**Root canal microbiota as an augmented reservoir of antimicrobial**

**resistance genes in type 2 diabetes mellitus patients**

**Abstract**

Antimicrobial resistance is a global public health problem. Root canal microbiota associated with apical periodontitis (AP) represents a well-known reservoir of antimicrobial resistance genes (ARG). However, it is unknown the effect of Type 2 diabetes mellitus (T2DM) in this reservoir. The aim of this study was to establish if root canal microbiota associated with AP in T2DM patients is an augmented reservoir through identifying the prevalence of nine common ARG and comparing it with the prevalence in non-diabetic patients. Methodology: This cross-sectional study included two groups: A T2DM group conformed by 20 patients with at least ten years of living with T2DM, and a control group of 30 non-diabetic participants. Premolar or molar tooth with pulp necrosis and AP were included. A sample was collected from each root canal before endodontic treatment. DNA was extracted, and ARG were identified by polymerase chain reaction. Results: *tetW* and *tetM* genes were the most frequent (93.3 and 91.6%, respectively), while the *ermA* was the less frequent (8.3%) in the total population. The distribution of the ARG was similar in both groups, but a significant difference (p<0.005) was present in *ermB*, *ermC*, *cfxA*, and *tetQ* genes, being more frequent in the T2DM group. Eighty percent of the T2DM patients presented a minimum of four ARG, while 76.6% of the control group presented a maximum of three. Conclusions: Root canal microbiota associated with AP in T2DM patients carries a higher prevalence of ARG hence, this pathological niche could be considered as an augmented reservoir.

**Keywords:** Root canal. Apical periodontitis. Type 2 diabetes mellitus. Antibiotic resistance genes**.**

**Introduction**

Apical periodontitis is a common oral disease.1 It is an inflammatory response of the host immune system to microorganisms from the oral microenvironment that have infected the tooth root canal,2 mainly due to caries. It is considered a biofilm-induced pathology3,4 that can be solved by tooth extraction or by eliminating and entombing microorganisms through cleaning and filling the root canal. Therefore, while the tooth is not extracted or the root canal endodontically treated, it represents a pathological niche harboring a well-established multispecies biofilm.5

Chronic hyperglycemic states, whether for defects in insulin secretion, insulin action, or both, impair the host's immune response, making the individual susceptible to different, recurrent, and severe infections, including processes in the oral cavity, which may worsen glycemic control inducing a vicious circle. Then, the use of antimicrobials becomes necessary and more frequent in these individuals, thus promoting greater antimicrobial resistance and negatively impacting any infection control.6,7 Type 2 diabetes mellitus (T2DM) and antimicrobial resistance are two leading global public health problems, their prevalence constantly increases worldwide, and the high risk of presenting difficulty to treat potentially deadly infections indicates synergistic coexistence of both conditions.6,8

Different mechanisms of bacterial resistance are carried and transferred by antimicrobial resistance genes (ARG). Their propagation in commensal and pathogenic microorganisms benefits from establishing multispecies biofilms on specific niches, which provides an ideal environment for horizontal gene transfer via plasmids, bacteriophages, or transposons9,10 from bacterial cells of the same and other species, even from bacteria to yeast.11,12 It is estimated that 40% of the horizontal gen transfer occurs between bacteria of the same niche (intra-niche); while in the remaining 60%, the mechanism is still unknown. However, one theory establishes that it occurs inter-niche from one body site to another; either during disease states or throughout the individual's life.12 This established interconnection of multispecies biofilms can produce new genetic combinations.11

Horizontal gene transfer can become clinically relevant when it occurs between niches; this happens mainly among the two body sites with the most significant amount of microorganisms, the oral cavity and the gastrointestinal tract. The genetic propagation in these niches is expected since the anatomical relationship between both; however, a connection between these two and systemic circulation suggests that genetic information could move directly to the human circulatory system and then to other locations,12 this connection is proposed in figure 1. In this regard, it is crucial to investigate ARG presentation patterns in distinct niches not only because of the possibility of local resistant infections but also because of the possible association with complex resistant infections in locations different from oral cavity.

