ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

Screening of in-vitro Biofilm Production by Bacterial Pathogens Isolated from Secondary Hospital Waste-water and their Antibiotic Susceptibility Pattern

Bibek Aryal[1*], Shristi Tharu[1], Richa Chaudhary[1], Shashi Bhushan Chaturwedi[1]

[1]D.A.V. College, Dhobighat, Lalitpur, Nepal

Article Information

Received: 20 August 2024
Revised version received: 21 September 2024
Accepted: 23 September 2024
Published: 30 September 2024

Cite this article as:
B. Aryal et al. (2024) Int. J. Appl. Sci. Biotechnol. Vol 12(3): 145-150. DOI: 10.3126/ijasbt.v12i3.70156

***Corresponding author**
Bibek Aryal,
D.A.V. College, Dhobighat, Lalitpur, Nepal
Email: bibekaryal65@gmail.com

Peer reviewed under authority of IJASBT

Abstract

Objective: The main objective is to screen the in-vitro Biofilm Production by Bacterial Pathogens isolated from Secondary Hospital Wastewater and their Antibiotic Susceptibility Pattern.

Methods: A total of 10 hospital wastewater samples were collected within 3 consecutive days from a secondary hospital and research was carried out in the microbiological laboratory of D.A.V. College. Identification was done by performing Gram-staining followed by conventional biochemical tests. Screening of in-vitro biofilm production was done using the Congo Red Agar (CRA) method. Antibiotic Susceptibility Pattern (AST) was performed on Mueller-Hinton Agar (MHA) media by the Kirby-Bauer Disk Diffusion Method as per CLSI guidelines.

Results: A total of 25 bacterial isolates were identified during the identification process. Among all bacterial isolates, 12 (48%) were screened as biofilm producers, with dry crystalline black-centered colonies. In contrast, the remaining 13 (52%) were screened as non-biofilm producers with pink colonies. *Escherichia coli* (66.6%), were the most common biofilm-producing Gram-negative bacilli followed by *Citrobacter freundii* (16.66%), *Enterobacter spp* (8.37%), *Morganella morganii* (8.37%). During the antibiotic susceptibility pattern, a total of 16 (64%) bacterial isolates were recognized as Multidrug-resistant (MDR), and the remaining 9 (36%) were recognized as non-MDR.

Conclusion: The overall result showed that both MDR and non-MDR bacteria can form biofilms. However, Antimicrobial resistance patterns were observed higher in MDR biofilm producers than in non-MDR biofilm producers.

Keywords: Biofilm; secondary hospital; wastewater; Congo Red Agar; Disk Diffusion method.

Introduction

A biofilm is a complex structure in which microorganisms adhere to each other and/or to a surface, forming a thick layer. These microorganisms produce a slimy matrix called extracellular polymeric substances (EPSs). The EPS matrix consists of extracellular biopolymers, including proteins, polysaccharides, lipids, and eDNA (Sharma *et al*., 2019). Biofilms can form on living or non-living surfaces and are common in natural, industrial, and hospital settings. They play a role in infection persistence, especially in healthcare settings with indwelling devices. According to NIH (Sharma *et al*., 2023), Biofilms are responsible for 70% of all microorganism-related infections and has a significant role to healthcare-associated infections (HAIs) in humans. Bacteria have developed the ability to form biofilms, which are surface-adherent communities that allow survival in challenging environments. In clinical settings, bacteria encounter stressors like antibiotics, nutrient limitations, and heat shock which in turn triggers adaptive responses in bacterial cells, leading to the formation of highly resistant biofilm structures. Unfortunately, these biofilms are challenging to eradicate using conventional antimicrobial

agents designed for free-swimming bacteria. However, recent research has identified strategies specifically targeting biofilm growth, providing hope for future anti-biofilm therapies. Biofilms play a significant role in infections, making understanding their adaptive mechanisms crucial for developing effective treatments (De la Fuente-Núñez *et al*., 2013)

Biofilm formation is influenced by various factors, including the surrounding environment, nutrient availability, geographical origin, specimen type, surface attachment features, and genetic makeup. Clinically, common bacterial pathogens associated with biofilms include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Acinetobacter baumannii*, and *Escherichia coli*. Eradicating biofilm producers can be challenging due to their resilience. High antimicrobial concentrations may be necessary, but their toxicity and side effects limit their practical use in vivo. Early screening for biofilm production and effective antimicrobial susceptibility testing aid in selecting appropriate antibiotics (Neopane *et al*., 2018).

