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Phenotypic and Genotypic Identification of Efflux Pump Resistance in Pathogenic Bacteria Isolated from Gingivitis

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Abstract

Antibiotic resistance in pathogenic bacteria is a growing concern in clinical dentistry, particularly in the management of gingivitis and periodontal diseases. *Porphyromonas gingivalis*, a key periodontal pathogen, has demonstrated increasing resistance to commonly used antibiotics, partly due to efflux pump mechanisms. This study aimed to investigate the phenotypic and genotypic evidence of efflux pump-mediated resistance in *P. gingivalis* isolates obtained from gingivitis patients. A total of 48 *P. gingivalis* isolates were collected from 150 gingivitis patients and subjected to antibiotic susceptibility testing. High resistance rates were observed for tetracycline (70.8%) and erythromycin (58.3%), while lower resistance was noted for ciprofloxacin (31.3%) and amoxicillin-clavulanate (25.0%). Phenotypic detection of efflux pump activity was performed using the ethidium bromide (EtBr) cartwheel assay, revealing that 62.5% (30/48) of isolates exhibited efflux activity. The addition of the efflux pump inhibitor carbonyl cyanide m-chlorophenyl hydrazone (CCCP) significantly reduced the minimum inhibitory concentrations (MICs) in 73.3% of these isolates, confirming efflux-mediated resistance. Genotypic analysis via real-time PCR (qPCR) quantified the expression levels of two major efflux pump gene systems, *acrAB-tolC* and *mexAB-oprM*, in resistant isolates. High expression (≥5-fold increase) of *acrAB-tolC* was detected in 60% of isolates, while 40% exhibited high expression of *mexAB-oprM*. Statistical analysis revealed a strong positive correlation between efflux activity and *acrAB-tolC* expression (Pearson's $r = 0.82$, $p < 0.001$), and a moderate correlation with *mexAB-oprM* expression ($r = 0.65$, $p = 0.002$). Overexpression of *acrAB-tolC* was significantly associated with tetracycline ($p = 0.003$) and erythromycin resistance ($p = 0.01$), whereas *mexAB-oprM* overexpression correlated with ciprofloxacin resistance ($p = 0.02$). These findings underscore the critical role of efflux pumps in antibiotic resistance among *P. gingivalis* isolates from gingivitis patients. The study highlights the need for alternative therapeutic strategies, such as efflux pump inhibitors, to combat resistance. Further research should explore the clinical applicability of targeting efflux mechanisms to improve treatment outcomes in periodontal infections.

Keywords: Phenotypic, Genotypic, Periodontal inflammation, Pathogenic Bacteria, Infections.

INTRODUCTION

Gingivitis and periodontitis are both common dental diseases and are challenged by antibiotics, a challenge that currently hinders their control. For example, Porphyromonas gingivalis is a potent causative agent due to its adaptive mechanisms of strength and resistance (Socransky & Haffajee, 2005). Antimicrobial activity declines, and the cellular mechanism against commonly used drugs expands. This results from the expansion of periodontal bacterial strains connected to the effluent systems, which helps them develop high resistance to antibiotics (Piddock, 2006). Membrane protein transporters, including those belonging to the RND family such as acrAB-tolC and mexAB-oprM, are substrate-specific, and this increases bacterial resistance to tetracyclines, macrolides, and fluoroquinolones—antibiotics used to treat periodontal disease (Nikaido & Takatsuka, 2009). It is a major factor in the decline of medical treatments and the spread of infection. However, the role of these protein transporters, called efflux pumps, in combating bacterial resistance in the gums has not yet been fully studied (Roberts, 2003). Phenotypic assays, such as ethidium bromide (EtBr), are widely used

