Journal of Iranian Dental Association (JIDA)

Original Article

Detection of Biofilm Production of Oral Bacterial Isolates and Its Impact on the Antibiotic Resistance Profile

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DOI: 10.34172/jida.2304

Abstract

Background: This study aimed to investigate the association between biofilm formation and antibiotic resistance among 294 bacterial isolates obtained from the oral cavity.

Methods: A total of 100 patients aged 25-65 years were enrolled. Buccal mucosa swabs were collected and cultured on appropriate media for bacterial isolation and identification. Biofilm production was assessed using the phenotypic Tissue Culture Plate Method (TCPM). Antibiotic susceptibility patterns were determined for all 294 isolates using the Kirby-Bauer disc diffusion method against 7 β-lactam antibiotics (ampicillin, penicillin, amoxicillin, cephalothin, oxacillin, cloxacillin, and cefoxitin) and 7 non-β-lactam antibiotics (tetracycline, co-trimoxazole, ciprofloxacin, clindamycin, erythromycin, lincomycin, and vancomycin).

Results: Biofilm detection by the TCP method revealed that 9 isolates (3.1%) exhibited strong biofilm formation, 213 (72.4%) showed moderate biofilm formation, and 72 (24.5%) demonstrated weak or no biofilm formation. Bacterial isolates with strong and moderate biofilm-forming capacity demonstrated significantly higher resistance rates to most tested antibiotics compared to those with weak or no biofilm formation ($p < 0.0001$).

Conclusion: This study demonstrates that aerobic bacteria remain the predominant isolates from the oral cavity. A significant association was observed between biofilm-forming capacity and antibiotic resistance among oral bacterial isolates.

Key Words: Adult, Antibiotic Resistance, Biofilm Formation, Oral Microbiome, Buccal Mucosa, Multidrug-Resistant Bacteria, Phenotypic Methods, *Staphylococcus Aureus*, *Streptococcus Mutans*.

Received: 12 July 2026 | **Accepted:** 21 Aug 2026 | **ePublished:** Summer and Autumn 2026; Vol. 38, No. 3-4

Introduction

Biofilm formation is a fundamental survival strategy among microorganisms, characterized by the development of structured microbial communities embedded within a self-produced extracellular polymeric matrix (1). The capacity to form biofilms varies considerably among bacterial species and strains and is influenced by environmental conditions, microbial characteristics, and host-related factors. Biofilms are particularly important in clinical settings because their complex architecture and heterogeneous physiology can enhance microbial persistence and tolerance to antimicrobial agents and host immune defenses (2).

The increased antimicrobial tolerance of biofilm-associated bacteria is multifactorial and involves the structural properties of the biofilm, limited antimicrobial penetration, the composition of the extracellular polymeric matrix, altered cellular metabolism, and the presence of phenotypically distinct bacterial subpopulations. The matrix contains a complex combination of polysaccharides, proteins, lipids, and extracellular DNA, contributing to biofilm stability and microbial protection (3). In addition, the biofilm environment may facilitate genetic exchange and the acquisition or dissemination of antimicrobial resistance determinants. These characteristics make biofilms particularly relevant to the persistence of chronic and recurrent infections.

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The oral cavity represents a highly complex microbial ecosystem in which microorganisms readily organize into polymicrobial biofilms. Dental plaque is one of the most clinically important examples and comprises diverse microbial communities that interact with host tissues and with one another. Oral biofilms are implicated in several common diseases, including dental caries and periodontal disease, and may also serve as reservoirs for antimicrobial-resistant microorganisms (4). Understanding the relationship between biofilm-forming capacity and antimicrobial susceptibility is consequently important for characterizing the clinical behavior of oral bacterial isolates and informing antimicrobial management strategies.