Bacterial biofilms are often resistant to broad-spectrum antibiotics in their typical concentrations or even in higher doses. Increasing multi-drug resistance in biofilms, which are responsible for emerging life-threatening nosocomial infections, is a serious health concern. However, they can be affected by environmental factors, their ability to cause infections depends on their specific characteristics like virulence. Understanding biofilm formation, structure and physiology can help us find better ways to fight them, such as using different antibiotics or their control by novel therapy approaches, such as anti-biofilm molecules, effective gene editing, drug-delivery systems, and probiotics (Mirghani *et al*., 2022)

Biofilm formation in healthcare poses significant challenges, contributing to increased morbidity, mortality, and financial strain on healthcare systems. These resilient microbial communities resist standard antimicrobial treatments, leading to persistent infections. Consequently, novel strategies beyond conventional antibiotics are urgently needed to combat biofilm-related issues. There are two propositions that have been applied so far to supervise biofilm formation in healthcare systems: one is the enhancement of biofilm inhibitors based on the understanding of the molecular mechanism of biofilm formation, structure, and physiology and the other is the modification of the biomaterials which are used in medical devices to prevent biofilm formation. Recent advances include targeting quorum-sensing communication systems and multidrug efflux pumps, both of which play crucial roles in biofilm formation. Continued research in these areas holds promise for improved anti-biofilm therapies beyond conventional treatments (Subhadra *et al*., 2018). Thus, screening of in-vitro biofilm production followed by an Antimicrobial Susceptibility Test is a necessary protocol to be performed in the future to enhance more insights into biofilm production, biofilm producers, and their antimicrobial resistance patterns. So, MDR biofilm producers can be analyzed earlier.

Materials and Methods

Sample Collection

Ten wastewater samples were aseptically collected from the Secondary hospital of Lalitpur district in sterile bottles with caps, each of 100 ml capacity. The bottles were labeled with a code corresponding to the location site and date of collection (within 3 consecutive days), and were immediately stored in an icebox for transportation and transferred to the microbiology laboratory of D.A.V College, Department of Microbiology, and microbiological analysis was performed within one hour of arrival adopting the methods of Rice & Bridgewater (2012).

Isolation Of Bacterial Pathogens

Bacteria present in the samples were isolated from wastewater samples by spread plate technique on MacConkey Agar medium by incubating aerobically at 37°C for 24 hours. Then, the isolated colony of bacteria was further streaked on sterile Nutrient Agar plates to obtain a pure culture. Media was prepared according to the manufacturers' instructions and sterility of the prepared media was achieved by autoclaving at 121°C for 15 minutes Tille (2015).

Identification Of Isolated Bacteria

Identification of pure cultured isolated bacteria was done by characterization of colony morphology and with gram-staining. Conventional biochemical tests, like catalase and oxidase tests, were used for further characterization of the bacteria, as per methods described by Tille (2015). Additionally, enzymatic tests and various biochemical tests (MR-VP, Indole, Citrate, TSIA, oxidative/fermentative) were performed for final analysis.

Screening The In-Vitro Biofilm Production

After the proper identification of isolates, screening of the in-vitro biofilm production was carried out qualitatively by using Congo Red Agar (CRA) medium as per the guidelines of the Journal of Clinical Pathology (Freeman *et al*., 1989). The test organism was then inoculated onto CRA plates and then incubated aerobically at 37°C for 24 hours and looked for the development of red colonies interpreted by strains as non-biofilm producers whereas dry crystalline black centered distinguished as biofilm producers (Fig.1).

