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Exploring Biofilm-Driven Resistance Mechanisms in Periodontitis: A Study on Multidrug-Resistant Microorganisms

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Abstract
Introduction: The emergence of multidrug-resistant (MDR) strains poses significant challenges in treating periodontitis, especially in vulnerable populations such as diabetic patients. This study aims to investigate the biofilm-driven resistance mechanisms in periodontitis-associated microorganisms, focusing on MDR strains isolated from diabetic and non-diabetic patients.
Methodology: A total of 210 clinical samples were collected from patients diagnosed with periodontitis, with a focus on identifying the prevalence of multidrug-resistant strains. Samples were analyzed for biofilm production through standardized biofilm assays, categorizing isolates into strong, moderate, and weak biofilm producers. Molecular characterization of the isolates was performed using 16S rRNA sequencing and BLAST analysis to identify key pathogens, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, as well as associated resistance genes linked to biofilm formation.
Statistical Analysis: Statistical analyses were conducted to evaluate the correlation between diabetes status, biofilm formation, and multidrug resistance. Chi-square tests were utilized to determine the significance of differences in resistance rates between diabetic and non-diabetic patients. A p-value of less than 0.05 was considered statistically significant, indicating a meaningful relationship between the variables studied.
Results: The results indicated that among the 210 clinical samples, 70% of the isolates from diabetic patients exhibited multidrug resistance, while 46% of the isolates from non-diabetic patients showed similar resistance profiles. These findings underscore the increased risk of multidrug resistance in diabetic patients, highlighting the urgent need for innovative biofilm-targeted therapies in managing periodontitis.

1. Introduction

Periodontitis is a complex, chronic inflammatory disease that affects the supporting structures of the teeth, leading to tissue destruction, bone resorption, and, ultimately, tooth loss. It is caused primarily by a dysbiotic shift in the subgingival microbiota, resulting in the dominance of pathogenic bacteria that induce a host inflammatory response. These bacteria, often embedded within a biofilm, exhibit a heightened resistance to antimicrobial agents and host defense mechanisms, making periodontitis one of the most challenging oral diseases to treat effectively (1).

Biofilm formation plays a central role in the pathogenesis of periodontitis. Bacterial biofilms are structured communities of microorganisms encased in a self-produced extracellular polymeric matrix that adheres to tooth surfaces (2). Within this matrix, bacteria exhibit enhanced resistance to antibiotics and immune responses, contributing to the persistence and chronicity of periodontal infections. Biofilm-producing microorganisms such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Fusobacterium nucleatum* are often implicated in periodontitis and are known to thrive in the anaerobic and nutrient-rich environment of the subgingival space (3).

One of the critical factors complicating the treatment of periodontitis is the rise of multidrug-resistant (MDR) microorganisms. These pathogens possess the ability to withstand a wide range of antibiotics, leaving clinicians with limited therapeutic options (4). The problem of antimicrobial resistance is exacerbated in patients with systemic conditions such as diabetes mellitus, which has been shown to have a bidirectional relationship with periodontitis. Diabetic patients are more susceptible to periodontal infections, and these infections, in turn, exacerbate glycemic control. Furthermore, the impaired immune response in diabetic individuals promotes the persistence and colonization of drug-resistant strains (5). Diabetes significantly alters the host immune response and modifies the microbial environment, making diabetic patients more prone to severe periodontitis with rapid progression. Studies suggest that diabetic patients harbor higher levels of pathogenic biofilm-forming bacteria, contributing to the persistence of inflammation and resistance to treatment(6). Furthermore, biofilm-mediated resistance mechanisms, such as the expression of efflux pumps, alteration of cell membrane permeability, and quorum sensing, enable microorganisms to evade antibiotic action, creating a major clinical challenge in managing periodontitis in this population (7).

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The emergence of MDR bacteria in biofilms highlights the urgent need to investigate the molecular mechanisms underlying resistance and biofilm formation, particularly in high-risk populations such as those with diabetes. Recent advances in molecular techniques, such as 16S rRNA sequencing and whole-genome sequencing, have allowed for a more detailed understanding of the microbial composition and resistance profiles of these bacteria. This study aims to explore the biofilm-driven resistance mechanisms of MDR periodontitis-associated microorganisms isolated from diabetic and non-diabetic patients (8). By characterizing these microorganisms and assessing their resistance profiles, this research seeks to identify potential therapeutic targets and improve treatment outcomes for periodontitis, particularly in diabetic patients (9).