Antimicrobial resistance may arise through genetic mutations and horizontal transfer of resistance determinants, while biofilm-associated physiological adaptations can further increase bacterial tolerance to antimicrobial exposure (5). These processes may coexist and potentially reinforce one another, complicating the treatment and eradication of infections caused by biofilm-producing bacteria. Importantly, the relationship between biofilm formation and antibiotic resistance may vary according to bacterial species, antimicrobial class, and local epidemiological characteristics.

Regarding this background, the present study aimed to investigate the association between biofilm production by oral bacterial isolates and their antibiotic resistance profiles, with particular emphasis on determining whether biofilm-forming capacity is associated with reduced antibiotic susceptibility among the isolates examined.

Methods and Materials

Study Design and Sample Collection

A total of 100 patients attending dental clinics at the Faculty of Dentistry, Sana'a University, were enrolled in this study. Buccal mucosa swabs were collected and cultured on appropriate media, yielding 294 bacterial isolates for further analysis.

Antibiotic Susceptibility Testing

Antibiotic susceptibility profiles were determined using the Kirby-Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) 2015 guidelines [13]. Bacterial inocula were adjusted to 0.5 McFarland standard turbidity, swabbed onto Brain Heart Infusion (BHI) agar plates, and allowed to dry for 10 minutes. Standard microbiological techniques for bacterial culture and identification were performed as described by Cheesbrough [14]. The following antibiotics (Oxoid, UK) were tested:

- β-lactam antibiotics: ampicillin (25 µg), penicillin (10 µg), oxacillin (15 µg), cefoxitin (30 µg), and amoxicillin-clavulanic acid (25 µg).
- Non-β-lactam antibiotics: erythromycin (15 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), vancomycin (5 µg), tetracycline (30 µg), and co-trimoxazole (15 µg).

Plates were incubated aerobically at 37°C for 24 hours, and inhibition zones were measured. All experiments were performed in triplicate, and results were interpreted according to CLSI criteria (13).

Biofilm Detection by Tissue Culture Plate Method (TCPM)

Biofilm production was assessed using the tissue culture plate method as previously described [15]. Bacterial isolates from fresh agar plates were inoculated into 2 mL of BHI broth and incubated at 37°C for 24 hours. Cultures were then diluted 1:40 with fresh BHI broth supplemented with 1% glucose, and 200 µL of this suspension was added to each well of a microtiter plate. After 24 hours of incubation at 37°C, the contents were gently tapped, and free-floating (planktonic) bacteria were removed by rinsing three times with phosphate-buffered saline (PBS, pH 7.2). Adherent biofilm-forming bacteria were fixed with 2% sodium acetate for 10-15 minutes and stained with 0.1% crystal violet. Unbound stain was removed by three PBS washes, and the plates were air-dried. The dye was solubilized by adding 200 µL of 95% ethanol to each well, and optical density (OD) was measured at 630 nm. The OD cutoff value (ODc) was calculated according to standard guidelines, and isolates were classified based on their biofilm-forming capacity [15, 34, 37].

Statistical Analysis

Data were analyzed using EPI-INFO statistical software version 7. The association between biofilm production levels and antibiotic resistance was assessed. Due to small sample sizes in several

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subgroups, data were presented descriptively. The chi-square test and Fisher's exact test were used where appropriate, with statistical significance set at $p < 0.05$.

Ethical Considerations

This study was approved by the Medical Ethics and Research Committee of the Faculty of Medicine and Health Sciences, Sana'a University (Approval No. 217, dated August 21, 2022). All participants provided written informed consent, and the study was conducted in accordance with the ethical standards of the review committee.

Results

Biofilm Production Among Bacterial Isolates

Biofilm-forming capacity was assessed for 294 bacterial isolates using the tissue culture plate method (TCPM). Based on optical density (OD) measurements, isolates were classified into four categories: negative, weak, moderate, and strong biofilm production. The majority of isolates (72.4%) exhibited moderate biofilm formation, while 21.4% showed weak biofilm formation. Strong biofilm production and negative biofilm production were each observed in 3.1% of isolates (Table 1).