Antimicrobial Susceptibility Test

Antimicrobial susceptibility test of different isolates was performed using by Kirby-Bauer Disk Diffusion Method as per CLSI guidelines on MHA plates against different class antibiotics like Amoxycillin (AMX) (10µg), Nalidixic acid (NA) (30µg), Ceftazidime (CAZ) (30µg), Erythromycin(E)

(15µg), and Gentamicin (GEN) (10µg) and the zone of inhibition diameter was measured in millimeter and strains were reported as Resistant, Intermediate and Susceptible. Isolates resistant to three or more antibiotics were termed Multidrug resistance (MDR) as per CLSI (2013).

Results and Discussion

Among 10 wastewater samples collected from the secondary hospital of Lalitpur district for isolation and identification. All isolated bacteria i.e. 25, were Gram-negative bacteria, where *Escherichia coli* (16, 64%) was the most predominant followed by *Citrobacter freundii* (3, 12%), *Enterobacter spp* (2, 8%), *Morganella morganii* (2, 8%), *Klebsiella pneumoniae* (1, 4%) and *Proteus vulgaris* (1, 4%). In the study conducted in Northwest Ethiopia (2014), 91.8 % of isolated bacteria were Gram-negative bacteria and 8.2 % were Gram-positive bacteria (Moges *et al*., 2014). In a similar trend study conducted in Hawassa, Sidama Regional State (2021), 89.2% of Gram-negative bacteria and 10.8% of Gram-positive bacteria were isolated (Mekengo *et al*., 2021). A similar kind of result was also obtained in the present study, which showed a predominance of gram-negative bacteria in wastewater samples. This is the acceptable environment for Gram-negative bacteria that are adapted to low osmolarity environments (high water content). So, this might be the reason behind the isolation of a significantly large number of Gram-negative bacteria in Hospital wastewater (Stobnicka-Kupiec *et al*., 2024)

Screening of biofilm producers qualitatively by Congo Red Agar (CRA) method is shown in Table 1. In-vitro biofilm production was screened qualitatively on CRA medium, where 12 (48%) isolates were able to produce biofilm and 13 (52%) isolates were non-biofilm producers (Fig. 1 & 2). *Escherichia coli* (8,66.6%), were the most common biofilm-producing Gram-negative bacilli followed by *Citrobacter freundii* (2, 16.66%), *Enterobacter spp* (1, 8.37%), *Morganella morganii* (1, 8.37%). However, isolated *Klebsiella pneumoniae* and *Proteus vulgaris* were unable to produce biofilms. Biofilms were screened based on development of red colonies interpreted by strains as non-biofilm producers whereas dry crystalline black centered distinguished as biofilm producers on CRA medium. The data of the present study was not similar to the previous study, which showed all isolated bacteria were biofilm producers. Since all bacteria do not have equal potential for biofilm formation. There are many factors responsible for biofilm formation. Biofilms are a form of existence for bacteria in the habitat, due to which bacteria can expand the boundaries of their surroundings during contamination of various surfaces under varying circumstances. The most crucial factors affecting biofilms include temperature, osmolarity, amount of ferrous iron ions, presence of nutrients, quality of material, light, and ambient acidity (Ponomareva *et al*., 2018).

To form a biofilm, bacteria should respond actively to these factors. When the interaction between microbes and the surface is absent, the bacteria is unable to form biofilm. So, the relationship between microbes and surfaces is a crucial phenomenon. Therefore, the ratio of biofilm producers is significantly lower than non-biofilm producers. This suggests that all the bacteria isolated from hospital wastewater are not able to produce biofilm.

