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Original Research Article

Antibiotic Resistance Genes Acquired in Endodontic *Enterococcus faecalis* Isolates: Implications for Epidemiological Surveillance

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Abstract: ***Background:*** In recent years, there has been an increase in the prevalence of *Enterococcus faecalis* strains with acquired antimicrobial resistance in the oral environment, a phenomenon associated with horizontal gene transfer. The oral cavity can act as a reservoir and a critical point for the persistence and transmission of multidrug-resistant microorganisms, posing a challenge to public health. ***Aim:*** To identify genes associated with acquired antimicrobial resistance and determine their prevalence against selected antibiotics as epidemiological surveillance markers in *Enterococcus faecalis* strains isolated from patients who attended endodontic consultations. ***Methods:*** Microbiological identification and antimicrobial susceptibility profiling were evaluated using the Dade/MicroScan Pos ID PC34 system (West Sacramento, CA, USA), in accordance with CLSI standards. PCR using specific primers allowed the detection of acquired resistance genes for epidemiologically relevant antibiotic markers in the oral flora, including erythromycin (ERY), high-level gentamicin resistance (HLGR), high-level streptomycin resistance (HLSR), tetracycline (TET), and vancomycin (VAN). *Results:* 35.3% of *Enterococcus faecalis* isolates showed acquired resistance, with particular emphasis on resistance to GEN (11.8%), HLSR (14.7%), TET (5.9%) and ERY (2.9%). VAN resistance was absent. The genes mainly associated with this resistance were *erm*B, *tet*M and *aac*(6')-Ie-*aph*(2")-Ia. ***Conclusion:*** The presence of acquired resistance genes in *E. faecalis* from endodontic infections was evident, signalling the oral cavity as an antimicrobial reservoir. These findings underscore the need for microbiological surveillance in dentistry.

Keywords: *Enterococcus faecalis*, Horizontal Gene Transfer, Drug Resistance, Bacterial, Epidemiological Monitoring, Endodontics.

INTRODUCTION

Antimicrobial resistance has become one of the biggest challenges to global public health. Although historically associated with hospital-acquired or systemic infections, the oral cavity has now emerged as a significant reservoir of resistant bacteria, including *Enterococcus faecalis*, a pathogen

frequently involved in endodontic treatment failures and persistent or recurrent infections [1, 2].

Enterococcus faecalis acts as an opportunistic pathogen in endodontic infections due to its ability to colonise, survive in previously treated root canals, and form biofilms that protect it from the immune response and antimicrobial agents [3].

The increase in the prevalence of resistant strains of *Enterococcus faecalis* is closely related to various factors such as the indiscriminate use of antibiotics in clinical practice, their incorporation into the food industry, oral-faecal contamination, and poor oral hygiene, which favour the colonisation and spread of these resistant bacteria in the oral cavity [4].

In recent years, an increase in antimicrobial resistance of *Enterococcus faecalis* has been documented at the oral level, especially in cases refractory to conventional treatments [5]. Systematic reviews have shown significant variations in minimum inhibitory concentrations (MICs) associated with the intrinsic resistance of *Enterococcus faecalis* to various antibiotics. A progressive increase in the frequency of acquired resistance to new antimicrobial agents has also been observed. [6]. Although the direct therapeutic impact of this resistance is controversial due to the limited use of some of these antibiotics in dental clinical practice [7, 8], from an epidemiological perspective, it is essential to monitor acquired resistance to certain antibiotics considered epidemiological markers, as these resistances indicate selective pressure and potential for genetic dissemination. Among these, the best markers are VAN (*van*A, *van*B), ERY (*erm*B, *mef*A), TET (*tet*M, *tet*L), and high-concentration GEN (*aac*6`*aph*2), because they allow the identification of the presence and possible spread of resistant strains in the oral cavity, which is a potential reservoir. Given the dynamic and conducive nature of the oral environment for horizontal gene transfer, this reservoir may facilitate the transmission of resistance to susceptible strains, posing a significant threat to public health [9, 10].

One of the mechanisms contributing to the spread of acquired antimicrobial resistance is the formation of biofilms, which, in addition to providing a physical barrier, promotes horizontal gene transfer (HGT) and the integration of transferable genetic material such as plasmids and transposons. This horizontal gene transfer allows *Enterococcus faecalis* to acquire antibiotic resistance through processes such as conjugation and transduction, facilitating its rapid adaptation to antimicrobial pressure [11-13].