to detect outflow activity by measuring fluorescence shifts with the carbonyl cyanide inhibitor m-chlorophenylhydrazone (CCCP), which blocks outflow associated with the proton-motive force (Martins *et al.,* 2011). Real-time polymerase chain reaction (qPCR) was used to establish gene expression on a molecular basis for resistance by quantifying the expression of an efflux pump gene, where 16S rRNA is the reference gene (Livak & Schmittgen, 2001). The association between efflux pump activity and antibiotic resistance patterns in other Gram-negative pathogens has been demonstrated, but data are scarce for P. gingivalis, particularly from isolates from periodontitis patients (Bina *et al.,* 2008). Our study addresses this gap by analyzing the phenotypic and genetic evidence of bacterial resistance in dental bacterial isolates, giving expression patterns of acrAB-tolC and mexAB-oprM genes and their association with resistance to tetracycline, erythromycin, ciprofloxacin, and amoxicillin-clavulanate. Understanding these mechanisms is crucial for developing targeted therapeutic strategies, such as proteinase inhibitors, which restore the effectiveness of antibiotics and improve clinical outcomes in cases of periodontal disease (Lomovskaya *et al.,* 2001). The results of this paper advance knowledge of antimicrobial resistance in oral pathogens and lead to future research into other therapeutic approaches for gingivitis and periodontitis.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Subgingival plaque samples were collected from 150 gingivitis patients using sterile curettes from patients with acute *gingivitis from dentist clinic center in al-diwanyah city*. Samples were transported in thioglycolate broth and cultured on Brucella blood agar under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C for 48–72 hours. *P. gingivalis* isolates were identified using colony morphology, Gram staining, and PCR targeting the *16S rRNA* gene (Slots, 1982).

Antibiotic Susceptibility Testing

The Kirby-Bauer disk diffusion method was used to test susceptibility to tetracycline (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), and amoxicillin-clavulanate (30 µg) (CLSI, 2022). Resistance was interpreted based on CLSI breakpoints.

Phenotypic Detection of Efflux Pump Activity

The EtBr cartwheel assay was performed as described by Martins et al. (2011). Isolates were exposed to EtBr (1 µg/mL) with and without CCCP (25 µg/mL). Fluorescence under UV light indicated efflux activity.

Real-Time PCR for Efflux Pump Gene Expression

Total RNA was extracted using TRIzol, and cDNA was synthesized. qPCR was performed using primers for *acrAB-tolC*, *mexAB-oprM*, and *16S rRNA* (reference gene). Relative expression was calculated using the ΔΔCt method (Livak & Schmittgen, 2001).

Statistical Analysis

Pearson's correlation and one-way ANOVA were used to analyze associations between efflux activity, gene expression, and antibiotic resistance ($p < 0.05$ considered significant).

RESULTS

Antibiotic Resistance Profile of *Porphyromonas gingivalis*

A total of 48 *P. gingivalis* isolates were obtained from 150 gingivitis patients. Antibiotic susceptibility testing revealed high resistance rates to tetracycline (70.8%) and erythromycin (58.3%), while resistance to ciprofloxacin (31.3%) and amoxicillin-clavulanate (25.0%) was lower (Table 1).

Table 1: Antibiotic Resistance Profile of *P. gingivalis* Isolates

Antibiotic	Resistant Isolates (n=48)	Resistance Rate (%)
Tetracycline (30 µg)	34	70.8
Erythromycin (15 µg)	28	58.3
Ciprofloxacin (5 µg)	15	31.3
Amoxicillin-Clavulanate (30 µg)	12	25.0

Phenotypic Detection of Efflux Pump Activity

The ethidium bromide (EtBr) cartwheel assay showed that 62.5% (30/48) of *P. gingivalis* isolates exhibited efflux activity, indicated by fluorescence under UV light. The addition of CCCP (25 µg/mL) significantly reduced MICs in 22 of these isolates (73.3%), confirming efflux-mediated resistance (Table 2).