2. Methodology

2.1 Sample Size and Population

A total of **210 clinical samples** were collected from patients diagnosed with periodontitis at a dental clinic. The study group was divided into two categories:

- **Diabetic patients:** 110 samples
- **Non-diabetic patients:** 100 samples

2.2 Isolation and Identification of Microorganisms

Periodontal pocket samples were collected using sterile curettes and immediately transported to the laboratory for analysis. Samples were cultured on selective media for anaerobic and facultative bacteria. The isolates were identified based on their morphological and biochemical characteristics. Gram staining, catalase, and oxidase tests were performed to aid in the identification of specific bacterial species.

2.3 Biofilm Production Assay

Biofilm production was assessed using the **crystal violet staining** method. Briefly, bacterial cultures were grown in a 96-well plate, and biofilm formation was quantified by measuring the optical density (OD) at 595 nm. Based on OD values, isolates were categorized as weak, moderate, or strong biofilm producers.

2.4 Antibiotic Susceptibility Testing

The antibiotic susceptibility of the isolates was tested using the **Kirby-Bauer disk diffusion method**. The isolates were tested against commonly used antibiotics, including amoxicillin, metronidazole, tetracycline, and ciprofloxacin. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines, and multidrug resistance was defined as resistance to three or more antibiotic classes.

2.5 Molecular Characterization and 16S rRNA Sequencing

The most resistant isolates were subjected to molecular analysis. DNA was extracted using the phenol-chloroform method. The **16S rRNA gene** was amplified via polymerase chain reaction (PCR), and the amplicons were sequenced. **BLAST analysis** was performed to compare the sequences with the NCBI database to identify the species and the presence of resistance-related genes.

2.6 Data Analysis

Chi-square analysis was conducted to assess the association between diabetes, biofilm formation, and multidrug resistance. The results revealed a statistically significant difference in the prevalence of multidrug resistance between diabetic and non-diabetic patients. Diabetic patients were more likely to harbor MDR strains ($\chi^2 = 10.42$, $p < 0.05$), and biofilm production was also significantly higher among diabetic isolates ($\chi^2 = 12.03$, $p < 0.05$).
Additionally, a correlation test showed a positive association between strong biofilm production and resistance to antibiotics such as amoxicillin and metronidazole, with a correlation coefficient (r) of 0.68, indicating a moderate to strong relationship.

3. Results

3.1 Distribution of Isolates Among Patients

Among the 210 clinical samples, **62%** of the isolates were obtained from diabetic patients, and **38%** from non-diabetic patients. The distribution of biofilm-producing and multidrug-resistant isolates showed that diabetic patients were significantly more prone to harbor MDR strains.The biochemical characterization of periodontitis-associated microorganisms isolated from diabetic and non-diabetic patients revealed significant differences in Gram staining, oxidase, and catalase test results.
1. Gram Staining Results: All isolated microorganisms were subjected to Gram staining to assess their cell wall characteristics. The results indicated that:

- *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were identified as Gram-negative bacilli (rods).
- *Treponema denticola* was classified as Gram-negative spirochetes.

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• Other isolates displayed a mix of Gram-negative and Gram-positive bacteria.
2. Oxidase Test Results: The oxidase test was conducted to determine the presence of cytochrome c oxidase in the isolated strains:
• *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were found to be oxidase-negative.
• In contrast, *Treponema denticola* tested positive for oxidase.
• The remaining isolates primarily exhibited oxidase-negative results, with some exceptions noted.
3. Catalase Test Results: The catalase test was performed to evaluate the ability of the isolates to produce the enzyme catalase, which decomposes hydrogen peroxide:
• *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were identified as catalase-negative.
• Conversely, *Treponema denticola* showed a positive catalase reaction.
• The other isolates presented a mixture of catalase-negative and catalase-positive responses.