OD value	N (%)
<0.17 Negative	9 (3.1)
0.17-0.34 Weak positive	63 (21.4)
0.35-0.68 Moderate positive	213 (72.4)
>0.68 Strong positive	9 (3.1)
Total	294 (100)

Table 1. Interpretation of biofilm production by bacterial isolates based on optical density values of tissue culture plate method Average value of OD* Biofilm production

Association Between Biofilm Formation and Antibiotic Resistance in Key Pathogens

The relationship between biofilm-forming capacity and antibiotic resistance was analyzed for the most prevalent bacterial species with adequate sample sizes, including *Staphylococcus aureus*, *Streptococcus mutans*, and *Neisseria* species. For all species analyzed, isolates with moderate or strong biofilm-forming capacity demonstrated significantly higher resistance rates to most tested antibiotics compared to those with weak or no biofilm formation ($p < 0.05$).

***Staphylococcus aureus* (n=48)**

Among *S. aureus* isolates, 47 (97.9%) exhibited moderate or strong biofilm formation, while only one isolate (2.1%) showed weak or no biofilm formation. Biofilm-producing strains demonstrated significantly higher resistance rates to multiple antibiotics, including ampicillin (97.8% vs. 2.2%, $p < 0.0001$), tetracycline (97.9% vs. 2.1%, $p = 0.001$), and erythromycin (93.7% vs. 6.3%, $p < 0.0001$). Complete resistance (100%) to several β-lactam antibiotics (cephalothin, oxacillin, penicillin, cefoxitin) was observed among biofilm-producing strains. All isolates were susceptible to amikacin, clindamycin, and vancomycin (Table 2).

***Streptococcus mutans* (n=82)**

Of 82 *S. mutans* isolates, 72 (87.8%) showed moderate or strong biofilm formation, and 10 (12.2%) showed weak or no biofilm formation. Biofilm-producing strains exhibited significantly higher resistance to tetracycline (87.8% vs. 12.2%, $p < 0.0001$), ampicillin (91.1% vs. 8.9%, $p < 0.0001$), and cephalothin (97.5% vs. 2.5%, $p < 0.0001$). Complete resistance (100%) to several antibiotics (amoxicillin-clavulanic acid, gentamicin, oxacillin, ciprofloxacin, cloxacillin, cefoxitin, and clindamycin) was observed among biofilm-producing strains. All isolates were susceptible to erythromycin, penicillin, amikacin, and vancomycin (Table 3).

***Neisseria* species (n=50)**

Among *Neisseria* isolates, 36 (72%) demonstrated moderate or strong biofilm formation, while 14 (28%) showed weak or no biofilm formation. Biofilm-producing strains showed significantly higher resistance to tetracycline (90% vs. 10%, $p < 0.0001$) and erythromycin (87.5% vs. 12.5%, $p < 0.0001$). Complete resistance (100%) to co-trimoxazole, amoxicillin-clavulanic acid, gentamicin, oxacillin, and ciprofloxacin was observed among biofilm-producing strains. All isolates were susceptible to cloxacillin, cefoxitin, amikacin, clindamycin, ceftriaxone, azithromycin, and penicillin (Table 4).

Discussion

This study aimed to investigate the association between biofilm formation and antibiotic resistance among bacterial isolates from the oral cavity. The