All the isolated bacteria showed resistance against Amoxycillin (AMX) (10µg), Nalidixic acid (NA) (30µg), and Erythromycin(E) (15µg), whereas *Proteus vulgaris* only showed resistance against Gentamicin (GEN) (10µg). Among 16 multi-drug resistant (MDR) isolates, *Escherichia coli* (75%) was the most predominant followed by *Citrobacter freundii* (12.5%), *Morganella morganii* (6.25%), and *Proteus vulgaris* (6.25%). However, *Enterobacter spp* and *Klebsiella pneumoniae* did not show MDR. Out of 25 bacteria identified, 64% were MDR, which is higher than MDR reported from Thapathali Hospital, Kathmandu (51.58%) (Sigdel *et al*., 2023), Ethiopia (57.1%) (Wabe *et al*., 2020), and Ghana (55.4%) (Baah *et al*., 2022). Antibiotic Susceptibility test (AST) by Disk diffusion method on Mueller Hinton Agar (MHA) plates is shown in Table 2. In this study, MDR-*Escherichia coli* was isolated in large numbers (75%) which shows similar data in comparison with previous research conducted in Biratnagar with 40% (Mahato *et al*., 2019) and 61% in Bangladesh (Rabbani *et al*., 2017). From the past and present research, the prevalence of large numbers of MDR-*Escherichia coli* in hospital wastewater can be due to several factors like frequent exposure to antibiotics, patient colonization, and its adaptability allowing it to easily acquire resistance genes and spread in different settings, including hospital wastewater.

This study also compares biofilm producers and MDR (Table 1), to determine whether all the biofilm producers exhibit MDR or not. According to this study, a total of 11 isolates (44%, n=25) were biofilm producers showing MDR patterns and 5 isolates (20%, n=25) were non-biofilm producers showing MDR. In the total of 16 isolates (64%, n=25) of *Escherichia coli*, 4 isolates were non-biofilm producers with MDR. Likewise, only 4% isolate of *Proteus vulgaris* was the non-biofilm producer with MDR. This study was similar to the research conducted in University Hospital of Campania, Italy where 12% of non-biofilm producers were MDR strains (Folliero *et al*., 2021). Therefore, it showed that non-biofilm can also be MDR strains.

Thus, screening of in-vitro biofilm production followed by an Antimicrobial Susceptibility Test is a necessary protocol to be performed in the future to enhance more insights into biofilm production, biofilm producers, and their antimicrobial resistance patterns (Table 2). So, MDR biofilm producers can be analyzed earlier. The reported

levels of antibiotic susceptibility reflect worrying scenarios in health practice in Nepal. At present, there are no properly implemented guidelines for clinicians to treat biofilm-related MDR associated with critical cases. We suggest factual research-based antibiotic treatment based on localized epidemiological data. Our study provides information about the current condition of Hospital waste-water settings in Kathmandu, novel strategies, biofilm-producing capacity, and MDR of different strains of clinical isolates.

Fig. 1: Congo Red Agar (CRA) plate showing biofilm-producing bacterial isolates with dry crystalline black-centered colonies.

Fig. 2: Congo Red Agar (CRA) plate showing non-biofilm producer with red colonies

Fig. 3*:* Antibiotic Susceptibility Test of biofilm-producing *Escherichia coli*
[Gentamicin (GEN)-S, Amoxycillin (AMX)-R, Erythromycin(E)-R, Ceftazidime (CAZ)-R and Nalidixic acid (NA)-R]

Fig. 4*:* Antibiotic Susceptibility Test of biofilm-producing *Morganella morganii*
[Gentamicin (GEN)-S, Amoxycillin AMX)-R, Erythromycin(E)-R, Ceftazidime (CAZ)-S and Nalidixic acid (NA)-R]

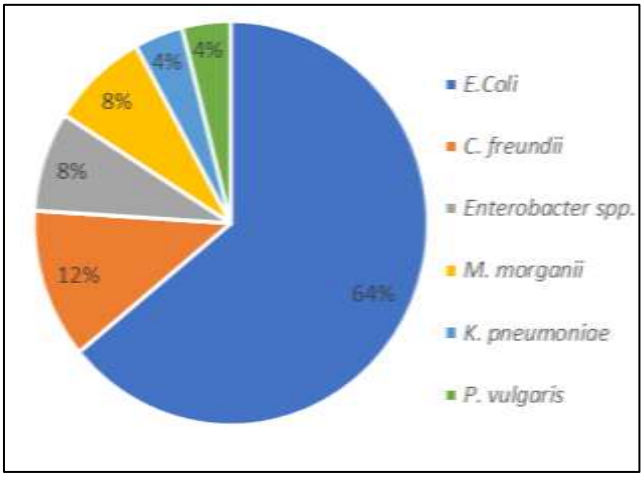

Fig. 5: Distribution of isolated bacteria in a sample

Table 1: Screening of biofilm producers qualitatively by Congo Red Agar (CRA) method.