In this context, the oral cavity acts as a potential reservoir for resistance genes, facilitating their transfer and promoting the spread of microorganisms both within and outside the oral ecosystem. This phenomenon increases the risk of oral and systemic infections, including mediastinitis, brain abscesses, sepsis, and infectious endocarditis [14, 15].

The increase in the prevalence of strains carrying virulence factors and acquired antimicrobial resistance poses a significant health risk, both locally and systemically, which underscores the importance of monitoring antimicrobial resistance in the dental field. To address this surveillance, it is essential to identify and characterise the phenotypic and genotypic resistance profiles of *Enterococcus faecalis* strains isolated from endodontic infections. This approach allows for a more accurate assessment of the associated epidemiological risk.

Given this situation, there is a need for studies that accurately characterise the resistance profiles of *Enterococcus faecalis* in the oral environment, especially in clinical contexts such as endodontic infections. Phenotypic antimicrobial susceptibility testing allows for the determination of the clinical effectiveness of antibiotics, while the detection of specific resistance genes provides information on the molecular mechanisms involved and the ability of these genes to spread [16].

This information is relevant for designing strategies to prevent the spread of multidrug-resistant strains and implementing epidemiological surveillance systems for antimicrobial resistance in dentistry, an aspect that is not currently addressed in dental clinics and hospital dental centres [12-16]. Therefore, this study aimed to detect acquired resistance genes for epidemiological marker antibiotic resistance in *Enterococcus faecalis* strains isolated from endodontic infections, to provide evidence about the role of the oral cavity as a reservoir for these genes and to highlight the need for epidemiological surveillance in dentistry.

METHODOLOGY

Bacterial Strain

A total of 170 isolates of *Enterococcus faecalis* collected between 2015 and 2022 from patients attending endodontic consultations were the research object. Clinical sampling follows the guidelines [17], and included swabs from the cheek, palate, tongue, buccal sulcus and gums. Additionally, samples taken from ejectors, paper cones, gutta-percha filling material, and K-type files used during treatment were analysed. Strains ATCC 70802 and ATCC 29212 served as controls in the microbiological and molecular assays.

Microbiological Identification / Susceptibility Testing

Microbiological identification and susceptibility testing were performed using the Dade/MicroScan Pos ID PC34 MicroScanSystem (West Sacramento, CA, USA), with readings taken on the autoSCAN-4 system (American MicroScan, Inc., Mahwah, NJ, USA) [18, 19]. The minimum inhibitory concentration (MIC) was determined in accordance with the standards [20], in force at the time of analysis.

Marker Antibiotic Genes

For this study, only antibiotics with epidemiological marker functions were selected, based on their relevance in monitoring bacterial resistance. Unlike conventional clinical antibiograms, this methodology has no therapeutic purpose. The inclusion criteria for the antibiotics analysed were documented evidence of acquired resistance in *E. faecalis*, resistance mechanisms associated with horizontal transfer (plasmids, integrons or transposons), resistance associated with persistent oral or endodontic flora, and antibiotics whose epidemiological value is considered high. Antibiotics not routinely used in dentistry, with mainly intrinsic resistance, whose resistance mechanisms are not related to horizontal transfer, and antibiotics whose epidemiological value is considered moderate or low were not included. The antibiotics evaluated and their minimum inhibitory concentrations (MIC) were, ERY (0.5, 1.0, 2.0 and 4.0 µg/mL); High-Level STR Resistance (HLSR, 1000 µg/mL); High-Level GEN Resistance (HLGR, 500 µg/mL); TET (4.0 - 8.0 µg/mL); VAN (0.25 - 16.0 µg/mL).

DNA Extraction and Detection of Resistance Genes

Genomic DNA was extracted using the DNeasy Blood & Tissue 69504 isolation kit (Qiagen, Santa Clarita, California) following the manufacturer's recommended instructions. DNA concentration was determined spectrophotometrically at 260/280 nm (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA) [19]. The detection of acquired resistance genes was performed following previously standardised and reported protocols [21], for the *erm*(A), *erm*(B), and *erm*(C) genes in ERY resistance, those reported for the *tet*M/tetL genes in TET resistance [22], and those reported for the *van*A/*van*B in VAN resistance [23]. Antibiotics such as HLSR and HLGR, the guidelines previously proposed [24], served for the *aac*(6')-Ie-*aph*(2")-Ia and *ant*6 genes. Amplification products were then visualised on 1% (w/v) agarose gels in 1X TBE buffer –Syber Green [25], and analysed in a photo documenter (ChemiDocTM MP Imaging System BioRad Laboratories Inc.).