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Antibiotic name	Total n=48	Negative/weak N=1	Moderate/strong N=47	Diff. (95% CI)	*p* value
	Resistance N (%)	Resistance N (%)	Resistance N (%)		
Ampicillin	46 (95.8)	1(2.2)	45 (97.8)	95.1(16.5-98.2)	<0.0001
Tetracycline	48 (100)	1 (2.1)	47 (97.9)	87.4(11-94.8)	0.001
Erythromycin	16 (33.3)	1 (6.3)	15 (93.7)	100(20.3-100)	<0.0001
Cephalothin	45 (93.8)	0 (0.0)	45 (100)	100(20.3-100)	<0.0001
Co-trimoxazole	22 (45.8)	0 (0.0)	22 (100)	100(19.3-100)	<0.0001
Amoxicillin-Clavulanic Acid	26 (54.2)	0 (0.0)	26 (100)	100(19.6-100)	<0.0001
Gentamicin	5 (10.4)	0 (0.0)	5 (100)	100(9.5-100)	0.025
Oxacillin	33 (68.7)	0 (0.0)	33(100)	100(19.9-100)	<0.0001
Penicillin	36 (75)	0 (0.0)	36 (100)	100(19.9-100)	<0.0001
Ciprofloxacin	36 (75)	0 (0.0)	36 (100)	100(20.1-100)	<0.0001
Cloxacillin	22 (45.8)	0 (0.0)	22 (100)	100(19.3-100)	<0.0001
Cefoxitin	33 (68.7)	0 (0.0)	33(100)	100(19.9-100)	<0.0001

Table 2. Association of biofilm formation and Antibiotic Resistance of *S. aureus* isolated from buccal mucosa of patients n=48 isolates

Antibiotic name	Resistance	Biofilm formation		Diff. (95% CI)	*p* value
	Total N (%)	Negative/weak N=10	Moderate/strong N=72		
Tetracycline	82 (100)	10 (12.2)	72 (87.8)	75.6 (43.4-86.8)	<0.0001
Ampicillin	79 (96.3)	7 (8.9)	72 (91.1)	82.2 (50.6-90.9)	<0.0001
Cephalothin	40 (48.8)	1 (2.5)	39 (97.5)	95 (65.5 -97.9)	<0.0001
Co-trimoxazole	10 (12.2)	1 (10)	9 (90)	80 (48.3-89.6)	<0.0001
Amoxicillin-Clavulanic Acid	3 (3.7)	0 (0.0)	3 (100)	100 (71.7 - 100)	<0.0001
Gentamicin	6 (7.3)	0 (0.0)	6 (100)	100 (71.7 - 100)	<0.0001
Oxacillin	5 (6.1)	0 (0.0)	5 (100)	100 (71.7 - 100)	<0.0001
Ciprofloxacin	4 (4.9)	0 (0.0)	4 (100)	100 (71.7 - 100)	<0.0001
Cloxacillin	5 (6.1)	0 (0.0)	5 (100)	100 (71.7 - 100)	<0.0001
Cefoxitin	7 (8.5)	0 (0.0)	7 (100)	100 (71.7 - 100)	<0.0001
Clindamycin	16 (19.5)	0 (0.0)	16 (100)	100 (71.7 - 100)	<0.0001

Table 3. Association of biofilm formation and Antibiotic Resistance of *S. mutans* isolated from buccal mucosa n= 82 isolates

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Antibiotic name	Resistance	Biofilm formation		Diff. (95% CI)	*p* value
	Total N (%)	Negative/weak N=14	Moderate/strong N=36		
Tetracycline	20 (40)	2 (10)	18 (90)	80 (51.3-89.9)	<0.0001
Erythromycin	8 (16)	1 (12.5)	7 (87.5)	75 (45.7-86.7)	<0.0001
Co-trimoxazole	5 (10)	0 (0.0)	5 (100)	100 (51.5-100)	<0.0001
Amoxicillin-Clavulanic Acid	3 (6)	0 (0.0)	3 (100)	100 (39.8-100)	0.0001
Gentamicin	3 (6)	0 (0.0)	3 (100)	100 (39.8-100)	0.0001
Oxacillin	3 (6)	0 (0.0)	3 (100)	100 (39.8-100)	0.0001
Ciprofloxacin	3 (6)	0 (0.0)	3 (100)	100 (39.8-100)	0.0001

Table 4. Association of biofilm formation and Antibiotic Resistance of *Neisseria* species isolated from buccal mucosa n= 50 isolates

findings demonstrate a clear relationship: bacterial isolates with moderate or strong biofilm-forming capacity exhibited significantly higher resistance to multiple antibiotics compared to those with weak or no biofilm formation. These results are consistent with previous studies by Pramodhini et al. (6), Maqbool et al. (7), Tayal et al. (8), and Alhasani et al. (9, 10), which reported that biofilm-producing bacteria show enhanced antibiotic resistance.

