

**ANTIBIOTIC RESISTANT GENE PREVALENCE IN PRIMARY AND SECONDARY ENDODONTIC  
INFECTIONS: A WHOLE-METAGENOME SHOTGUN BASED STUDY**

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## **DEDICATION**

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**DISCLAIMER**

The opinions and statements contained herein are those of the author(s) and do not reflect the opinions and views of the University of Minnesota School of Dentistry.

## **ABSTRACT**

**PURPOSE:** The purpose of this study was to assess the prevalence of antibiotic resistant genes in primary and secondary endodontic infections using whole-metagenome shotgun sequencing. This study was also used to determine if a difference exists between the counts per million of antibiotic resistant genes present in primary versus secondary endodontic infections.

**MATERIALS AND METHODS:** Twenty-five samples from patients with primary root canal infections, and twenty samples obtained from previously treated teeth currently diagnosed with apical periodontitis were analyzed with whole-metagenome shotgun sequencing at a depth of 15–20 M reads. Taxonomic and functional gene annotations were made using MetaPhlan3 and HUMAnN3 software. We profiled a manually curated list of antibiotic resistant genes in our metagenomic data using the KEGG BRITE database (<https://www.genome.jp/brite/ko01504>). To assess if a significant difference exists between infections belonging to primary and secondary endodontic infections, a Mann-Whitney U test non-parametric test was used. P-value was two tailed and significance was determined at  $P < 0.05$ .

**RESULTS:** Thirty-seven total samples yielded high-quality DNA, 21 and 16 samples that belonged to primary and secondary infection samples, respectively. The most prevalent antibiotic resistant genes present included the metallo-beta-lactamase family protein, MFS transporter (DHA1 family, bicyclomycin/chloramphenicol), multidrug resistance protein (MATE family), membrane fusion protein (multidrug efflux system), and multiple antibiotic resistance protein. The bacteria encoding for antibiotic resistant genes that have the highest prevalence in primary and secondary endodontic infections included *Eubacterium infirmum*, *Tannerella forsythia*, *Pseudopropionibacterium propionicum*, *Dialister pneumosintes*, *Prevotella denticola*, and *Selenomonas sputigena*. Mann-Whitney U analysis showed no significant difference between counts per million of primary and secondary endodontic infections ( $P = 0.6532$ ).

**CONCLUSIONS:** Numerous antibiotic resistant genes are prevalent and encoded for in both primary and secondary endodontic infections. No significant difference was found between the prevalence in counts per million of antibiotic resistant genes between the microbial composition in cases with primary or secondary endodontic infections.

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## **INTRODUCTION**

The first documented use of antibiotics occurred in 1910 when Salvarsan was first deployed. In the 100 years to follow, antibiotics have greatly changed modern medicine and have dramatically extended the average human lifespan by 23 years. The research and discovery regarding antibiotics peaked in the 1950s and since that time, a gradual decline in antibiotic discovery has occurred (Hutchings et al., 2019). In the wake of the decrease in development and evolution of antibiotics, a new problem has been encountered.

Antimicrobial resistance has been declared a global public health threat by the U.S. Center for Disease Control and the World Health Organization (Dantas et al., 2010). Antibiotics are essential medications but their overuse and misuse are creating resistant bacteria that are not susceptible to any antibiotics. Each year at least two million people in the U.S. become infected with multidrug resistant bacteria and 23,000 deaths have been attributed to these infections (Dellit et al., 2007; Fridkin et al., 2015). A significant issue lies in identifying which antibiotic resistant genes pose a threat to human health. Genes that are believed to confer antibiotic resistance including those predicted by sequence homology are ubiquitous among bacteria, fulfilling numerous biological roles. Only a small fraction of these genes pose a threat to human health. Thus, identifying high-risk resistance genes among thousands of presumptive antibiotic resistant genes is a critical step to tackle this global problem with cost-effective approaches (Dantas et al., 2010).

This widespread use of antibiotics can be observed in both medicine and dentistry. A common area in which antibiotics are utilized in dentistry is in the field of endodontics.

Endodontics is the branch of dentistry concerned with diseases and injuries of the dental pulp whether acute or chronic (Abbott, 2012). As a result, the use and overuse of antibiotics is often encountered when considering endodontic diagnosis and treatment. The spectrum of endodontic pathosis includes many conditions for which dentists and endodontists determine that are appropriate to prescribe antibiotics. Some of these conditions are purely inflammatory in origin, and some involve various stages of infection. This infection may be localized to the pulp and periapical tissues, or it may spread to regional lymph nodes, or systemically (Abbott, 2012).

The use of antibiotics in endodontics is primarily aimed at controlling infections and preventing complications. However, the use of antibiotics in endodontic treatment has been associated with several concerns, including antibiotic resistance, side effects, and the potential for antibiotic misuse (Segura-Egea et al., 2017). To prevent antibiotic misuse in endodontics, clinical guidelines for the appropriate use of antibiotics should be employed, and education for dental professionals and patients can be provided. Alternative approaches to the use of antibiotics, such as the use of non-antibiotic medications, can also help reduce overall use in endodontics (Bansal et al., 2019).

Despite efforts to decrease antibiotic use in endodontics with the release of position statements by the AAE (2017), antibiotics continue to be overprescribed and pose a great threat in the future regarding antibiotic resistance (Segura-Egea et al., 2017). Currently, the knowledge base on antibiotic resistance genes within primary and secondary endodontic infection is limited. However, next generation sequencing methods such as shotgun sequencing

metagenomics provides a necessary means to discover which antibiotic resistant genes are present in both primary and secondary endodontic infections.

## **REVIEW OF LITERATURE**

### **SHOTGUN SEQUENCING**

Whole-genome shotgun sequencing is a technique used to determine the complete DNA sequence of an organism's genome. This method involves randomly breaking the genome into smaller fragments, sequencing these fragments, and then using computational methods to reassemble the sequence into a contiguous whole (Venter et al., 2001). This method is in contrast to traditional sequencing methods that rely on mapping genomic information to a reference genome. The whole-genome shotgun sequencing approach was first described by DNA sequencing pioneer Craig Venter and his colleagues in 1995, and was used to sequence the first human genome in 2000. Since then, whole-genome shotgun sequencing has become the preferred method for sequencing large genomes, as it provides a rapid and cost-effective means of sequencing large amounts of DNA (Thomas et al., 2012).

Next-generation sequencing technologies have advanced the metagenomic approach of studying microbes in their natural environments without the need for isolation and cultivation of individual species. Culture-based studies are unable to provide this comprehensive view of the oral microbiota, as <1% of bacteria from some environments can currently be grown on solid culture media (Wade, 2011). Investigation of antibiotic resistant genes (ARGs) by culture-independent amplification-based methods, such as polymerase chain reaction (PCR) and DNA microarrays, is restricted by low throughput, limited availability of primers (generally targeting

known pathogens and ARGs), and amplification bias. High-throughput sequencing-based metagenomic analysis overcomes a number of these limitations (Li et al., 2015), allowing for the screening of ARGs in both culturable and nonculturable bacteria and, importantly, the detection of novel ARGs. Furthermore, next-generation sequencing is fast, robust, and cost-effective (Thomas et al., 2012). There are two metagenomic approaches to investigating antibiotic resistance: sequence-based and functional studies (Schmieder and Edwards 2012; Mullany 2014). Sequence-based metagenomics involves the extraction and random (shotgun) sequencing of DNA directly from an environment, such as the oral cavity. The short sequence reads that overlap are assembled to make longer contiguous sequences known as contigs, which are compared with reference sequences in a database (Schmieder and Edwards 2012).