Therefore, the present investigation aimed to establish if root canal (oral pathological niche) microbiota associated with pulp necrosis and apical periodontitis is an augmented ARG reservoir in T2DM patients through identifying the prevalence of nine ARG and comparing it with the present prevalence in non-diabetic patients.

**Methodology**

***Patient selection and clinical evaluation***

This cross-sectional study included 50 patients who attended the XXXXXXXXXXX. Before clinical examination and sample collection, informed and voluntary written consent was obtained, according to the ethical criteria described by the Declaration of Helsinki. This study was approved by the Ethical Committee of the XXXXXXXXX.

Participants were divided into two groups: a T2DM group conformed of 20 patients with at least ten years of living with T2DM and glucose at a maximum of 300mg/dL at the time of sampling, and a Control group of 30 non-diabetic participants with glucose between 70 to 126 mg/dL.​​​​​​​ Smokers and those who have taken antimicrobials in the last three months and pregnant or lactating women were excluded. The teeth inclusion criteria were: Premolar or molar tooth with a diagnosis of pulp necrosis and apical periodontitis following the American Association of Endodontists guideline and a periapical radiolucency classified ≥ 3 according to the periapical index (PAI) score.13 On the other side, teeth with a gingival abscess, endo-periodontal disease, and non-restorable were excluded.

Clinical (including height, weight, and blood glucose concentration) and radiographic examinations, as well as all the procedures were done by a resident of the endodontic specialization program and supervised by an expert.

***Sample collection***

After anesthesia and rubber dam isolation, disinfection of the tooth was done using 2.5% sodium hypochlorite (NaOCl). Restauration, caries, and weak tissues were removed, and the endodontic access was prepared with a high-speed N°4 carbide bur. The operative field was cleaned with 3% hydrogen peroxide and a 2.5% NaOCl solution. A number 3 Gates–Glidden drill was used for coronal flaring with copious irrigation of 2.5% NaOCl solution, then neutralized with 5% sodium thiosulfate solution and final irrigation with distilled sterile water. To take the root canal sample associated with apical periodontitis, a new sterile size 15 stainless-steel hand file was introduced into the canal to determine a tentative working length with a Root ZX II electronic apex locator (J. Morita USA., Irvine, CA). The file was then introduced into a microtube containing sterile phosphate-buffered saline and was vortexed. Two sterile absorbent paper tips were consecutively introduced into the canal to soak up the fluid and then were transferred to the same microtube. The root canal treatment was routinely continued, and the sample was kept at − 80 °C until DNA extraction.

***DNA extraction and polymerase chain reaction (PCR)***

DNA was extracted by phenol-chloroform purification and [isopropanol](https://www-sciencedirect-com.ezproxy.javeriana.edu.co/topics/medicine-and-dentistry/2-propanol) precipitation method. Specific oligonucleotides were selected to identify nine common ARG in oral bacteria. PCR assays were carried out in 25µL reactions using the parameters reported in Table 1. PCR products were analyzed by electrophoresis in a 2% agarose gel and a 100-bp DNA ladder marker. Each gel was stained and examined in ultraviolet light.

***Statistical analyses***

Quantitative data were analyzed using Student’s t-test. For qualitative variables, Fisher’s exact test was applied. GraphPad Prism V8.0 (GraphPad Software, San Diego, CA) was used. Statistical significance was considered at p ˂ 0.05.

**Results**

The clinical characteristics of the patients are shown in Table 2. No significant difference was observed in the distribution by age, sex, or type of affected tooth, but a large body mass index (BMI) in the T2DM group was observed (p<0.001). The mean age of the study population was 46 years, and the patients were predominantly female (73.3%).

