Escherichia spp.*, *Klebsiella spp.*, *Pseudomonas spp.*, *Staphylococcus spp.*, *Salmonella spp.*, *Enterococcus spp.*, *Vibrio spp.*, *Shigella spp.*, and *Proteus spp.*

Isolation and Phenotypic Identification

Obtained bacterial isolates were inoculated onto selective and differential agar media (HiMedia, India). The isolates were phenotypically characterized and identified based on colony morphology, Gram staining, and standard biochemical profiling according to the criteria defined in Bergey's Manual of Systematic Bacteriology and standard microbiological protocols. The primary biochemical hallmarks used for generic verification are summarized in Table 1.

Table 1: Biochemical characterization profiles for the identification of clinical isolates.

Organism	Primary Biochemical Characteristics
<i>Escherichia spp.</i>	Indole (+), Methyl red (+), Citrate (-), Lactose fermenter
<i>Klebsiella spp.</i>	Citrate (+), Urease (+), Lactose fermenter, Non-motile
<i>Pseudomonas spp.</i>	Oxidase (+), Non-lactose fermenter, Citrate (+)
<i>Staphylococcus spp.</i>	Catalase (+), Coagulase (+/-)
<i>Salmonella spp.</i>	H ₂ S production (+), Motile, Citrate (+)
<i>Enterococcus spp.</i>	Bile esculin (+), Catalase (-)
<i>Vibrio spp.</i>	Oxidase (+), Motile, Sucrose fermenter
<i>Shigella spp.</i>	Non-motile, Non-lactose fermenter

<i>Proteus spp.</i>	Urease (+), Swarming motility, H2S production (+)
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Detection of Biofilm Formation

Biofilm-producing capabilities were assessed qualitatively using the tube method (TM) described by Christensen et al. (1985). Briefly, isolates were cultured in glass tubes, and the development of a visible, continuous film on the bottom and inner walls of the tube was scored as biofilm-positive. The absence of a discernible film or the presence of a ring formation at the liquid-air interface alone was considered negative.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility profiling was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA). The isolates were screened against a panel of clinically relevant antibiotics spanning multiple classes: cephalosporins, aminoglycosides, carbapenems, tetracyclines, quinolones, and sulfonamides. Zones of inhibition were measured and categorized as Sensitive (S), Intermediate (I), or Resistant (R).

3. RESULTS

Phenotypic screening of the 36 clinical isolates revealed substantial variations in biofilm architecture and production capacity across different genera. Strong phenotypic biofilm positivity was predominantly clustered among *Klebsiella spp.*, *Pseudomonas spp.*, *Staphylococcus spp.*, *Enterococcus spp.*, *Vibrio spp.*, and *Shigella spp.* Moderate biofilm phenotypes were detected in *E. coli* and *Proteus spp.* Conversely, 100% of the evaluated *Salmonella spp.* isolates were strictly non-biofilm-forming.

The phenotypic resistance profiles revealed a strong positive correlation with biofilm-forming capability across most genera. Among the Gram-negative enterics *Escherichia spp.* and *Klebsiella spp.*, 25% of *Escherichia spp.* isolates were biofilm-positive. These biofilm-forming strains demonstrated pervasive resistance (up to 100%) against erythromycin, tetracycline, nalidixic acid, chloramphenicol, ciprofloxacin, and penicillin. *Klebsiella spp.* exhibited severe multi-drug resistance phenotypes; 50% of the isolates were biofilm-positive, displaying critical resistance rates ranging from 75% to 100% against imipenem, tetracycline, polymyxin, and co-trimoxazole.

Among the non-fermenters, fifty percent (50%) of *Pseudomonas spp.* isolates were confirmed biofilm producers. These strains displayed concurrent, high-level resistance to erythromycin, tetracycline, novobiocin, chloramphenicol, and penicillin.

Among the Gram-positive cocci, *Staphylococcus spp.* demonstrated an alarming resistance profile, with 100% resistance observed against imipenem, erythromycin, tetracycline, novobiocin, chloramphenicol, polymyxin, and penicillin.

Atypical resistance vectors such as *Salmonella spp.* and *Proteus spp.* were also notable: *Salmonella spp.* isolates lacked biofilm-forming capacities entirely, yet maintained pronounced resistance to erythromycin, tetracycline, nalidixic acid, and penicillin. In contrast, *Proteus spp.* displayed the highest overall antimicrobial susceptibility and significantly lower phenotypic resistance rates compared to the other investigated genera.

Overall, biofilm-positive isolates displayed significantly higher rates of multidrug resistance compared to their non-biofilm-forming counterparts, highlighting a robust co-selection of virulence and resistance traits.