This method can be used to detect and quantify ARGs in the microbiome as well as predict the function of these genes. In addition, high-throughput sequencing can, if the assembly is satisfactory, suggest which bacteria within the sampled microbiome contain which ARGs. This is possible because long contigs that contain either the whole ARG or a part of it will also contain DNA flanking the ARG, which can be used to determine the likely bacterial source due to homology with sequenced genomes in the database (Sukumar et al., 2016). To demonstrate that targeted sequences actually confer resistance, functional metagenomic studies are required.

### **PRIMARY ENDODONTIC INFECTION**

Endodontic infections are caused by bacterial invasion of the pulp tissue within a tooth, resulting in inflammation and tissue destruction. These infections can be primary, meaning that

it is the initial infection of the tooth as the pulp becomes necrotic, or secondary which is recontamination of the root canal system. The presence and absence of a microbial flora is the major determinant in the healing of exposed pulp tissue. If microbiota are left in untreated pulp exposures, pulp necrosis and apical periodontitis will result (Kakehashi et al., 1965). Furthermore, necrotic pulp tissue will not cause periradicular inflammation unless bacteria are introduced into the canal (Lin et al., 2006).

Primary endodontic infections are caused by various bacterial species and can range in number of species present. A study showed that the number of bacterial species in the samples ranged from 1 to 33 (mean 5.9). The most prevalent species found were: *Bacteroides forsythus* (29.6% of cases), *Porphyromonas gingivalis* (29.6%), *Streptococcus constellatus* (25.9%), *Prevotella intermedia* (22.2%), *Prevotella nigrescens* (22.2%), *Fusobacterium periodonticum* (18.5%), *Fusobacterium nucleatum* (18.5%), and *Eikenella corrodens* (18.5%) (Siquiera et al., 2001).

Another study identified that endodontic infections are mixed infections of polymicrobial etiology. The most prevalent species were *Bacteroides forsythus* (39.3%), *Haemophilus aphrophilus* (25%), *Corynebacterium matruchotii* (21.4%) and *Porphyromonas gingivalis* (17.9%) (Siquiera et al., 2000). These findings highlight the role of anaerobic bacteria in primary endodontic infections, as they are able to survive and thrive in the low oxygen environment of the root canal system. This study also notes that *Streptococcus sp.* and *Prevotella sp.* are among the most commonly isolated bacteria from these infections.

In addition to the species of bacteria present, there has been an association with the types of bacteria as it relates to time present in endodontic infections. The relative number of obligate anaerobes increased with time. Final root canal samples from the apical region showed a predominance of obligatory anaerobic non-sporulating bacteria; 85-98% of the bacterial cells were anaerobic. The most frequently found species were *Bacteroides* and gram positive anaerobic rods (Fabricus et al., 1982). Understanding the bacterial profile of primary endodontic infections can aid in the development of effective treatment strategies and improve clinical outcomes for patients.

### **SECONDARY ENDODONTIC INFECTION**

Secondary endodontic infections occur when bacteria are present in the root canal system after the initial endodontic treatment. There are two predominant types of secondary endodontic infections which occur. The first is reinfection which occurs when bacteria re-invade the root canal system after an incomplete or inadequate root canal treatment (Neelakantan et al., 2017). Bacteria may enter through untreated canals, missed accessory canals, or via leakage around the coronal restoration, contaminating the obturation material (Nair et al., 1999). The second type is a persistent infection which refers to the presence of bacteria or other microorganisms within the root canal system that were not completely eliminated during the initial root canal treatment, was also described by Nair et al., (1999). These residual microorganisms can multiply over time and cause persistent or recurrent symptoms. Persistent infections can arise from various sources and include intraradicular infection persisting in the complex apical root canal system, extra radicular infection generally in the form of

actinomycosis, extruded root canal filling or other exogenous materials that can cause foreign body reactions, accumulation of endogenous cholesterol crystals that irritate periapical tissues, true cystic lesions, and scar tissue healing of the lesion (Nair et al., 1999).

These infections can cause persistent symptoms and can lead to the failure of the initial endodontic treatment. An aspect in which secondary infections differ from primary infections is microbial diversity. A study by Chugal et al., (2011) showed that primary infections were significantly more diverse than secondary infections with an average of 33 compared to 16 identified species. Different roots of the same teeth with secondary infections displayed low similarity in bacterial composition whereas an equivalent sample collected from primary infection contained almost identical populations. This study also shows that many secondary infections contained bacteria not common to the oral microflora that may represent opportunistic environmental pathogens.

One of the most common bacteria involved in secondary endodontic infections is *Enterococcus faecalis*. A study published in the Journal of Endodontics by Sundqvist et al., (1998) found that in previously treated teeth, the microbial flora within the canals could be characterized as mono infections of predominantly gram positive microorganisms. *E. faecalis* was isolated from 38% of teeth that had recoverable microorganisms. 74% of cases healed after endodontic retreatment. This study also showed that infection at the time of root filling and size of the lesion were significant factors that had a negative influence on the outcome. A second study showed similar results in that the microflora of the obturated canal differs from that found normally in the untreated necrotic dental pulp, quantitatively as well as

qualitatively. One or two strains of bacteria are most commonly isolated from root-filled teeth with apical periodontitis (85%), the most common of which were *Enterococcus* (69%) (Molander et al., 1998).

### **ANTIBIOTICS IN DENTISTRY**

Antibiotics find widespread use within dental practices with approximately 10% of all prescriptions being attributed to dental infections (Thompson et al., 2022). Dentists primarily favored amoxicillin or amoxicillin-clavulanate combination as the most commonly prescribed medications (Roda et al., 2007). The dental profession's approach to antibiotic usage involves a reliance on clinical and bacteriological epidemiological factors, leading to the administration of broad-spectrum antibiotics for brief durations (Ramu & Padmanabhan 2012). This practice is further limited by a restricted selection of antibiotics. Additionally, the oral cavity presents an increasing number of bacterial strains that exhibit resistance to conventional antibiotics especially when considering their ability to form biofilms (Rath et al., 2021). According to Roda et al., (2007), dentists prescribe antibiotics for various purposes, including treating odontogenic infections, oral non-odontogenic infections, as prophylaxis against both focal and local infections, and as the prevention of their spread to adjacent tissues and organs. This study also notes situations such as pregnancy, kidney failure, and liver failure that necessitate the clinician's careful consideration when deciding upon antibiotic treatment.

When considering antibiotics for prophylactic measures, there are various guidelines to follow. According to the ADA Chairside Guide published by Sollecito et al., (2015), for patients with a history of complications associated with their joint replacement surgery who are

undergoing dental procedures that include gingival manipulation or mucosal incision, prophylactic antibiotics should only be considered after consultation with the patient and orthopedic surgeon; in cases where antibiotics are deemed necessary, it is most appropriate that the orthopedic surgeon recommend the appropriate antibiotic regimen and, when reasonable, write the prescription. For infective endocarditis prophylaxis, the American Heart Association Guidelines published by Wilson et al., (2021) support premedication for a relatively small subset of patients. This is based on a review of scientific evidence which showed that the risk of adverse reactions to antibiotics generally outweigh the benefits of prophylaxis for many patients who would have been considered eligible for prophylaxis in previous versions of the guidelines.

Another way antibiotics are used in dentistry and more specifically endodontics is through the use of topical antibiotics. A review by Segura-Egea et al., (2017) showed that topical antibiotics have been proposed and used in several endodontic treatments such as pulp capping, root canal treatment, regenerative endodontic procedures, and tooth avulsion. This review states that although several clinicians and researchers have used topical antibiotics in pulp capping there is no scientific evidence to support the use of antibiotics in pulp capping procedures. Taking into account that endodontic infections are polymicrobial, a group of broad-spectrum antibiotics that are effective against a wide range of microorganisms, have been proposed as intracanal topical antibiotics in both conventional root canal treatment as well as regenerative endodontic procedures in the form of ciprofloxacin, metronidazole, and minocycline (Diogenes et al., 2013). Topical antibiotic application on a tooth to be replanted

after avulsion is also advocated to enhance healing. Moreover, the use of topical antibiotics has been reported to be more beneficial compared with systemic antibiotics in avulsion cases (Hinckfuss & Messer, 2009).