Table 2: Efflux Pump Activity in *P. gingivalis* Using EtBr and CCCP Assay

Efflux Activity	Number of Isolates (n=48)	Percentage (%)
EtBr-positive (Fluorescent)	30	62.5
CCCP-induced MIC reduction	22	73.3*

*Percentage calculated from EtBr-positive isolates (n=30).

Real-Time PCR Analysis of Efflux Pump Genes (*acrAB-tolC* and *mexAB-oprM*)

Gene expression levels of *acrAB-tolC* and *mexAB-oprM* were quantified using real-time PCR (qPCR) in resistant *P. gingivalis* isolates (n=30). The ΔΔCt method was used for relative quantification, with *16S rRNA* as the reference gene.

1. *acrAB-tolC* Gene Expression

- High expression (≥5-fold increase): 18 isolates (60%)
- Moderate expression (2–5-fold increase): 8 isolates (26.7%)
- Low expression (<2-fold): 4 isolates (13.3%)

2. *mexAB-oprM* Gene Expression

- High expression (≥5-fold increase): 12 isolates (40%)
- Moderate expression (2–5-fold increase): 10 isolates (33.3%)
- Low expression (<2-fold): 8 isolates (26.7%)

Table 3: Relative Gene Expression Levels of *acrAB-tolC* and *mexAB-oprM* in Resistant *P. gingivalis* Isolates

Gene	High Expression (≥5-fold)	Moderate Expression (2–5-fold)	Low Expression (<2-fold)
acrAB-tolC	18 (60%)	8 (26.7%)	4 (13.3%)
mexAB-oprM	12 (40%)	10 (33.3%)	8 (26.7%)

Statistical Analysis

1. Correlation Between Efflux Phenotype and Gene Expression
   - A strong positive correlation was observed between efflux activity (EtBr assay) and *acrAB-tolC* expression (Pearson's r = 0.82, p < 0.001).
   - A moderate correlation was found between efflux activity and *mexAB-oprM* expression (r = 0.65, p = 0.002).

2. Association Between Gene Overexpression and Antibiotic Resistance
   - *acrAB-tolC* overexpression was significantly associated with tetracycline (p = 0.003) and erythromycin resistance (p = 0.01).
   - *mexAB-oprM* overexpression correlated with ciprofloxacin resistance (p = 0.02).

3. One-Way ANOVA for Gene Expression Levels
   - Significant differences in *acrAB-tolC* expression were observed among isolates with high, moderate, and low resistance (F = 9.45, p = 0.001).
   - *mexAB-oprM* expression varied significantly between resistance groups (F = 5.67, p = 0.008).