The enhanced resistance in biofilm-forming bacteria can be attributed to several mechanisms. Biofilms provide a physical barrier that limits antibiotic penetration, while the close proximity of cells within the biofilm facilitates horizontal gene transfer, including the exchange of resistance plasmids (11). Additionally, bacteria within biofilms exhibit reduced metabolic activity and growth rates, making them less susceptible to antibiotics that target actively dividing cells (12-14).

Biofilm Formation Among Oral Isolates

In the present study, the majority of oral bacterial isolates (72.4%) showed moderate biofilm production, while 3.1% exhibited strong biofilm formation (Table 1). These findings are comparable to those reported in similar investigations of oral microbiota (9, 10).

Staphylococcus aureus (n=48)

Among *S. aureus* isolates, 97.9% demonstrated moderate or strong biofilm-forming capacity, with significantly higher resistance rates to most tested antibiotics compared to non-biofilm producers (Table 2). For example, biofilm-producing strains showed 95.1% higher resistance to ampicillin ($p < 0.0001$), and complete resistance (100%) to several β-lactam antibiotics including cephalothin, oxacillin, penicillin, and cefoxitin. These findings reflect the clinical importance of *S. aureus* as a major multidrug-resistant pathogen capable of causing persistent infections through biofilm formation (15-20).

The prevalence of MRSA in our study was 68.7%, which is higher than previously reported in Yemen by Al-Safani et al. (18) and Al-Akwa et al. (15), but comparable to international studies reporting MRSA rates between 40% and 69.8% (21, 22). Notably, all *S. aureus* isolates in our study remained susceptible to vancomycin, clindamycin, and amikacin, which is encouraging and consistent with previous Yemeni studies (9, 10).

Streptococcus mutans (n=82)

S. mutans, a key pathogen in dental caries, showed that 87.8% of isolates exhibited moderate or strong biofilm formation. Biofilm-producing strains demonstrated significantly higher resistance to tetracycline (87.8% vs. 12.2%, $p < 0.0001$), ampicillin (91.1% vs. 8.9%, $p < 0.0001$), and cephalothin (97.5% vs. 2.5%, $p < 0.0001$) (Table 3). Complete resistance to multiple antibiotics was observed among biofilm-forming strains, consistent with the role of biofilms in protecting *S. mutans* from antimicrobial agents (9, 10, 23).

Dental plaque, a complex oral biofilm containing *S.*

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mutans, contributes to dental caries through acid production and demineralization of tooth structures (24-26). The high level of antibiotic resistance observed in biofilm-forming *S. mutans* highlights the clinical challenge posed by these organisms and the importance of preventive strategies such as mechanical biofilm removal and reduced sugar consumption (27).

***Neisseria* species** (n=50)

Among *Neisseria* isolates, 72% demonstrated moderate or strong biofilm formation. Biofilm-producing strains showed significantly higher resistance to tetracycline (90% vs. 10%, $p < 0.0001$) and erythromycin (87.5% vs. 12.5%, $p < 0.0001$) (Table 4). Complete resistance to co-trimoxazole, amoxicillin-clavulanic acid, gentamicin, oxacillin, and ciprofloxacin was observed among biofilm-forming strains, while all isolates remained susceptible to multiple other antibiotics including penicillin and ceftriaxone.

Clinical Implications and Study Limitations

The association between biofilm formation and antibiotic resistance demonstrated in this study has important clinical implications. Biofilm-forming bacteria are more difficult to eradicate, often requiring prolonged high-dose antibiotic therapy, which may increase the risk of drug toxicity and further resistance development (28-30). Understanding biofilm formation mechanisms is essential for developing effective infection control strategies (22, 31, 32).