Data Analysis

Descriptive statistical methods (frequency and percentage) were used for the analysis and presentation of the resistance genes identified using IBM SPSS Statistics V.25.0 statistical software.

RESULTS

Characterisation of Isolates

The 170 isolates included in the study were 100% viable and pure at the time of reactivation. All strains were sensitive to amoxicillin/clavulanic acid (AMX/CLA) and moxifloxacin (MXF). Resistance profiles, specific and expected for the genus and species, such as ampicillin (AMP), cephalosporins, clindamycin (CLI), low-level aminoglycosides, ciprofloxacin (CIP), sulfonamides, and trimethoprim (TMP), were observed; resistances that correspond to intrinsic resistance mechanisms or are related to antibiotics that are not considered epidemiological markers, and therefore were not included in the analysis of this study.

Resistance Profile

According to the established inclusion criteria, 35.3% (60/170) of isolates showed acquired resistance to at least one of the antibiotics evaluated. The resistances detected were to ERY 2.9% (5/170), HLGR 11.8% (20/170), HLSR 14.7% (25/170) and TET 5.9% (10/170). VAN resistance did not occur.

Detection of Acquired Resistance Genes

Molecular analysis using PCR identified genes associated with acquired resistance. In erythromycin-resistant isolates, the predominant gene was *erm*B (100%), in TET-resistant strains, the *tet*M gene was present (90%), while VAN resistance genes were absent. HLGR resistance was associated with the *aac*(6')-Ie-*aph*(2") gene, and in HLSR with the *ant*6` gene;

Table 1 summarises the observed resistance frequencies, the minimum inhibitory concentration (MIC) values obtained for each antibiotic, and the transferable resistance mechanisms detected. All of these are associated with mobile genetic elements, suggesting a potential for horizontal spread between strains.

Table 1: Frequency of resistance, minimum inhibitory concentration (MIC) and resistance genes detected in the isolates analysed

Antibiotic	Class	Resistance % (n=170)	*n/N*	MIC_{90} (mg/mL)	Detected gene	Resistance Mechanism Information			Ref
ERY	Macrolide	2.9	5/170	≥ 8.0	*erm*B (100%)	ERM	Erythromycin ribosomal methylase	EC 2.1.1.- (methyltransferase)	[26]
HLGR	Aminoglycoside	11.8	20/170	≥ 500	*aac*(6')-Ie-*aph*(2")-Ia (100%)	AAC	Aminoglycoside N-acetyltransferase	EC 2.3.1.- (acetyltransferase)	[27]
						APH	Aminoglycoside O-phosphotransferase	EC 2.7.1.- (phosphotransferase)	
HLSR	Aminoglycoside	14.7	25/170	≥ 1000	*ant*6 (100%)	ANT	Aminoglycoside nucleotidyltransferase	EC 2.7.7.- (nucleotidyltransferase)	
						aadA, strA, strB	Streptomycin-specific nucleotidyltransferases	EC 2.7.7.- (nucleotidyltransferase)	[27]
TET	Tetracycline	5.9	10/170	≥ 4.0	*tet*M (90%) *tet*L (10%)	TET(A), TET(B)	Efflux pumps or ribosomal protection proteins (not classical enzymes)	Not applicable	[26, 28]
VAN	Glycopeptide	0	0/170	< 2.0	No detected	VAN(A), VAN(B)	Peptidyl ligases modifying target peptide (D-Ala-D-Lac ligases)	EC 6.3.2.- (ligases)	[29]
Total resistance	35.3	60/170							

DISCUSSION

International policies for controlling antimicrobial resistance in dentistry are due to the increase in the inappropriate use of antibiotics in oral infections. Organisations such as the WHO, CDC, ADA and FDI have developed antibiotic stewardship strategies specific to the dental field, aimed at reducing selective pressure on the oral microbiota, improving clinical outcomes and preventing the spread of resistance genes [30].

In this context, it is essential to address acquired resistance, considering that the oral microbiota can harbour and transfer resistance genes through mobile genetic elements such as plasmids, integrons, and transposons. This phenomenon favours the circulation of genetic determinants among bacterial species in the oral ecosystem, with both local and systemic clinical implications [31].