### **MISUSE OF ANTIBIOTICS IN DENTISTRY**

The use of antibiotics in endodontic treatment is primarily aimed at controlling infections and preventing complications. Endodontic infections can be caused by a variety of bacteria, including facultative and anaerobic bacteria (Siquiera et al., 2000). Antibiotics are used in endodontics to control bacterial growth and prevent the spread of infection. However, the use of antibiotics in endodontics and dentistry as a whole has been associated with several concerns, including antibiotic resistance, side effects, and the potential for antibiotic misuse (Segura-Egea et al., 2017). Antibiotic resistance is a growing concern in dentistry, and the overuse and misuse of antibiotics are significant contributors to the development of antibiotic-resistant bacteria. To prevent antibiotic misuse in endodontics, several strategies have been employed.

A position statement from the AAE was released in 2017 in an attempt to decrease the use in antibiotics by placing guidelines on when antibiotics should be used. This document was intended to present the available evidence related to prescribing antibiotics, highlight appropriate clinical recommendations and identify gaps in knowledge for which personal judgment is the best guide for assessing risks and benefits in this practice. The drug distribution is restricted to the surrounding vascularized tissues. However, in cases of apical abscess, the presence of purulence limits vascular supply, and contains cellular debris and proteins that can

bind and sequester antibiotics making these drugs less effective in the absence of adequate drainage (Konig et al., 1998). Thus, when it comes to odontogenic etiology of pathosis, antibiotics should only be used as adjuvant therapies in cases with evidence of systemic involvement such as fever, malaise, cellulitis, or lymphadenopathy following adequate endodontic disinfection and abscess drainage if swelling is present (Mathews et al., 2003; Aminoshariae & Kulild 2016). In addition, patients who are immunocompromised or have predisposing conditions such as previous endocarditis should be medicated as a prophylactic measure. It is important to note that administration of antibiotics in the absence of the above-mentioned reasons has no evidence of therapeutic benefit. Lastly, in the cases of a therapeutic indication, the choice of the antibiotic agent, dosage and duration is typically made in an empirical fashion (Fouad et al., 1996; Walton & Chiappinelli 1993).

Despite efforts to decrease antibiotic use in endodontics with the release of position statements, antibiotics continue to be overprescribed and pose a great threat in the future regarding antibiotic resistance in the form of multiple genes (Fiore et al., 2017).

### **ANTIBIOTIC RESISTANCE MECHANISMS**

Bacteria can become resistant to antibiotics through the acquisition of one or multiple genes. The mechanisms for antibiotic resistance can involve changes to the target site of the antibiotic, alteration of the antibiotic transport system, or production of enzymes that inactivate the antibiotic (Reygaert, 2018). In addition, this study notes that the number of genes involved can vary depending on the type of resistance mechanism and the antibiotic being used. Some

resistance mechanisms can be encoded by a single gene, while others may require multiple genes working together.

When determining antibiotic resistance, the different mechanisms must be reviewed. Within this context, there are two principal types of resistance: natural, which can be further categorized into intrinsic or induced, and acquired which involves gene transfer and mutation (Reygaert, 2018). The natural intrinsic resistance phenotype is when bacterial species are naturally resistant to certain classes of antibiotics through mechanisms which can be conferred by genes themselves or efflux pumps (Sandner-Miranda et al., 2018). Natural resistance in bacteria can also be induced by the activation of genes as a result of exposure to clinical amounts of antibiotics (Ben et al., 2019). The acquired resistance can occur through two distinct processes: by a mutation that occurs in the DNA of the cell during the replication process or by horizontal gene transfer which involve mechanisms of transformation, transduction, and conjugation (Mancuso et al., 2021).

There are several types of gene mutations that can occur in bacteria, with some mutations leading to antibiotic resistance. The most common types of mutations include point mutations, frameshift mutations, insertion and deletion mutations, and gene duplication. Regardless of the type of mutation that occurs within bacteria, mutant strains are capable of transferring the mutation to the progeny via the vertical pathway which may ultimately lead to antibiotic resistance (Reygaert, 2018; Friedrich, 2019).

Horizontal gene transfer (HGT) is a process of genetic exchange between organisms that occur outside of the typical parent-to-offspring inheritance pattern. In other words, it is the

transfer of genetic material between different organisms that are not related by descent. This can happen through three main mechanisms: transformation, transduction, and conjugation (Smillie et al., 2011).

Transformation is the uptake of exogenous DNA from the environment and has been reported in bacteria (Johnston et al., 2014; Chimileski et al., 2014). Transduction is the delivery of genetic material through phage predation owing to the integration of exogenous host genetic material into a phage genome. This phenomenon has been observed in both bacteria and archaea (Chiang et al., 2019). There are two types of transduction: generalized, in which a random piece of the host DNA is incorporated during cell lysis; and specialized, in which a prophage imprecisely excises itself from a host genome and incorporates some of the flanking host DNA (Soucy et al., 2015). Conjugation is restricted to bacterial cells as the donor and recipient (Norman et al., 2009; Kyndt, 2015). The majority of antibiotic resistance in human commensals and pathogens is acquired through HGT. In fact, it has been demonstrated that in some circumstances, the presence of low levels of antimicrobials in the environment is a key signal that promotes HGT of ARGs (Berendonk et al., 2015). The above mechanisms can be deployed in combinations and contribute to resisting various antibiotics which are used in dentistry and medicine.

### **AMINOGLYCOSIDE MECHANISM AND RESISTANCE**

Aminoglycosides (AGs) are broad-spectrum antibiotics effective against both gram negative and some gram positive bacteria (Krause et al., 2016). AGs have long been known to exert their antibacterial action by binding to the bacterial ribosome and interfering with bacterial protein

translation according to Ramirez et al., (2013). The study notes that AGs are used as potential treatments for fungal infections, parasitic infections, and for genetic diseases arising from premature termination of codons. Furthermore, the main factor which contributes to aminoglycoside resistance revolves around the enzymatic modification of aminoglycosides. Enzymatic modification is the most common type of aminoglycoside resistance (Ramirez et al., 2013). Acetyltransferases, adenylyltransferases and phosphotransferases are three classes of enzymes that play a critical role in the resistance to aminoglycosides. The genes encoding for aminoglycoside modifying enzymes can be transferred through plasmids and transposons (Chen et al., 2021).

### **MACROLIDE MECHANISM AND RESISTANCE**

Macrolide antibiotics belong to the protein synthesis inhibitors and are divided into 12, 14, 15, 16, and 18 membered ring groups based on the structures of the atoms in the lactone ring and show antimicrobial activity against both gram positive and some gram negative bacteria (Katz et al., 2009; Arsic et al., 2018). This study notes that 14 membered and 16 membered ring macrolide antibiotics are the main contributors to effective antibiotic medications but consequently give rise to resistant mutants. Macrolide antibiotics work by binding to the ribosomal components and prevent protein biosynthesis. However, the binding mode is controlled discreetly in the molecular-species-specific manner (Wilson, 2009; Vasquez-Laslop & Mankin, 2018). Mechanisms of macrolide resistance primarily arise from inducible macrolide resistant bacteria. As a result, overuse of antibiotics contributes to an increase in the numbers

of resistance genes (Nakajima, 1999). Examples of different genes that indicate macrolide resistance include *ermA*, *ermB*, *ermC*, *ermE*, and *ermV* (Moraes et al., 2015).