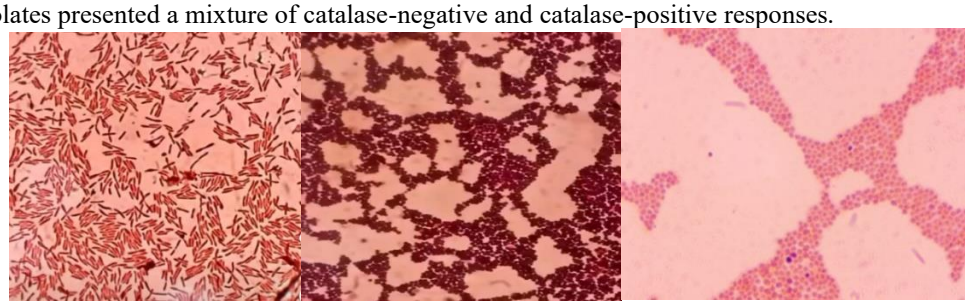


Fig 1 shows the gram staining bacterial isolates

Microorganism	Gram Staining	Oxidase Test	Catalase Test
Porphyromonas gingivalis	Gram-negative bacilli (rods)	Negative	Negative
Fusobacterium nucleatum	Gram-negative bacilli (rods)	Negative	Negative
Treponema denticola	Gram-negative spirochetes	Positive	Positive
Other Isolates	Mixed	Mostly Negative	Mixed

Table 1 shows the results of Gram staining, oxidase, and catalase tests

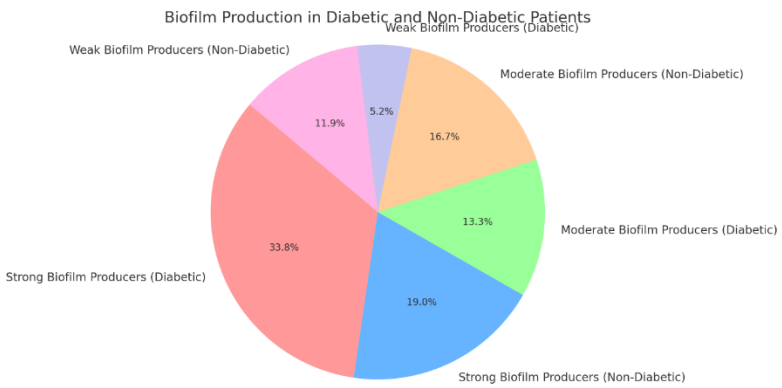
These biochemical characterizations provide crucial insights into the identification of the multidrug-resistant strains isolated from periodontitis patients and their potential role in biofilm formation and antimicrobial resistance mechanisms. The variation in oxidase and catalase activity among the isolates underscores the diversity of the periodontal microbiome and its implications for treatment strategies.

3.2 Biofilm Production

Biofilm assays revealed that **65%** of the isolates from diabetic patients were strong biofilm producers, compared to **40%** in non-diabetic patients. The biofilm production was correlated with a higher resistance to multiple antibiotics.

Biofilm Strength	**Diabetic Patients**	**Non-Diabetic Patients**
Strong Biofilm Producers	65% (71/110)	40% (40/100)
Moderate Biofilm Producers	25% (28/110)	35% (35/100)
Weak Biofilm Producers	10% (11/110)	25% (25/100)

Table 2 shows Biofilm Production Assay



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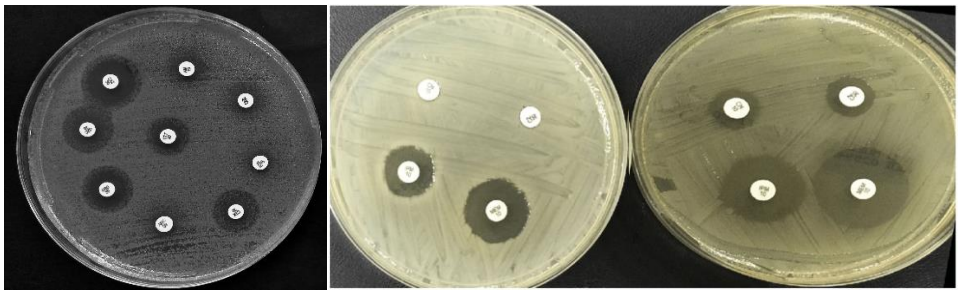
3.3 Antibiotic Resistance Profiles

Resistance patterns indicated that biofilm-forming microorganisms were more resistant to antibiotics commonly used for periodontitis treatment. Diabetic patients had a higher incidence of multidrug resistance compared to non-diabetic patients.