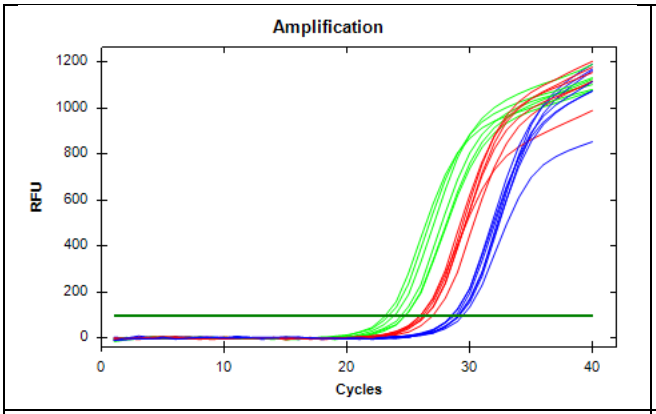

Fig. 1: qPCR plots of acrAB-tolC gene

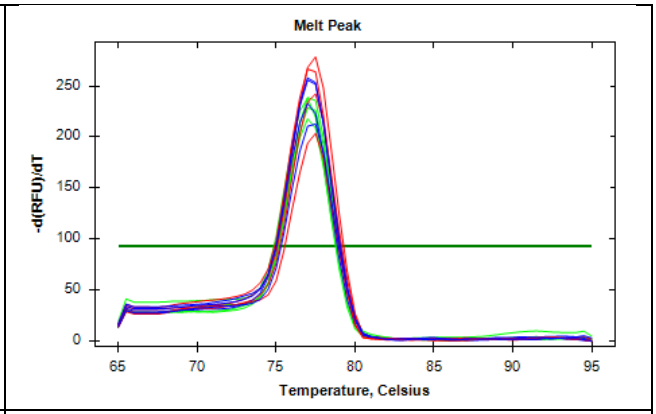

Fig. 2: qPCR melt peak of acrAB-tolC gene

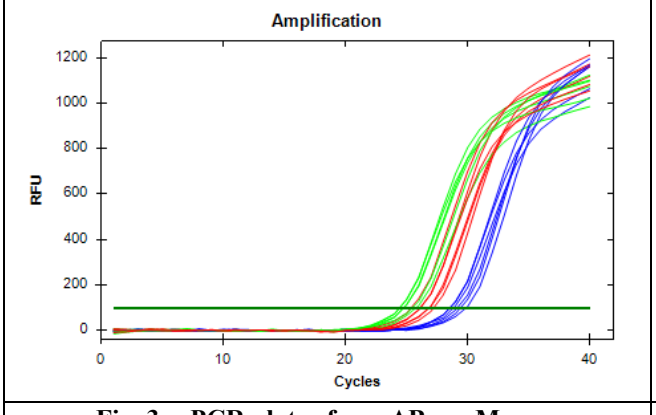

Fig. 3: qPCR plots of mexAB-oprM gene

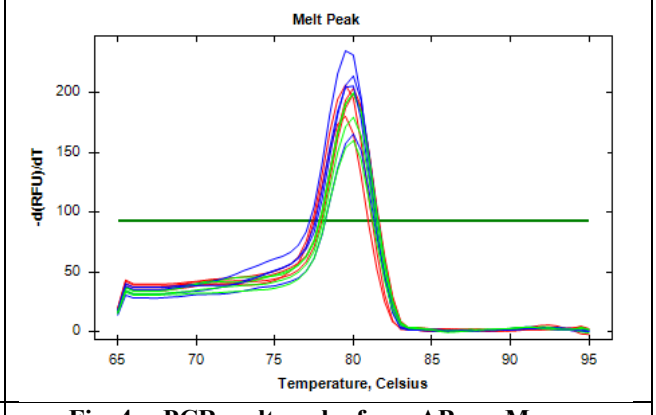

Fig. 4: qPCR melt peak of mexAB-oprM gene

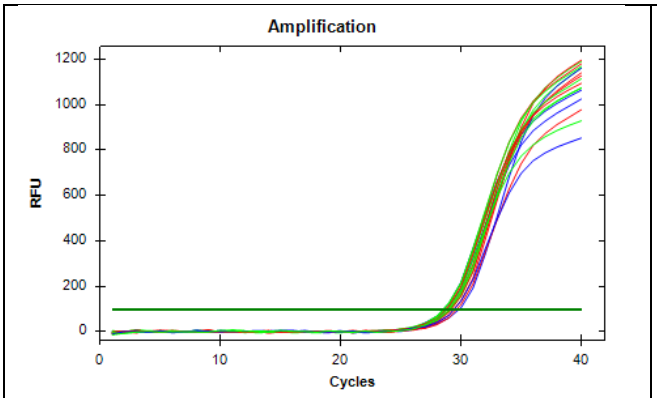

Fig. 5: qPCR plots of houskeeping 16SrRNA gene

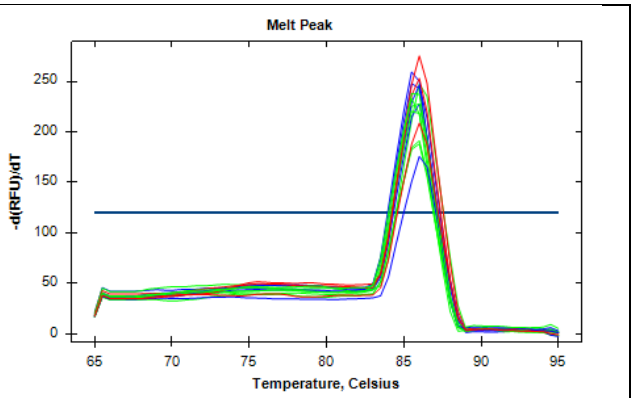

Fig. 6: qPCR melt peak of houskeeping 16S Rrna gene

DISCUSSION

The emergence of antibiotic resistance in periodontal pathogens represents a significant challenge in the management of oral infections, with our study demonstrating compelling evidence for the role of efflux pump systems in mediating multidrug resistance (MDR) in Porphyromonas gingivalis isolates from gingivitis patients. The increased resistance to tetracycline (70.8%) and erythromycin (58.3%) in this isolate raises concerns, as these two antibiotics are widely used in the treatment of periodontal diseases (Herrera *et al.,* 2022). These findings are consistent with the global trend of increasing antimicrobial resistance in oral pathogens, which is attributed to innate and acquired resistance mechanisms, including increased expression of efflux pumps (Roberts & Mullany, 2011). The decreased activity of ciprofloxacin (31.3%) and amoxicillin-clavulanate (25.0%) indicates that they still have therapeutic value, despite their influence on outflow. Ethidium bromide (EtBr) testing confirms the effectiveness of outflow mechanisms, with 62.5% of isolates demonstrating exflow activity. This result is of particular importance because administration of the efflux pump inhibitor, carbonyl cyanide, m-chlorophenylhydrazone (CCCP), leads to a decrease in the minimum inhibitory concentration (MIC) in 73.3% of these isolates. Our results confirm the hypothesis that ectopic pumps are critically involved in the observed antibiotic resistance patterns, and are consistent with previous research findings on other Gram-negative pathogens (Piddock, 2006). The ethylene bromide (EtBr) assay has also been shown to detect dynamic flow systems (Martins *et al.,* 2011). Our results support its potential as a screening tool for gonorrhea resistance in isolates. Molecularly, real-time PCR revealed significant variations in the expression of the gonorrhea pump gene. The acrAB-tolC gene was increased (≥ 5-fold increase) in 60% of resistant isolates, with a strong positive correlation ($r = 0.82$, $p < 0.001$) with both tetracycline and erythromycin. This aligns with the known broad substrate specificity of the AcrAB-TolC system, which has