### **BETA-LACTAM MECHANISM AND RESISTANCE**

Beta-lactam group antibiotics including semi-synthetic penicillins and cephalosporins are the most commonly used antibiotics in the clinic for the treatment of gram positive as well as gram negative bacterial infections, and they have been used for almost a century. They are classified into five groups according to their chemical structures: penicillins, cephalosporins/cephamycins, clavulanic acid, thienamycin, nocardicin A and sulfazecin (Ogawara, 2019). Beta-lactam resistance derives from the production of enzymes that degrade beta-lactam antibiotics in the form of beta-lactamases. The beta-lactamases (originally called penicillinases and cephalosporinases) inactivate beta-lactam drugs by hydrolyzing a specific site in the beta-lactam ring structure, causing the ring to open. The open-ring drugs are not able to bind to their target proteins (Reygaert, 2018). Resistance to the beta-lactam drugs occurs through three general mechanisms: (1) preventing the interaction between the target and the drug, usually by modifying the ability of the drug to bind to the penicillin binding protein, (2) the presence of efflux pumps that can extrude beta-lactam drugs; (3) hydrolysis of the drug by beta-lactamase enzymes (Pfeifer et al., 2010; Bush & Bradford, 2016). Different genes that have been shown to cause resistance are as follows: *blaTEM*, *blaZ*, *cfxA*, *cfxA2*, *blaCMY2*, *ampC*, *mecA*, *pbp1A*, *pbp2X*, and *pbp2B* (Moraes et al., 2015).

## **TETRACYCLINE MECHANISM AND RESISTANCE**

Tetracyclines have been used for the treatment of a wide variety of gram positive and gram negative bacterial infections and for animal feeds and aquaculture since the 1940s (Chopra & Roberts, 2001). Now, third and fourth generation compounds have rejuvenated clinical prospects for this drug class. Tetracyclines inhibit bacterial protein biosynthesis by binding to the 16S rRNA, preventing the delivery of tRNA to the A-site (Wilson, 2009; Chopra & Roberts, 2001). As for the resistance to tetracyclines in pathogenic bacteria, at least four mechanisms have been reported, that is, binding site mutations, ribosomal protection proteins, efflux pumps, and enzymatic inactivation (Thacker et al., 2010; Nguyen et al., 2014). Different genes that have been shown to contribute to tetracycline resistance are *tetA*, *tetB*, *tetC*, *tetD*, *tetK*, *tetL*, *tetM*, *tetO*, *tetQ*, *tetS*, *tetW*, and *tet32* (Moraes et al., 2015).

## **VANCOMYCIN MECHANISM AND RESISTANCE**

Glycopeptide and lipoglycopeptide antibiotics such as vancomycin and teicoplanin show antibacterial activity against gram positive bacteria through binding to the D-alanyl-D-alanine terminus of the lipid II bacterial cell wall precursor and sequestering the lipid II substrate, resulting in the inhibition of peptidoglycan biosynthesis (Ogawara, 2019). Natural resistance to the antibiotic can occur in which the vancomycin molecule is simply too large to pass through the porin channels in the outer membrane of the cell wall, thus rendering the antibiotic ineffective, resulting from a phenotypic change to the bacteria (Chen et al., 2009). Genes that have been shown to contribute to vancomycin resistance are *vanA*, *vanB*, *vanC1*, *vanC2/3*, *vanD*, and *vanE* (Moraes et al., 2015).

## **TRANSPORT SYSTEMS**

Bacteria possess chromosomally encoded genes for efflux pumps. Some are expressed constitutively, and others are induced or overexpressed under certain environmental stimuli or when a suitable substrate is present. The efflux pumps function primarily to rid the bacterial cell of toxic substances, and many of these pumps will transport a large variety of compounds and are categorized as multi-drug efflux pumps (Reygaert, 2018). The resistance capability of many of these pumps is influenced by what carbon source is available (Blair et al., 2014; Villagara et al., 2012). Most bacteria possess many different types of efflux pumps.

An overview published by Raygaert (2018) noted that there are five main families of efflux pumps in bacteria classified based on structure and energy source: the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division (RND) family. The overview also notes that most of these efflux pump families are single-component pumps which transport substrates across the cytoplasmic membrane. The RND family are multi-component pumps (found almost exclusively in gram negative bacteria) that function in association with a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP-porin) to efflux substrate across the entire cell envelope (Kumar & Schweizer, 2005; Piddock, 2006; Poole, 2007). There are also instances where other efflux family members act with other cellular components as multicomponent pumps in gram negative bacteria (Raygaert, 2018).

## **BIOFILM FORMATION**

A study by Hall-Stoodley (2004) showed that biofilms provide bacterial increased survivability through three dimensional structure, exchange of metabolites, communication, and genetic exchange. The biofilm itself is a heterogeneous matrix-enclosed microcolony interspersed with open water channels divided into four stages of transient surface association: robust adhesions, aggregation of cells into microcolonies, and subsequent growth and maturation. The study further states that biofilm formation does seem to have an indirect relationship with ARGs as their formation poses three different mechanisms that can confer resistances against antibacterial agents and are as follows: barrier properties of slime matrix that dilutes antimicrobials, the physiological state of biofilms which creates dormant zones that are resistant to antimicrobial agents, and the creation of subpopulations of resistant phenotypes (persisters).

## **SPECIFIC AIMS**

The purpose of this study was to assess the prevalence of antibiotic resistant genes in primary and secondary endodontic infections using whole-metagenome shotgun sequencing. This study was also used to determine if there was a difference between the counts per million of antibiotic resistant genes present in primary versus secondary endodontic infections.

## **MATERIALS AND METHODS**

This study was a secondary bioinformatics analysis of “The root canal microbiome diversity and function. A whole-metagenome shotgun analysis” by Ordinola-Zapata et al., (2023). The study

protocol was approved by the institutional board of the University of Minnesota (IRB #00011937, 143115).

### **PATIENT SELECTION**

Forty-five patients, 25 patients with primary infection and 20 patients with secondary infection, were included in the study. Inclusion criteria included the following: Eighteen years of age or older, tooth diagnosis of pulp necrosis and apical periodontitis by evaluation of radiograph and cold testing. Pathosis of primary and secondary endodontic infections were included in the study and all teeth were deemed to have a favorable restorative prognosis. Criteria which excluded patients from the study included patients who were pregnant at the time of sample collection, individuals under the age of 18 years old, teeth which had an unfavorable restorative prognosis, patients with periodontal disease (Stage III and IV) according to the Community Periodontal Index for Treatment Needs, combined endo-perio lesions, smokers, and inability of the patient to sign a written consent form. Preoperative periapical radiographs were obtained and interpreted using CareStream RVG6200 digital sensors and CareStream imaging software.

### **SAMPLING PROCEDURE**

Teeth with primary endodontic infections selected for sampling were first isolated with a non-latex rubber dam. Following caries excavation, the tooth and rubber dam field was scrubbed with sterile cotton saturated with 3% hydrogen peroxide for 1 minute. Then the tooth and rubber dam field were scrubbed with a sterile cotton pellet saturated with 5.25% sodium hypochlorite. Just before access into the pulp chamber, the sodium hypochlorite was inactivated by scrubbing the surfaces with sterile cotton saturated with 10% sodium thiosulfate.

The previous measures were used in an attempt to obtain isolation of root canal samples and to avoid false positives. Samples of the tooth surface were taken and processed for DNA extraction and qPCR analysis using universal primers to discard contamination. After access into the pulp chamber, the root canal space was sampled by instrumenting to the middle to apical third of the root canal using a sterile Vortex Blue 25.04 rotary file in a sterile handpiece approximately 1-2 mm short of working length. The rotary file was then removed from the handpiece using sterile instruments and placed in a sterile Eppendorf tube containing 1mL of 10 mM tris-HCl. The Eppendorf tubes with root canal samples were placed in a -80C freezer until processing. For ease of sample collection, the largest canal was sampled in molars, specifically the palatal root of maxillary molars and distal root of mandibular molars.