Table 2: Distribution of Biofilm Formation Phenotypes Across Clinical Isolates

S.No.	Bacterial Isolate	Total Tested (n)	Biofilm Positive (n,%)	Biofilm Negative (n,%)
1.	<i>Escherichia spp.</i>	4	1 (25.0%)	3 (75.0%)
2.	<i>Klebsiella spp.</i>	4	2 (50.0%)	2 (50.0%)
3.	<i>Pseudomonas spp.</i>	4	2 (50.0%)	2 (50.0%)
4.	<i>Staphylococcus spp.</i>	4	2 (50.0%)	2 (50.0%)
5.	<i>Salmonella spp.</i>	4	0 (0.0%)	4 (100.0%)
6.	<i>Enterococcus spp.</i>	4	3 (75.0%)	1 (25.0%)
7.	<i>Vibrio spp.</i>	4	2 (50.0%)	2 (50.0%)
8.	<i>Shigella spp.</i>	4	3 (75.0%)	1 (25.0%)
9.	<i>Proteus spp.</i>	4	2 (50.0%)	2 (50.0%)
	<i>Total Burden</i>	36	17 (47.2%)	19 (52.8%)

Table 3: Comparative Antibiotic Resistance Percentages (%) Across Genera (4 isolates for each genus)

Antibiotic	Esh spp (%)	Kleb spp. (%)	Pseudo spp. (%)	Staph spp. (%)	Salmon spp. (%)	Entero spp. (%)	Vibrio spp. (%)	Shigella spp. (%)	Proteus spp. (%)
CPD (Cefpodoxime)	100	0	75	0	75	0	25	0	25
GEN (Gentamicin)	50	25	25	0	0	0	0	0	0
IPM (Imipenem)	75	100	0	100	75	75	100	100	0
MRP (Meropenem)	0	0	75	0	0	25	25	25	50
E (Erythromycin)	100	75	100	100	100	100	100	100	25
TE (Tetracycline)	100	100	100	100	100	100	75	100	25
CXM (Cefuroxime)	0	0	75	0	75	0	50	100	0
LE (Levofloxacin)	100	0	0	0	75	0	0	0	0
NX (Norfloxacin)	100	0	0	100	100	0	100	0	25
NV (Novobiocin)	75	75	100	100	75	100	100	100	50
C (Chloramphenicol)	100	0	100	100	0	0	0	0	0
PI (Piperacillin)	75	100	0	100	100	0	100	100	25
CIP (Ciprofloxacin)	100	50	50	75	75	0	25	0	25
COT (Co-trimoxazole)	25	100	75	0	75	100	25	100	25
P (Penicillin)	100	100	100	100	100	100	100	100	100

4. DISCUSSION

The persistence of clinical pathogens within hospital environments is driven by a complex interplay between virulence factor expression and antimicrobial resistance. In this study, we evaluated the phenotypic distribution of biofilm formation and resistance patterns among 36 clinical isolates from Namakkal District, India. Our findings demonstrate a high prevalence of biofilm-forming capacities among key clinical pathogens, notably *Klebsiella spp.*, *Pseudomonas spp.*, *Staphylococcus spp.*, *Enterococcus spp.*, *Vibrio spp.*, and *Shigella spp.* These observations align with the foundational work of Donlan and Costerton (2002), which established that biofilm architecture serves as a primary driver for chronic, recurrent, and device-associated nosocomial infections.

The notable increase in minimum inhibitory concentrations (MICs) and overall multidrug resistance among our biofilm-positive isolates can be mechanistically explained by the structural dynamics of the extracellular polymeric substance (EPS) matrix. The EPS matrix functions as a robust physical and chemical diffusion barrier, retarding the penetration of diverse antimicrobial classes and exposing embedded cells to sub-inhibitory antibiotic concentrations (Hall-Stoodley et al., 2004). Furthermore, this altered microenvironment fosters the development of metabolically quiescent “persister cells,” which exhibit transient tolerance to conventional therapies and escape standard bactericidal mechanisms (Hall-Stoodley et al., 2004).

In our study, *Klebsiella spp.* and *Pseudomonas spp.* stood out due to their simultaneous expression of strong biofilm phenotypes and severe multidrug resistance (including resistance to reserve carbapenems like imipenem in *Klebsiella*). This phenotypic synergy agrees with Mah and O'Toole (2001), who demonstrated that biofilm-associated *Pseudomonas* cells exhibit up to a 1000-fold increase in antibiotic tolerance compared to genetically identical planktonic cells.

An interesting exception was observed in *Salmonella spp.* isolates, which lacked biofilm-forming capabilities but retained high resistance to erythromycin, tetracycline, nalidixic acid, and penicillin. This decoupling of resistance and biofilm formation indicates that antibiotic resistance in these specific *Salmonella* strains is likely mediated by classical genetic determinants—such as chromosomal mutations, active multi-drug efflux pumps (e.g., the AcrAB-TolC system), or plasmid-mediated resistance genes—rather than physical matrix protection.

Conversely, *Proteus spp.* displayed lower phenotypic resistance and minimal biofilm capacity, suggesting a lower degree of virulence adaptation within this specific clinical cohort, a trend previously noted by Costerton et al. (1999).

5. CONCLUSION

In conclusion, this study demonstrates a strong positive correlation between biofilm production and multidrug resistance profiles among clinical bacterial isolates. The high prevalence of MDR strains among biofilm-forming *Klebsiella spp.*, *Pseudomonas spp.*, and *Staphylococcus spp.* underscores the clinical challenge these pathogens present. These findings highlight the need for systematic epidemiological surveillance of biofilm-producing phenotypes in clinical settings and the development of targeted anti-biofilm therapeutic strategies to disrupt the EPS matrix and restore antibiotic efficacy.