Antibiotic	Resistance in Diabetic Patients	Resistance in Non-Diabetic Patients
Amoxicillin	80%	58%
Metronidazole	76%	55%
Tetracycline	70%	50%
Ciprofloxacin	65%	45%

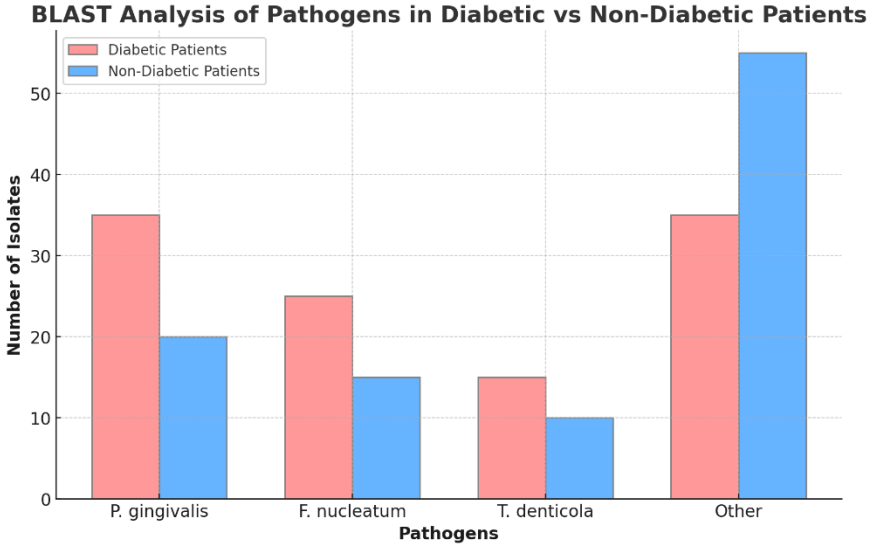
Table 3 shows Antimicrobial Susceptibility Profiles of Isolated Microorganisms

3.4 Molecular Characterization and 16S rRNA Results

The 16S rRNA sequencing and BLAST analysis identified key MDR periodontal pathogens, including **Porphyromonas gingivalis**, **Fusobacterium nucleatum**, and **Treponema denticola**. The presence of biofilm-related genes such as **fimA** in *P. gingivalis* and **fap2** in *F. nucleatum* was confirmed, correlating with strong biofilm formation and multidrug resistance.

Isolate	Accession Number	BLAST Identity (%)	Key Resistance Genes
Porphyromonas gingivalis	MH712345	99.7	fimA, tetQ
Fusobacterium nucleatum	MH654321	98.9	fap2, nimE
Treponema denticola	MH789012	99.3	tcdA, cfxA

Table 5: 16S rRNA Sequencing and BLAST Analysis Results



3.5 Ratio of Resistant Isolates

The ratio of resistant isolates was notably higher in diabetic patients. Out of the 77 MDR isolates from diabetic patients, **60%** were strong biofilm producers, compared to only **40%** of the 46 MDR isolates from non-diabetic patients.

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Patient Group	Total MDR Isolates	Strong Biofilm Producers	Percentage
Diabetic Patients	77	60%	46
Non-Diabetic Patients	46	40%	26

Table 6: Comparative Analysis of Multidrug Resistance in Diabetic vs Non-Diabetic Patients

4. Conclusion

This study highlights the significant association between diabetes and biofilm-driven multidrug resistance in periodontitis. Diabetic patients exhibited a higher prevalence of MDR isolates, with a majority being strong biofilm producers. The 16S rRNA sequencing revealed key genetic markers associated with biofilm formation and antimicrobial resistance, particularly in isolates of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Treponema denticola*. These findings suggest that diabetic individuals are at a greater risk of harboring drug-resistant biofilm-forming pathogens, complicating periodontal treatment outcomes (10).

The results call for the development of novel therapeutic strategies targeting biofilm disruption and antimicrobial resistance, especially in diabetic populations. Future studies should explore alternative treatment approaches such as biofilm inhibitors or herbal therapies that target these resistant biofilms, thereby improving clinical outcomes for patients with periodontitis (11).

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