Teeth with secondary endodontic infections selected for sampling were scheduled for endodontic surgery. Following anesthesia, surgical flap was incised using a 15c scalpel blade and reflected. After osteotomy was performed to gain access to the periapical lesions and root apices, the root apices were resected. Root apex sample was delivered using sterile tissue forceps and placed in a sterile Eppendorf tube with 1mL of 10 mM tris-HCl. The Eppendorf tubes with root apex samples were placed in a -80C freezer until processing. Prior to DNA extraction procedure, Root apices were pulverized via cryogenic grinding in a DNA-free glass mortar in liquid nitrogen before the DNA extraction procedure.

### **DNA EXTRACTION AND SEQUENCING ANALYSIS**

Microbial composition was characterized using methods routinely employed in the University of Minnesota Genomics Center (UMGC). Shotgun sequencing was done to determine the

distributions of bacterial species and functional genes. DNA was initially extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen). For this project, DNA quality was measured by absorbance. Samples with low quality (determined as 260/230 nm absorbance ratio <1.5 using Nanodrop and a total concentration <0.4 ng/μL) were subjected to AMPure XP bead cleanup (Beckman Coulter, Inc.) following the manufacturer's instructions. Libraries were made using ¼ Nextera XT reactions (Hillman et al., 2018) and sequencing was done on the NovaSeq with a target of 15–20 M reads per sample. Taxonomic and functional annotations were made using MetaPhlan3 and HUMAnN3 (Beghini et al., 2021), after removal of host DNA using kneaddata, with normalization to copies per million (cpm). Gene annotations were made against KEGG, and UniRef databases. Raw data were deposited in the Sequence Read Archive.

### **STATISTICAL ANALYSIS**

Abundances of predominant taxa and functional genes were analyzed to confirm findings from two different populations (primary vs. secondary infection). Functional genes included protein families classified to tier 1 KEGG orthology (KO) categories of metabolism, genetic and environmental information processing and signaling and cellular processes (Kanehisa et al., 2017). We profiled a manually curated list of antibiotic resistant genes in the metagenomic data using the KEGG BRITE database (<https://www.genome.jp/brite/ko01504>). To assess if a significant difference exists between infections belonging to primary and secondary endodontic infections, a Mann-Whitney U test non-parametric test was used to compare the abundance of antibiotic resistant genes in counts per million (cpm). P-value was two tailed and significance was determined at  $P < 0.05$ .

## **RESULTS**

Thirty-seven total samples yielded high-quality DNA, 21 and 16 samples that belonged to primary and secondary infection samples, respectively. Eight samples were excluded from the final analysis because of low quality DNA or contamination of the samples.

The total number of antibiotic resistant genes detected in the samples were twenty-eight. Prevalence of the twenty-eight genes per patient was calculated as a percentage in nonsurgical and surgical cases (N=37). The 5 most prevalent antibiotic resistant genes present included metallo-beta-lactamase family protein at 54.0%, MFS transporter (DHA1 family, bicyclomycin/chloramphenicol) at 45.9%, multidrug resistance protein (MATE family) at 40.5%, membrane fusion protein (multidrug efflux system) at 40.5%, and multiple antibiotic resistance protein at 37.8%. The complete list of genes with their respective prevalence are included in **Table 2**. Prevalence and abundance ( $\log_2$ ) of the above antibiotic resistant genes as the genes relate to each patient in the non-surgical and surgical root canal treatments can be viewed in **Figure 2**.

The total number of traceable bacteria presenting ARGs was thirty-five respective species and can be seen in **Figure 1**. The **Figure 1** heatmap also documents the number of ARGs present in each bacteria in which an ARG was present representing the ability of bacteria to have multiple genes. The bacteria encoding for antibiotic resistant genes that have the highest prevalence in primary and secondary endodontic infections included *Eubacterium infirmum* at 43.24%, *Tannerella forsythia* 21.62%, *Psuedopropionibacterium propionicum* 18.92%, *Dialister*

*pneumosintes* 16.22, *Prevotella denticola* 13.51%, and *Selenomonas sputigena* at 10.81%. The additional bacteria and their prevalence are included in **Table 3**.

When comparing the counts per million (cpm) between primary endodontic infections and secondary endodontic infections with regard to antibiotic resistant genes the median value for primary infections was 0.6313 (N=21) and the median value for secondary infections was 0.5131 (N=16). Mann-Whitney U analysis showed no significant difference between counts per million of primary and secondary endodontic infections (P = 0.6532).

## **DISCUSSION**

The present study shows that the most prevalent bacteria detected encoding for ARGs are anaerobic bacteria. Although the role of antibiotic resistance is difficult to demonstrate in mixed infections, there have been multiple studies isolating anaerobic bacteria showing their role in medical complications (Dubreuil et al., 2021). One instance is a study by Salonen et al., (1998) in which a single anaerobic strain was isolated from blood culture and was shown to be the pathogen involved in systemic bacteremia. A similar study on anaerobic bacteremia was done by Kim et al., (2016) which looked at blood cultures from 70 patients. The research concluded that the survival rate of anaerobic bacteremia was significantly worse in patients who received inappropriate therapy compared with those who underwent correct therapy based on antibiotic resistance determination. This research shows that *Eubacterium infirmum*, *Tannerella forsythia*, *Pseudopropionibacterium propionicum*, *Dialister pneumosintes*, *Prevotella denticola*, and *Selenomonas sputigena* are of high prevalence and all contribute to the anaerobic microbiome of primary and secondary endodontic infection.

The ARG with the highest prevalence was the metallo-beta-lactamase family protein (K07576). In recent years, metallo-beta-lactamases have received considerable attention because these particular genes have the potential to inactivate most of the beta-lactam antibiotics including carbapenems (Parimelzaghan et al., 2016). This is of great concern as carbapenems are a very effective antibiotic agent commonly used for the treatment of severe bacterial infections. In addition, carbapenems are usually reserved for known or suspected multidrug-resistant bacterial infections. Increase in dissemination of metallo-beta-lactamase encoding antibiotic resistance genes in pathogenic bacteria often results in unsuccessful treatments (Parimelzaghan et al., 2016). In addition, due to the structural and functional diversity of metallo-beta-lactamase family proteins, the understanding of their catalytic mechanism has been limited, therefore thwarting advancements to better understand the gene. With limited understanding, this further increases the virulence of bacteria which successfully express the gene (Crowder et al., 2006). There are no clinically approved inhibitors of this protein, making them a serious threat to human health (King & Strynadka, 2013). Therefore, given the virulence potential, this current research hopes to make strides to further understand the gene in an attempt to target it in the future. This current research shows that bacteria with a high prevalence in primary and secondary endodontic infections which code for metallo-beta-lactamase family proteins (K07576) are *Eubacterium infirmum*, *Desulfobulbus oralis*, and *Selenomonas sputigena*.

Genes encoding for MFS transport systems have a high prevalence in the current study presenting in 45.9% of primary and secondary endodontic infections. MFS transport systems

can aid as a crucial mechanism for bacteria to express antibiotic resistance. A study by De Rossi et al., (2002) shows that MFS transporters are an important aspect of the mechanism of *Mycobacterium tuberculosis* in pumping antibiotics from the cell inferring antibiotic resistance. Given the potentially life threatening infection that *M. tuberculosis* can present, targeting genes coding for MFS transport systems such as K07552 can aid in diminishing the deleterious effects the transport system can pose. Another study showed the pathogenic potential of MFS transport systems in relation to *S. aureus* and the contribution to forming methicillin-resistant *S. aureus* (MRSA) (Stephen et al., 2023). Due to the repertoire of mechanisms *S. aureus* has to fight against antimicrobial agents, MFS transport systems add another layer of resistance complexity which needs to be further explored to limit the spread of potentially life threatening diseases such as MRSA. Based off the present data, *P. propionicum*, *M. immunigenum*, *D. oralis*, *C. metallidurans*, *C. curvus*, and *A. acidipropionici* are among bacteria which encode for major facilitator superfamily transport systems.