REFERENCES

1. Ali A, Zahra A, Kamthan M, Husain FM, Albalawi T, Zubair M, Alatawy R, Abid M, Noorani MS. Microbial Biofilms: Applications, Clinical Consequences, and Alternative Therapies. *Microorganisms*. 2023 Jul 29;11(8):1934.
2. Jamal M., Khan A.W., Andleeb S. Bacterial Biofilm: Its Composition, Formation and Role in Human Infections. *J. Microbiol. Biotechnol.* 2015; 4:3.
3. Grari O, Ezrari S, Yandouzi IE, Benaissa E, Lahlou YB, Lahmer M, Saddari A, Elouennass M, Male A. A comprehensive review on biofilm-associated infections: Mechanisms, diagnostic challenges, and innovative therapeutic strategies. *The Microbe*. Volume 8, September 2025; 100436.
4. Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: a focus on anti-biofilm

- agents and their mechanisms of action. 2018; *Virulence*, 9, pp. 522-554.
5. Römmling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 2013; 77:1–52.
6. Rather MA, Gupta K, Mandal M. Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. *Brazilian Journal of Microbiology* 2021; 52:1701–1718.
7. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim H. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Med Chem* 2015; 7:493–512.
8. Toyofuku M, Inaba T, Kiyokawa T, Obana N, Yawata Y, Nomura N. Environmental factors that shape biofilm formation. *Biosci Biotechnol Biochem* 2016; 80:7–12.
9. Gashaw M, Berhane M, Bekele S, Melaku T, Lemmi G, Chelkeba L, Wakjira T, Tesfaw G, Mekonnen Z, Kroidl A, Gudina EK. Antibiotic Use Patterns at Jimma Medical Center in Southwest Ethiopia: A Call for Local Antibiogram-Guided Prescription. *Journal of Clinical Medicine* 2025; 14(7), 2413.
10. Spellberg B, Blaser M, Guidos RJ, et al. Combating antimicrobial resistance: policy recommendations to save lives. *Clin Infect Dis*. 2011; 52(Suppl 5): S397–428. [PubMed: 21474585]
11. Magiorakos AP, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; 18, 268–281.
12. Shiyamala G, Mathanmohun M, Lakshmanan A, Sundaram S. Antimicrobial Resistance Mechanisms of Drug-resistant Bacterial Pathogens: A Narrative Review. *Uttar Pradesh Journal of Zoology*. 2026; Volume 47, Issue 2, Page 75-87.
13. Chakraborty AK. Multi-Drug Resistant Genes in Bacteria and 21st Century Problems Associated with Antibiotic Therapy. *Biotechnol Ind J*. 2016;12(12):114.
14. Yasir M, et al. Genomic and antimicrobial resistance genes diversity in multidrug-resistant CTX-M-positive isolates of *Escherichia coli* at a health care facility in Jeddah. *Journal of Infection and Public Health* 2020; Volume 13, Issue 1, Pages 94-100.
15. Kirtikliene T, Mierauskaitė A, Razmienė I, Kuisiene N. Genetic Characterization of Multidrug-Resistant *E. coli* Isolates from Bloodstream Infections in Lithuania. *Microorganisms*, 2022;10(2):449.
16. Bharadwaj A, Rastogi A, Pandey S, Gupta S, Sohal JS. Multidrug-Resistant Bacteria: Their Mechanism of Action and Prophylaxis. *Biomed Res Int*. 2022 Sep 5;2022:5419874.

17. Pai L, Patil S, Liu S, Wen F. A growing battlefield in the war against biofilm-induced antimicrobial resistance: insights from reviews on antibiotic resistance. *Front. Cell. Infect. Microbiol.* 2023; 13:1327069.
18. Saffari Natanzi A, Poudineh M, Karimi E, et al. Innovative approaches to combat antibiotic resistance: integrating CRISPR/Cas9 and nanoparticles against biofilm-driven infections. *BMC Med* 2025; 23, 486.
19. Almatroudi A. Biofilm Resilience: Molecular Mechanisms Driving Antibiotic Resistance in Clinical Contexts. *Biology (Basel)*. 2025 Feb 6;14(2):165.
20. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.* 2002;15(2):167–193.
21. Percival SL, Suleman L, Vuotto C, Donelli G. Healthcare-associated infections, medical devices and biofilms. *J Med Microbiol.* 2015;64(Pt 4):323–334.
22. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 2002. 15(2), 167–193.
23. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*, 2004. 2(2), 95–108.
24. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, 2001. 9(1), 34–39.
25. van Leeuwen, J. (ed.): *Computer Science Today. Recent Trends and Developments. Lecture Notes in Computer Science*, Vol. 1000. Springer-Verlag, Berlin Heidelberg New York (1995)
26. Michalewicz, Z.: *Genetic Algorithms + Data Structures = Evolution Programs*. 3rd edn. Springer-Verlag, Berlin Heidelberg New York (1996)