Enterococcus faecalis is a microorganism prevalent in endodontic infections, representing a therapeutic challenge due to its persistence and resistance. The use of antibiotics with value as epidemiological markers, such as TET, ERY, VAN, HLSR, and HLGR, among others, serves for monitoring trends in resistance and potential sources of dissemination [32, 33].

In this study, phenotypic resistance occurred in 35.3% of isolates, with rates oscillating from 11.8 to 14.7% for aminoglycosides (HLSR and HLGR) and between 2.9 and 5.9% for ERY and TET. VAN resistance did not occur. These findings, although moderate compared to international reports, confirm the presence of resistant strains in patients without recent antibiotic consumption, suggesting the community circulation of these resistance genes. Some systematic reviews indicate variations in the geographical distribution of *E. faecalis* resistance, with prevalences from 40 to 50% for ERY and TET [34]. The absence of resistance to VAN in the isolates in this study is an encouraging finding, considering its use as a last-line treatment [35]. However, the emergence of enterococci resistant to this antibiotic in other contexts necessitates continuous surveillance.

At the molecular level, *erm*B, tetM, *aac*(6′)-Ie-*aph*(2″)-Ia and *ant*(6)-Ia genes were present in the isolates, all previously described in the literature. *erm*B induces resistance to macrolides by ribosome methylation; *tet*M acts by ribosomal protection against tetracyclines; *aac*(6′)-Ie-*aph*(2″) -Ia modifies aminoglycosides by inactivating them, and *ant*(6)-Ia confers resistance to STR by adenylation [26, 27]. The resistance mechanisms against each antibiotic are previously known. The *erm*B gene confers resistance to macrolides by methylating the ribosome, preventing the antibiotic from binding to its target site. The *tet*M gene, meanwhile, protects the ribosome from the action of TET, facilitating protein synthesis in the presence of the antibiotic. As for aminoglycosides, the *aac*(6′)-Ie-*aph*(2″) -Ia gene encodes a bifunctional enzyme that structurally modifies the antibiotic, inactivating it, and is commonly associated with high-level resistance to GEN (HLGR), while the *ant*(6)-Ia gene is related to resistance to STR through adenylation of the drug [26, 27], (Table 1).

The global trend indicates a progressive increase in the detection of strains carrying genes such as *erm*B, *tet*M, and *aac*(6′)-Ie-*aph*(2″)-Ia in various clinical settings, with reports ranging from 60 to 90%. At the oral level, molecular studies are scarce, limiting comparisons and the establishment of clear epidemiological patterns. [34]. From a clinical perspective, it is crucial to note that resistance to aminoglycosides due to the presence of the *aac*(6′)-Ie-*aph*(2″)-Ia gene prevents the usual synergy with β-lactam antibiotics. Similarly, the simultaneous detection of *erm*B and *tet*M poses an additional risk, as both genes are often associated with mobile genetic elements such as Tn916/Tn1545 transposons, which favours their horizontal co-transfer between different bacterial species [36], this risk increase is due to superficial or invasive oral procedures, such as endodontic and periodontal treatments, which facilitate the horizontal transfer to other commensal or pathogenic species, thereby increasing the community spread of resistance [37].

E. faecalis has demonstrated a high genetic plasticity, allowing it to adapt and persist in niches such as the oral cavity. This adaptability requires oral microbiology to be considered not only from a clinical perspective, but also from an epidemiological one. Systematic surveillance of resistance genes in dental settings is key to anticipating the spread of multidrug-resistant strains and protecting long-term therapeutic efficacy [19-38].

CONCLUSION

The present study demonstrated the existence of acquired resistance genes in *E. faecalis* originating from endodontic infections, reinforcing the role of the oral cavity as a reservoir of antimicrobial resistance. The detection of genes such as *erm*B, *tet*M, *aac*(6′)-Ie-*aph*(2″)-Ia, and *ant*(6)-Ia suggests the existence of selective pressure and potential for dissemination. These findings highlight the necessity for implementing microbiological surveillance strategies in dentistry.

DECLARATIONS

Conflict of Interest Statement

The authors declare no potential conflicts of interest concerning the research, authorship or publication of this article.

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Ethics Committee

The present study was evaluated and approved by both the Ethics Committees of the Faculties of Science and Dentistry. Research doesn´t include human subjects, materials or data. Bacterial isolates were from an institutional screening, in compliance with national and international ethical guidelines.

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