Regarding ARG distribution in the total population, *tetW* and *tetM* were the most frequent (93.3 and 91.6%, respectively), while the *ermA* was the less frequent (8.3%). ARG distribution was similar in both groups, but a significant difference (p<0.005) was present in *ermB*, *ermC*, *cfxA*, and *tetQ,* being more frequently in the T2DM group (Table 3). Eighty percent of the T2DM patients presented a minimum of four ARG (p<0.01), while 76.6% of the control group presented a maximum of three (p<0.0001) (Table 4).

**Discussion**

Oral microbiota is considered a reservoir for several ARG;14,15,16 particularly root canals as pathological niches in individuals without systemic diseases have been reported as reservoirs for ARG that encode resistance mainly to tetracyclines, beta-lactams, and macrolides.17,18 However, there are no reports from this pathological niche in immune-compromised individuals who tend to consume more significant quantities and types of antimicrobials as the patients diagnosed with T2DM.

T2DM is one of the most prevalent diseases around the world. Individuals living with it are considered a risk group for local infections and are more prone to developing severe systemic conditions6 hence, their antimicrobial consumption is more remarkable.

There are few reports of ARG in T2DM individuals, mostly in urinary tract infections, sepsis, pneumonia, and diabetic foot wounds6 nevertheless, T2DM patients present a higher prevalence of apical periodontitis19,20 and considering the connection that may exist between niches, it is essential to investigate this niche too. This study presents novel information on the pattern of presentation of some common ARG in root canal microbiota associated with apical periodontitis in patients with T2DM.

Although a limitation of this study is the small number of participants, which may result in the low statistical power of the analyses, the included patients met strict inclusion criteria. Both groups presented a homogeneous distribution regarding age and sex. BMI was the only different variable, this was expected since obesity is frequently related to T2DM.21 Regarding the type of affected tooth, the distribution in both groups was similar too. All teeth had the same diagnosis, pulp necrosis and apical periodontitis with a forthright periapical radiolucency classified by the PAI index;13 this is especially relevant because it is necessary time for apical periodontitis to develop and become radiographically visible; thus the included teeth would represent a long-standing pathologic process caused by a well-established intraradicular biofilm infection.

It is outstanding that 80% of the T2DM group presented more than four ARG in their root canals microbiota, while the 76.6% of the control group presented a maximum of three. This is surely related to selective pressure due to a higher intake of antimicrobials by T2DM patients than in healthy ones because more frequent infections are associated with them. This in part, could be sustained by the information obtained from the patients during the clinical interview (data not shown). It was found that 100% of both groups ingested at least one antimicrobial from the beta-lactam group in the last five years, but the use of tetracyclines or macrolides was reported in the T2DM group by 17 (85%) and 12 (60%) patients respectively, but only in 6 (20%) and 5 (16.6%) patients in the control group.

Five of the nine ARG presented a similar distribution in both groups, but in the T2DM group, a significant high presentation was observed in four of them, *ermB,* *ermC,* *cfxA*, and *tetQ.* They occurred in at least 35% of the T2DM samples but only in a maximum of 10% of the non-diabetic samples.

The *tet* genes that encode for tetracyclines are found in diverse niches of the oral cavity,15 *tetW* and *tetM* were the most prevalent; this is coincident with a previous report in which *tetW* was present in 93.5% and *tetM* in 83.9% of root canal samples with primary infection of similar non-diabetic Mexican population.18 In comparison, *tetQ* was less carried in the non-diabetic group samples (6.6%), which coincides with previous reports in which it was not even found,18,22 due to this, the frequency with which it occurs in the T2DM group (35%) is remarkable since this moderate prevalence had not been reported before.

The *erm* genes which encode rRNA methylases were present in both groups in distinct frequencies; *ermA* was the least carried, this could be coincident with previous investigations in isolated species from primarily infected root canals of non-diabetic patients where it was not detected.22,23 *ermB* and *ermC* were less detected in the non-diabetic group (10 and 6.6%, respectively), while in the T2DM group they were moderately found (45 and 35%, respectively). Carriage of *ermB* in root canals had not been previously reported;22,23 interestingly the T2DM group presented it in almost half of the group. *ermC* has been previously reported with varying frequencies depending on the study: 10%,22 24%,16 3.2 and 51.5% in primary infection or post-treatment infection, respectively,18 the last being a more similar presentation percentage to the current and probably due, as previously mentioned, to better-established and overexposed to antimicrobials microbiota.