Efflux pumps also comprise a great prevalence in the bacterial strains of primary and secondary endodontic infections, in particular the membrane fusion protein, multidrug efflux system. Efflux pumps have been documented as antibiotic resistant mechanisms in *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Salmonella typhimurium* infections (Webber & Piddock, 2003). The study also notes that overexpression of efflux pumps can form mutations which can ultimately lead to resistance of more than one class of antibiotics. In addition to efflux pumps leading its mechanism of action to actively pumping antibiotics, the pumps have also been reported as one of the mechanisms responsible for the antimicrobial

resistance in biofilm structures (Webber & Piddock, 2003). This has been found in multiple microorganisms including *Escherichia coli* and *Pseudomonas*, as well as fungal infections of *Candida albicans* (Webber & Piddock, 2003; Ramage et al., 2002). This research shows that *Dialister pneumosintes* and *Prevotella denticola* have the potential to encode for this gene in primary and secondary endodontic infections.

The multi drug resistant protein MATE family comprised the same prevalence as the membrane fusion protein, multidrug efflux system (40.5%). MATE family proteins have been identified as being associated with *Staphylococcus aureus* infections as also shown in MFS transport systems. Just as with efflux pumps, the overexpression of MATE family proteins has led to mutations encoding for resistance to fluoroquinolone, norfloxacin, and ciprofloxacin for *S. aureus* (Kaatz et al., 2005). The ability of *S. aureus* to use the multi drug resistant protein MATE is one reason for its success as a virulent pathogen that is currently the most common cause of infections in hospitalized patients and is able to involve any organ system (Archer, 1998). In the present study, all bacteria coding for the drug resistant protein MATE family had a relatively low prevalence suggesting that MATE is not a main mechanism of potential resistance in bacteria of endodontic origin. *Streptococcus sanguinis*, *Porphyromonas gingivalis*, *Prevotella denticola*, *Prevotella enoeca*, and *Desulfobulbus oralis* are examples of bacteria from this study which encode for multi drug resistant protein MATE family.

Although *Eubacertium infirmum* has a great prevalence based on the present data, literature is quite limited with respect to antibiotic resistance or different potential human diseases this bacteria may cause. Further research is required to gain a better understanding of

the potential mechanisms that are expressed by *Eubacertium infirmum* and disease processes that the bacteria may utilize when it comes to resisting antibiotics and propagating human disease. Given the high prevalence in endodontic pathosis, the research can give insight to processes that can lead to potentially persistent disease.

*Tannerella forsythia* is a gram negative oral pathogen strongly associated with periodontitis. In this study, *T. forsythia* encodes for the multiple antibiotic resistant protein (K05595). Based on the KEGG database, the multiple antibiotic resistant protein is categorized as marC. However, research has shown that although marC was thought to show antibiotic resistance, there is no involvement of such activity within the gene (McDermott et al., 2008). The function of the protein family is unclear at this time. The study further states that MarC should no longer be classified as a multiple antibiotic resistance protein but only after a function is found (McDermott et al., 2008). As there is a high prevalence of *Tannerella forsythia* encoding for K05595, it would appear that encoding for this gene might not contribute to the overall virulence of the pathogen in the form of antibiotic resistance within the present study. Although *T. forsythia* may not code for antibiotic resistance directly, studies have shown that there is synergy between the bacteria and *Fusobacterium nucleatum* in biofilm formation (Sharma et al., 2005). Due to biofilm formation, *T. forsythia* still poses resistance given that biofilms can pose barrier properties of slime matrix that dilutes antimicrobials, physiological state of biofilms can create dormant zones that are resistant to antimicrobial agents, and the creation of subpopulations of resistant phenotypes in biofilms which can aid in the pathogens virulence and resulting human disease (Hall-Stoodley, 2004).

In the present study, *Pseudopropionibacterium propionicum* has a high prevalence when encoding for MFS transport systems in cases of primary and secondary endodontic infections. Given that the bacteria codes for a transport system which can interact with a multitude of antibiotics including macrolides, beta-lactams, and tetracycline, there is potential for *P. propionicum* to express antibiotic resistance (Suzuki et al., 2019). This should be considered as *P. propionicum* is able to cause actinomycosis-like disease and has been associated with lacrimal canaliculitis, osteomyelitis, endodontic infections, chronic mastoiditis, cervico-facial actinomycosis. In addition, the bacteria has been found in abscesses of the lung, brain, and kidneys (Suzuki et al., 2019). In the case of endodontic infections, *P. propionicum* has a strong association with extraradicular infections which leads to a higher probability of secondary endodontic infection (Grgurević et al., 2017). Recent research has provided more valuable insights into the significance of *Pseudopropionibacterium* species in human disease. For instance, there have been links to oropharyngeal microbiota alteration in patients with COVID-19. In these patients, there has been an enrichment of opportunistic pathogens such as *Pseudopropionibacterium* species (Ma et al., 2021). Given that *Pseudopropionibacterium propionicum* has the potential to encode for ARGs in the form of MFS transport systems, it is important to better understand the mechanism as *Pseudopropionibacterium* has the potential for serious health complications as shown in any of the human diseases listed above.

*Dialister pneumosintes* was first isolated from a patient with influenza virus infection (Olitsky et al., 1921). Although this bacteria is native to the oral cavity, it has the potential to cause severe infections such as peritonsillar and retropharyngeal abscesses which can lead to

Lemierre Syndrome (Hirai et al., 2022). According to Rousée et al., (2002) *D. pneumosintes* has also been associated with the formation of human brain abscesses suspected to be of nasopharyngeal or dental origin and adds that *Dialister pneumosintes* contained in a mixed flora can behave as a clinically and medically important pathogen. Although the study by Hirai et al., (2022) notes that *Dialister pneumosintes* is susceptible to multiple antibiotics including metronidazole, ampicillin, amoxicillin, cefmetazole, piperacillin, imipenem, meropenem, clindamycin, and moxifloxacin, the present study shows that there is a high prevalence of this bacteria encoding for membrane fusion protein, multidrug efflux system (K03585). As stated previously, efflux systems lower the intracellular antibiotic concentration allowing bacteria to survive at higher antibiotic concentrations. Overexpression of some efflux pumps can cause clinically relevant levels of antibiotic resistance in gram-negative pathogens (Blair et al., 2014). As efflux pumps are not all specific to certain types of antibiotics, the expression of the gene K03585 in *Dialister pneumosintes* may give the potential for this organism to be resistant to a multitude of the above mentioned antibiotics. This could lead to inability to treat more serious infections such as peritonsillar and retropharyngeal abscesses which could potentially lead to brain abscess and ultimately death (Rousée et al., 2002).

Oral *Prevotella* are known as anaerobic commensals on oral mucosa and in dental plaques from early life onwards, including pigmented *P. melaninogenica*, *P. nigrescens*, *P. pallens*, and non-pigmented *Prevotella* species (Könönen et al., 2022). Many *Prevotella* species contribute to oral inflammatory processes, being frequent findings in dysbiotic biofilms of periodontal diseases (Könönen et al., 2022). In the current study, *P. denticola* was found to

have a high prevalence of encoding for membrane fusion protein, multidrug efflux system (K03585) similar to *Dialister pneumosintes*. Implications for this can be seen in the medical community as *P. denticola* has been shown to cause necrotizing fasciitis (Ling and Hirase, 2022). Although *Prevotella* species are often implicated in periodontal and dental disease, they can be associated with soft tissue infections as well as other systemic complications such as cerebral abscess as well as endocarditis (Ling and Hirase, 2022). Furthermore, *Prevotella* species have been linked and isolated in patients with cystic fibrosis (Sherrard et al., 2013). In the present data, *Prevotella denticola* encodes for the membrane fusion protein (K03585) and for the multidrug resistant protein MATE family (K03327). Other *Prevotella* species in the current study including *P. dentalis*, *P. Enoeca*, and *P. intermedia* encode for additional genes of MFS transporter, beta-lactamase induction signal transducer (K018218) and ribosomal protection tetracycline resistance protein (K18220) giving the *Prevotella* species a potentially vast arsenal of antibiotic resistance if these genes are expressed and active.