S.N.	Name of isolates	Biofilm producers (n)	Non-biofilm producers (n)
1.	*Escherichia coli*	8	8
2.	*Citrobacter freundii*	2	1
3.	*Enterobacter spp.*	1	1
4.	*Morganella morganii*	1	1
5.	*Klebsiella pneumoniae*	-	1
6.	*Proteus vulgaris*	-	1
Total		12	13

Table 2: Antibiotic Susceptibility test (AST) by Disk diffusion method on Mueller Hinton Agar (MHA) plates

S.N.	Name of isolates	Multi-drug resistant (MDR) (n)	Multi-drug resistant (non-MDR) (n)
1.	*Escherichia coli*	12	4
2.	*Citrobacter freundii*	2	1
3.	*Enterobacter spp.*	-	2
4.	*Morganella morganii*	1	1
5.	*Klebsiella pneumoniae*	-	1
6.	*Proteus vulgaris*	1	-
Total		16	9

Table 3: Comparison of Biofilm producers and MDR

S.N.	Name of isolates	Total isolates	Biofilm producers	Multi-drug resistant (MDR)
1	*Escherichia coli*	16	8	12
2	*Citrobacter freundii*	3	2	2
3	*Morganella morganii*	2	1	1
Total		21	11	15

Conclusion

Biofilm can be present everywhere in nature and cause problems in medical and non-medical settings. All the biofilm producers were identified as MDR except some of the biofilm producers like *Enterobacter spp* which were identified as non-MDR. Thus, indicating that whether bacteria are MDR or non-MDR, they can produce biofilm. Antimicrobial resistance patterns were observed high in MDR biofilm producers than in non-MDR biofilm producers. Among all the antibiotics, Gentamicin (GEN) (10µg) was effective towards most of the bacteria. So, Gentamicin could be used to treat all isolated bacteria like *Escherichia coli Citrobacter freundii*, *Enterobacter spp*, *Morganella morganii*, *Klebsiella pneumoniae* except *Proteus vulgaris*.

Authors' Contribution

Bibek Aryal and Shristi Tharu both performed all the experimental research in the laboratory. The manuscript was prepared by Bibek Aryal. Shristi Tharu and Richa Chaudhary contributed to data analysis and the finalization of the manuscript. Shashi Bhushan Chaturwedi conceptualized the work plan, critically analyzed the findings, and finalized the manuscript. The final form of the manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with the present research work and publication.

Acknowledgment

Authors acknowledge Department of Microbiology, D.A.V. College, Dhobighat, Lalitpur, Nepal for providing Laboratory & all research facilities. Acknowledge also goes to Alka Hospital, Jawalakhel, Lalitpur, Nepal for cooperation in sample collections.

References

Addae-Nuku DS, Kotey FC, Dayie NT, Osei MM, Tette EM, Debrah P and Donkor ES (2022) Multidrug-Resistant Bacteria in Hospital Wastewater of the Korle Bu Teaching Hospital in Accra, Ghana. *Environmental Health Insights*, **16.** DOI: 10.1177/11786302221130613

CLSI. (2013) *Performance standards for antimicrobial susceptibility testing* (23 ed.). USA: Informational supplements.

De la Fuente-Núñez C, Reffuveille F, Fernández L and Hancock RE (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Current opinion in microbiology* **16**(5): 580-589. DOI: 10.1016/j.mib.2013.06.013

Folliero V, Franci G, Dell'Annunziata F, Giugliano R, Foglia F, Sperlongano R and Galdiero M. (2021) Evaluation of Antibiotic Resistance and Biofilm Production among Clinical Strain Isolated from Medical Devices.