*blaTEM* gen represented the third most prevalent in both groups (87.5% on average); this is consistent with a previous report from four years ago in patients from the same city (87.1%)18 and confirms a significant difference from what was reported in a US17 and a Brazilian16 populations about ten years ago, 43% and 24%, respectively. The *cfxA* gene was also relevant in the T2DM group since it was found consistently in only 6.6% of non-diabetic individuals, similar to the 2% found in root canal isolates22 and the 0% reported in samples of the root canal,16 especially in a similar population.18 However, in a notable way, it was found in 40% of the T2DM samples. *mecA* gene, was detected in one non-diabetic individual (3.3%). Although its prevalence is low, this is the first report on the oral microbiota of systemically healthy individuals.22,24 Then, it is remarkable that *mecA* was detected in three T2DM patients (15%). This higher frequency coincides in part with a recent study that included oral mucosa of diabetic individuals in Brazil25 in where *mecA* was present in 4.8% of the individuals. However, the presence of *mecA* in these oral cavities was underestimated because it was only detected in isolated *Staphylococcus aureus* excluding all other bacteria. Therefore, the contribution of different species in the complete microbiota was not evaluated. This is a strength of the present investigation; the direct screening of clinical samples for the presence of ARG is more appropriate for this purpose.

Antimicrobial resistance is a severe problem. Public health strategies have been implemented in many countries after the presentation of the action plan by the World Health Organization in 2015.26 It is well known that antimicrobial resistance results from an evolutionary process whose frequency is influenced by human, animal, and agricultural consumption of antimicrobials, sanitation, and water reuse, among others.

This study evidence that the oral health of an individual not only benefits them directly since microorganisms from a pathological oral niche could spread to aponeurotic planes of the head and neck and could cause severe septic conditions such as descending mediastinitis, Lemierre’s syndrome, cervical necrotizing fasciitis, orbital abscess, cavernous sinus thrombosis, cerebral abscess, and osteomyelitis27 which would be difficult to treat with antimicrobials. In addition, oral health plays an essential role in this global problem since maintaining pathological niches in the mouth further promotes the reservoir and spread of ARG. Therefore, eliminating these niches, especially in individuals with systemic conditions such as T2DM in where the ARG reservoir is more significant is essential to help in the control of this global problem since bacteria and their ARGs can be shared between humans or even with their pets.28 Therefore, the strategies to reduce the appearance and spread of resistant and multiresistant pathogens should also consider preventive dentistry to avoid the appearance of pathological niches, and when this has failed, the intervention of a dentist is crucial to eliminate these niches.

Root canal with pulp necrosis and apical periodontitis represents a common pathological niche in the human body in where the transference of ARG is favored and acts as a reservoir. This condition in patients with T2DM would represent an augmented presence of a wide variety of ARG. Therefore, eliminating these pathological niches is of utmost importance, especially in patients with T2DM, not only to avoid complications in their infection management but also to avoid the spread of ARG in different niches and among individuals in the community.

**Consent to participate**

All authors have provided consent to participate.

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**Conflicts of interest**

The authors declare that they have no conflict of interest.

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Figures and tables list

**Figure 1-** Influence of pathogenic root canal biofilms in individuals with poorly controlled type 2 diabetes mellitus on human antimicrobial resistance. ARGs: antimicrobial resistance genes; HGT: horizontal gene transfer

**Table 1-** Oligonucleotides employed to detect the nine antimicrobial resistance genes (ARG)

**Table 2-** Clinical characteristics of the included subjects and teeth in both groups

**Table 3-** Distribution of the nine antimicrobial resistance genes (ARG) in both groups

**Table 4-** Frequency of positive samples to a different number of assessed genes