*Selenomonas sputigena* is a gram negative bacteria that can be found in the oral cavity as well as the upper respiratory tract of humans. This bacteria has been shown to have the ability to form biofilms which forms a synergistic relationship with *S. mutans* in the formation of carious lesions (Nagal et al., 2016). Furthermore, this study shows *Selenomonas sputigena* has been noted in the formation of subgingival biofilms in chronic and aggressive periodontitis. Along with biofilm formation acting as a mechanism for antibiotic resistance, the present data shows that *Selenomonas sputigena* has a high prevalence of encoding for metallo-

beta-lactamase. This may allow for *Selenomonas sputigena* to express resistance to various beta-lactam antibiotics including carbapenem.

As this research was a screening study with shotgun genome sequencing, whether the genes are active during the disease process of primary and secondary endodontic infections is yet to be determined. In the instance of determining which genes are pathogenic, the gold standard remains to be culture studies which can determine if genes are active and contributing to the overall pathosis of a given bacteria as culturing can isolate microbial flora for antibiotic sensitivity and resistance profiles in cases of persistent infection (Yamane, 1998).

In the present study, it has been shown that numerous antibiotic resistant genes are prevalent in both primary and secondary endodontic infections. Future studies with greater sample sizes need to more closely examine the specific bacteria and how they relate to the ARGs that are present. This current research has detected which genes are encoded for, but further research needs to be done with RNA sequencing from prevalent bacteria in the form of transcriptomics to determine which antibiotic resistant genes are expressed by bacteria in primary and secondary endodontic infections. From there, proteomics can determine the enzymes and mechanisms which these genes work through for the ultimate goal of clinical confirmation of antibiotic resistance in bacteria of primary and secondary endodontic infections. Specifically, ones which are known to cause human disease and are capable of causing serious life threatening infections. In addition, further research can be conducted to determine the unclassified bacteria present in each different category of antibiotic resistant gene groups. Furthermore, by studying the genes which are expressed in primary and

secondary endodontic infections, targeted treatment could be used to limit or eliminate these infections before the requirement of antibiotics is needed which could be deemed ineffective due to the resistance which is being expressed.

## **CONCLUSION**

The five most prevalent antibiotic resistant genes in the present study included metallo-beta-lactamase family protein, MFS transporter (DHA1 family, bicyclomycin/chloramphenicol), multidrug resistance protein (MATE family), membrane fusion protein (multidrug efflux system), and multiple antibiotic resistance protein. The five most prevalent bacteria encoding for antibiotic resistant genes in the present study were *Eubacterium infirmum*, *Tannerella forsythia*, *Psuedopropionibacterium propionicum*, *Dialister pneumosintes*, *Prevotella denticola*, and *Selenomonas sputigena*. No significant difference was found between the prevalence in counts per million of antibiotic resistant genes between the microbial composition in cases with primary or secondary endodontic infections.

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## **APPENDIX**

**Table 1.** Detected antibiotic resistant genes categorized by type of resistance.

<b>Antibiotic Resistant Genes</b>
<u>Aminoglycoside Resistant Genes</u>
K00662: aminoglycoside N3'-acetyltransferase
K00897: aminoglycoside 3'-phosphotransferase
<u>Macrolide Resistant Genes</u>
K08217: MFS transporter, DHA3 family, macrolide efflux protein
K19350: lincosamide and streptogramin A transport system ATP-binding/permease protein
K18230: macrolide transport system ATP-binding/permease protein
K05685: macrolide transport system ATP-binding/permease protein [EC:3.6.3.-]
<u>Beta-Lactam Resistant Genes</u>
K17837: metallo-beta-lactamase class B [EC:3.5.2.6]
K01467: beta-lactamase class C [EC:3.5.2.6]
K08218: MFS transporter, PAT family, beta-lactamase induction signal transducer AmpG
K17836: beta-lactamase class A [EC:3.5.2.6]
K03585: membrane fusion protein, multidrug efflux system
K07576: metallo-beta-lactamase family protein
<u>Tetracycline Resistant Genes</u>
K18217: ATP-binding cassette, subfamily B, tetracycline resistant protein
K08151: MFS transporter, DHA1 family, tetracycline resistance protein
K18220: ribosomal protection tetracycline resistance protein
K08168: MFS transporter, DHA2 family, metal-tetracycline-proton antiporter
<u>Vancomycin Resistant Gene</u>
K18346: vancomycin resistance protein VanW
<u>Fosmidomycin Resistant Gene</u>
K08223: MFS transporter, FSR family, fosmidomycin resistance protein
<u>Transport Systems</u>
K16905: fluoroquinolone transport system permease protein
K19309: bacitracin transport system ATP-binding protein
K07788: multidrug efflux pump
K03297: small multidrug resistance protein, SMR family unclassified
K03446: MFS transporter, DHA2 family, multidrug resistance protein B
K18139: outer membrane protein, multidrug efflux system
K18138: multidrug efflux pump
K05595: multiple antibiotic resistance protein
K07552: MFS transporter, DHA1 family, bicyclomycin/chloramphenicol
K03327: multidrug resistance protein, MATE family

**Table 2.** Prevalence of the twenty-eight genes in primary (nonsurgical) and secondary (surgical) endodontic infections (N=37). Prevalence was calculated as a percentage.

Antibiotic Resistant Genes	% Prevalence
K00662: aminoglycoside N3'-acetyltransferase	8.108108108
K00897: aminoglycoside 3'-phosphotransferase	5.405405405
K01467: beta-lactamase class C [EC:3.5.2.6]	13.51351351
K03585: membrane fusion protein, multidrug efflux system	40.54054054
K05595: multiple antibiotic resistance protein	37.83783784
K05685: macrolide transport system ATP-binding/permease protein [EC:3.6.3.-]	27.02702703
K07576: metallo-beta-lactamase family protein	54.05405405
K07788: multidrug efflux pump	5.405405405
K08217: MFS transporter, DHA3 family, macrolide efflux protein	5.405405405
K08218: MFS transporter, PAT family, beta-lactamase induction signal transducer AmpG	10.81081081
K16905: fluoroquinolone transport system permease protein	13.51351351
K18138: multidrug efflux pump	32.43243243
K18139: outer membrane protein, multidrug efflux system	13.51351351
K18230: macrolide transport system ATP-binding/permease protein	13.51351351
K18346: vancomycin resistance protein VanW	8.108108108
K19350: lincosamide and streptogramin A transport system ATP-binding/permease protein	16.21621622
K19309: bacitracin transport system ATP-binding protein	13.51351351
K18220: ribosomal protection tetracycline resistance protein	29.72972973
K17836: beta-lactamase class A [EC:3.5.2.6]	21.62162162
K17837: metallo-beta-lactamase class B [EC:3.5.2.6]	2.702702703
K08223: MFS transporter, FSR family, fosmidomycin resistance protein	29.72972973
K03446: MFS transporter, DHA2 family, multidrug resistance protein B	8.108108108
K07552: MFS transporter, DHA1 family, bicyclomycin/chloramphenicol	45.94594595
K03297: small multidrug resistance protein, SMR family unclassified	2.702702703
K03327: multidrug resistance protein, MATE family	40.54054054
K08151: MFS transporter, DHA1 family, tetracycline resistance protein	2.702702703
K08168: MFS transporter, DHA2 family, metal-tetracycline-proton antiporter	10.81081081
K18217: ATP-binding cassette, subfamily B, tetracycline resistant protein	2.702702703