International journal of microbiology: 1-11. DOI: 10.1155/2021/9033278

Freeman D, Falkiner F and Keane C (1989) New method for detecting slime production by coagulase negative staphylococci. *Journal of clinical pathology* **42**(8): 872-874. DOI: 10.1136/jcp.42.8.872

Mahato S, Mahato A and Adhikari P (2019) Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in Effluents of Different Hospitals Sewage in Biratnagar, Nepal. *International Journal of Environment* ***8***(3): 53-67. DOI: 10.3126/ije.v8i3.26617

Mekengo BM, Hussein S and Ali MM (2021) Distribution and antimicrobial resistance profile of bacteria recovered from sewage system of health institutions found in Hawassa, Sidama Regional State, Ethiopia: A descriptive study. *SAGE open medicine*. DOI: 10.1177/20503121211039097

Mirghani R, Saba T, Khaliq H, Mitchell J, Chambi L and Rijal G (2022) Biofilms: Formation, drug resistance and alternatives to conventional approaches. *AIMS microbiology* 239-277. DOI: 10.3934/microbiol.2022019

Moges F, Endris M, Belyhun Y and Worku W. (2014) Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments, Northwest Ethiopia. *BMC Research Notes*, **7(**1): 215. DOI: 10.1186/1756-0500-7-215

Neopane P, Nepal H, Uehara R and Abiko Y (2018) In vitro biofilm formation by *Staphylococcus aureus* isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. *International journal of general medicine* 25-32. DOI: :10.2147/IJGM.S153268

Ponomareva AL, Buzoleva LS & Bogatyrenko EA (2018) Abiotic Environmental Factors Affecting the Formation of Microbial Biofilms. *Biology Bulletin* **45**(5): 490-496. DOI: 10.1134/S106235901805014X

Rabbani M, Howlader Z and Kabir Y. (2017). Detection of multidrug resistant (MDR) bacteria in untreated waste water disposals of hospitals in Dhaka City, Bangladesh. *Journal of Global Antimicrobial Resistance*, ***7***: 120-125. DOI: 10.1016/j.jgar.2017.04.009

Rice EW, and Bridgewater L (2012). *Standard methods for the examination of water and wastewater* (Vol. 10). Washington, DC: American Public Health Association.

Sharma D, Misba L and Khan A (2019). Antibiotics versus biofilm: an emerging battleground in microbial communities. *BMC*, 2-10. DOI: 10.1186/s13756-019-0533-3

Sharma S, Mohler J, Mahajan SD, Schwartz SA, Bruggemann L and Aalinkeel RK (2023). Microbial Biofilm: A Review on Formation, Infection, Antibiotic Resistance, Control Measures, and Innovative Treatment.*MDPI* **11**(6): DOI: 10.3390/microorganisms11061614

Sigdel S, Khanal A and Thakur SK (2023). Bacteriological Profile and Detection of β-Lactamase Producing Bacteria Isolated from Blood Samples of Neonates. *International Journal of applied sciences and biotechnology*, **11**(2): 66-77 DOI: 10.3126/ijasbt.v11i2.56116

Stobnicka-Kupiec A, Gołofit-Szymczak M, Cyprowski M, and Górny RL (2024). Monitoring of enteropathogenic Gram-negative bacteria in wastewater treatment plants: a multimethod approach. *Environmental Science and Pollution Research*, **31**(25): 37229-37224 DOI: 10.1007/s11356-024-33675-2

Subhadra B, Kim DH, Woo K, Surendran S and Choi CH (2018). Control of Biofilm Formation in Healthcare: Recent Advances Exploiting Quorum-Sensing Interference Strategies and Multidrug Efflux Pump Inhibitors. *Materials* 16-21. DOI: 10.3390/ma11091676

Tille P (2015). *Bailey & Scott's diagnostic microbiology-E-Book.* Elsevier Health Sciences.

Wabe YA, Reda DY, Abreham ET, Gobene BM and Ali MM (2020). Prevalence of asymptomatic bacteriuria, associated factors and antimicrobial susceptibility profile of bacteria among pregnant women attending Saint Paul's Hospital Millennium. *Therapeutics and clinical risk management* 923-932 DOI: 10.2147/TCRM.S267101