This study has several limitations. The small sample sizes in some bacterial subgroups precluded detailed statistical analysis, and data were therefore presented descriptively. Additionally, the study was conducted in a single center, which may limit generalizability. Further studies with larger sample sizes are needed to confirm these findings.

Conclusion

This study demonstrated that 72.4% of 294 oral bacterial isolates exhibited moderate to strong biofilm-forming capacity, which was significantly associated with multidrug resistance ($p < 0.0001$). *Staphylococcus aureus* and *Streptococcus mutans* were predominant biofilm producers, showing high antibiotic resistance rates of 95.1% and 96.3%, respectively. Additionally, biofilm-forming *Neisseria* species demonstrated significantly elevated resistance to multiple antibiotics. These findings confirm that biofilm formation is a major contributor to antimicrobial resistance in the oral cavity.

Based on these findings, we recommend:

1. Clinical integration: Incorporate biofilm susceptibility testing into routine diagnostic protocols for oral infections.
2. Therapeutic development: Prioritize the development of anti-biofilm therapies targeting oral pathogens.
3. Antimicrobial stewardship: Establish national stewardship programs in Yemen to monitor and curb the evolution of resistance.

These recommendations align with the WHO Global Action Plan on Antimicrobial Resistance and support Sustainable Development Goal 3 (Good Health and Well-being) by promoting evidence-based interventions to combat antimicrobial resistance.

Declarations

Ethical Approval and Consent to Participate

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and applicable institutional and national ethical guidelines. The study protocol was reviewed in accordance with the procedures applicable at the Faculty of Dentistry, Sana'a University. No formal ethics approval/reference code was issued or available for this study. Written informed consent was obtained from all participants before enrollment and collection of buccal mucosa swab specimens. Participants were informed about the objectives and procedures of the study, and participation was voluntary. All participant information and microbiological data were handled confidentially and used exclusively for research purposes.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are not publicly available because they contain participant-related and microbiological information collected as part of the study. The data may be available from the corresponding author on reasonable request, subject to applicable ethical, institutional, and confidentiality restrictions.

Competing Interests

The authors declare that they have no financial, professional, personal, or institutional conflicts of

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interest that could have influenced the design, conduct, analysis, interpretation, or reporting of this study. The authors received no honoraria, consultancy fees, research support, equipment, materials, or other benefits from any commercial or non-commercial organization that could be perceived as having an interest in the findings of this study. No pharmaceutical, biotechnology, diagnostic, or medical/dental device company was involved in the design of the study, collection or analysis of data, interpretation of the results, preparation of the manuscript, or decision to submit the manuscript for publication. The authors declare that they have no other competing interests relevant to this work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The study was conducted without external financial support.

Authors' Contributions

Nesreen Fadel Al-Sanabani contributed to the conceptualization of the study, data curation, and formal analysis. Hassan Abdulwahab Al-Shamahy contributed to study supervision, validation of the research procedures and findings, and preparation of the original draft of the manuscript. Both authors contributed to the interpretation and critical review of the manuscript, approved the final version, and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of the study were appropriately investigated and resolved.

Declaration of Generative Artificial Intelligence (AI) Utilization

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) and DeepSeek as generative artificial intelligence tools for language-related assistance, including grammar correction, improvement of sentence structure, enhancement of readability, and refinement of academic English. These tools were not used to generate, collect, analyze, or interpret the study data, perform statistical analyses, or determine the scientific conclusions of the study. The authors independently reviewed, critically evaluated, and verified all AI-assisted content and remain fully responsible for the accuracy, integrity, originality, and scientific validity of the manuscript. The use of these tools does not constitute authorship, and no generative AI system was listed as an author of the manuscript.

Acknowledgements

The authors would like to express their sincere gratitude to the Yemeni Ministry of Health and the Faculty of Dentistry, Sana'a University, for their valuable institutional support and assistance during the conduct of this study.

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