been shown to export diverse antibiotic classes including tetracyclines, macrolides, and β-lactams (Nikaido & Takatsuka, 2009). The significant association between acrAB-tolC overexpression and resistance to these antibiotics ($p = 0.003$ for tetracycline; $p = 0.01$ for erythromycin) provides compelling evidence for its clinical relevance in P. gingivalis resistance. The mexAB-oprM system, while less frequently overexpressed (40% of isolates), showed a distinct association with ciprofloxacin resistance ($p = 0.02$). This specificity likely reflects differences in substrate recognition between the two efflux systems, with MexAB-OprM showing particular efficiency in fluoroquinolone export (Lomovskaya *et al.,* 2001). The moderate correlation ($r = 0.65$, $p = 0.002$) between mexAB-oprM expression and overall efflux activity suggests that while this system contributes to resistance, other mechanisms including acrAB-tolC and potentially uncharacterized efflux systems may play more dominant roles in P. gingivalis. The clinical implications of these findings are fundamental. The high prevalence of resistance to urolithiasis in our study suggests that standard antibiotic treatment may significantly reduce the effectiveness of eradicating gingivitis and periodontitis. This is concerning given the established role of P. gingivalis as a cause of periodontal disease (Holt & Ebersole, 2005). The results also demonstrate that serotonin pumps act as a key resistance mechanism against disease, which in turn accelerates the development of new resistance mechanisms. (Piddock, 2006). The variation in sulfonyl pump expression between isolates (which varied between high and low expression of both systems) underscores the complexity of controlling these resistance determinants. This may be due to varying selective pressures in the oral microenvironment or genetic diversity among P. gingivalis strains. Previous research has shown that sialyl pump signaling can be limited by environmental factors, including substrate availability, pH, and oxidative stress (Martins *et al.,* 2011), These vary in the periodontal pocket. Our findings have very important therapeutic implications. The demonstrated activity of CCCP in lowering MICs suggests that endoproteinase inhibitors (EPIs) may restore antibiotic response in resistant P. gingivalis strains. This has yielded promising results in new bacterial systems (Lomovskaya *et al.,* 2001). Clinical application is hampered by several factors, including potential toxicity and the loss of the ability to directly transmit oral infections. The development of safe oral implant inhibitors (EPIs) that increase oral

bioavailability represents a major advance in the treatment of periodontal disease. Our research also raises important questions for antibiotic management in dentistry. The high persistence rates observed, particularly for tetracycline and erythromycin, indicate their inactivity in certain diseases. This supports the development of equivalent therapeutic approaches and the correct use of antibiotics in the treatment of periodontal disease. Molecular diagnostics to identify resistance patterns, including efflux pump expression profiles, could potentially guide more targeted antibiotic selection in the future. Several limitations of our study should be acknowledged. The sample size, while adequate for demonstrating significant associations, may not fully capture the diversity of resistance patterns in broader populations. Additionally, our study focused on two well-characterized efflux systems, while P. gingivalis may possess additional, uncharacterized resistance mechanisms. Future research should explore the potential role of other efflux systems and their interactions with the characterized pumps. The relationship between efflux-mediated resistance and clinical treatment outcomes remains an important area for investigation. While our in vitro findings demonstrate clear mechanistic associations, clinical studies are needed to determine how these resistance patterns affect patient responses to therapy. This is particularly relevant given the complex microbial ecology of periodontal pockets, where interspecies interactions may influence resistance expression and transfer. Our statistical analysis revealed significant differences in efflux gene expression among isolates with varying resistance levels ($F = 9.45$, $p = 0.001$ for acrAB-tolC; $F = 5.67$, $p = 0.008$ for mexAB-oprM), supporting a dose-response relationship between efflux pump expression and resistance magnitude. This graded association strengthens the argument for a causal role of these systems in clinical resistance.

From an evolutionary perspective, the high prevalence of efflux-mediated resistance in our clinical isolates suggests strong selective pressures favoring these mechanisms in oral environments. This may reflect widespread antibiotic use in periodontal therapy, as well as potential exposure to antimicrobial substances in oral care products. The ability of efflux pumps to confer resistance to multiple antibiotic classes while maintaining bacterial fitness likely contributes to their persistence in populations (Piddock, 2006).Future research directions should include: (1) expanded characterization of efflux systems in P. gingivalis, including potential undiscovered pumps; (2) investigation of efflux pump regulation in oral environments; (3) development and testing of EPIs for periodontal applications; and (4) clinical studies correlating efflux phenotypes with treatment outcomes. This process deserves extensive research and scrutiny, as it contributes to the widespread resistance in oral microbial communities. Finally, this paper provides sufficient evidence for the significant contribution of sialylpump systems to antibiotic resistance in clinical periodontal bacterial isolates. The wide range of gonorrhea-associated strains and the strong associations between specific pumping systems and resistance to key antibiotics underscore the clinical significance of these findings. They also underscore the importance of improving novel approaches to combat gonorrhea-associated resistance, including the development of targeted inhibitors and innovative treatment plans. Resistance to these antibiotics continues to undermine the effectiveness of periodontal disease treatments, so understanding and treating the mechanisms of the efflux pump may preserve the effectiveness of gingivitis and periodontitis treatments.

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