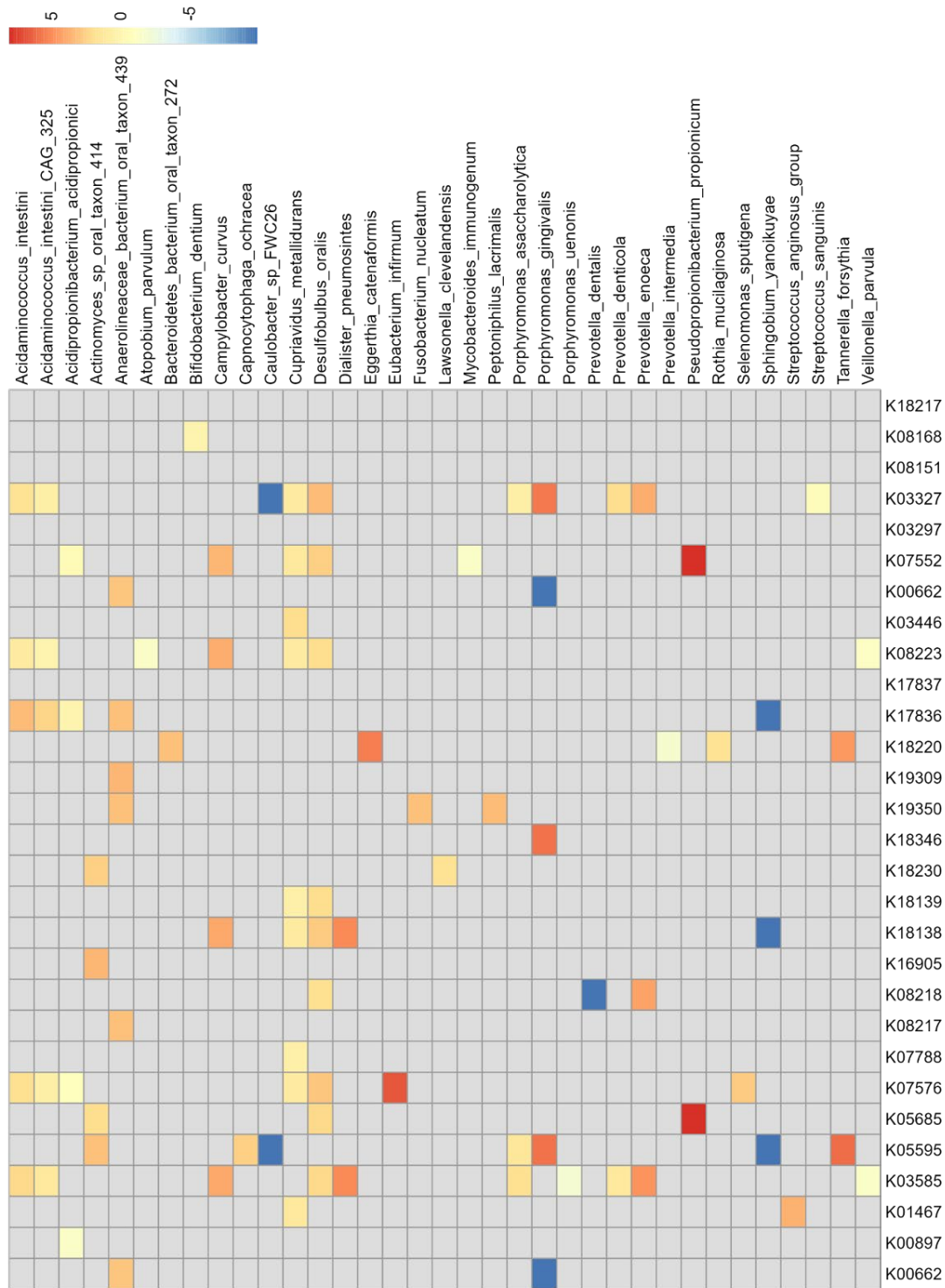
**Table 3.** Prevalence of bacteria encoding for antibiotic resistant genes in the 37 samples obtained from primary and secondary endodontic infections.

<b>Bacteria Encoding for Antibiotic Resistant Genes</b>	<b>% Prevalence</b>
K07576: metallo-beta-lactamase family protein (resist. Carbapemems)	54.05405405
K07576: <i>Acidaminococcus intestini</i>	2.702702703
K07576: <i>Acidaminococcus intestini</i> CAG 325	2.702702703
K07576: <i>Acidipropionibacterium acidipropionici</i>	2.702702703
K07576: <i>Eubacterium infirmum</i>	43.24324324
K07576: <i>Cupriavidus metallidurans</i>	2.702702703
K07576: <i>Desulfobulbus oralis</i>	8.108108108
K07576: <i>Selenomonas sputigena</i>	10.81081081
K07552: MFS transporter, DHA1 family, bicyclomycin/chloramphenicol	45.94594595
K07552: <i>Acidipropionibacterium acidipropionici</i>	2.702702703
K07552: <i>Campylobacter curvus</i>	5.405405405
K07552: <i>Cupriavidus metallidurans</i>	2.702702703
K07552: <i>Desulfobulbus oralis</i>	8.108108108
K07552: <i>Mycobacteroides immunogenum</i>	2.702702703
K07552: <i>Pseudopropionibacterium propionicum</i>	18.91891892
K03327: multidrug resistance protein, MATE family	40.54054054
K03327: <i>Acidaminococcus intestini</i>	2.702702703
K03327: <i>Acidaminococcus intestini</i> CAG 325	2.702702703
K03327: <i>Caulobacter</i> sp FWC26	0
K03327: <i>Cupriavidus metallidurans</i>	2.702702703
K03327: <i>Desulfobulbus oralis</i>	8.108108108
K03327: <i>Porphyromonas asaccharolytica</i>	2.702702703
K03327: <i>Porphyromonas gingivalis</i>	8.108108108
K03327: <i>Prevotella denticola</i>	8.108108108
K03327: <i>Prevotella enoeca</i>	8.108108108
K03327: <i>Streptococcus sanguinis</i>	2.702702703
K03585: membrane fusion protein, multidrug efflux system	40.54054054
K03585: <i>Acidaminococcus intestini</i>	2.702702703
K03585: <i>Acidaminococcus intestini</i> CAG 325	2.702702703
K03585: <i>Campylobacter curvus</i>	5.405405405
K03585: <i>Desulfobulbus oralis</i>	8.108108108
K03585: <i>Dialister pneumosintes</i>	16.21621622
K03585: <i>Porphyromonas asaccharolytica</i>	2.702702703
K03585: <i>Porphyromonas uenonis</i>	2.702702703
K03585: <i>Prevotella denticola</i>	13.51351351
K03585: <i>Prevotella enoeca</i>	5.405405405
K03585: <i>Veillonella parvula</i>	2.702702703
K05595: multiple antibiotic resistance protein	37.83783784
K05595: <i>Actinomyces</i> sp oral taxon 414	5.405405405
K05595: <i>Capnocytophaga ochracea</i>	5.405405405
K05595: <i>Caulobacter</i> sp FWC26	0
K05595: <i>Porphyromonas asaccharolytica</i>	2.702702703
K05595: <i>Porphyromonas gingivalis</i>	8.108108108
K05595: <i>Sphingobium yanoikuyae</i>	0
K05595: <i>Tannerella forsythia</i>	21.62162162

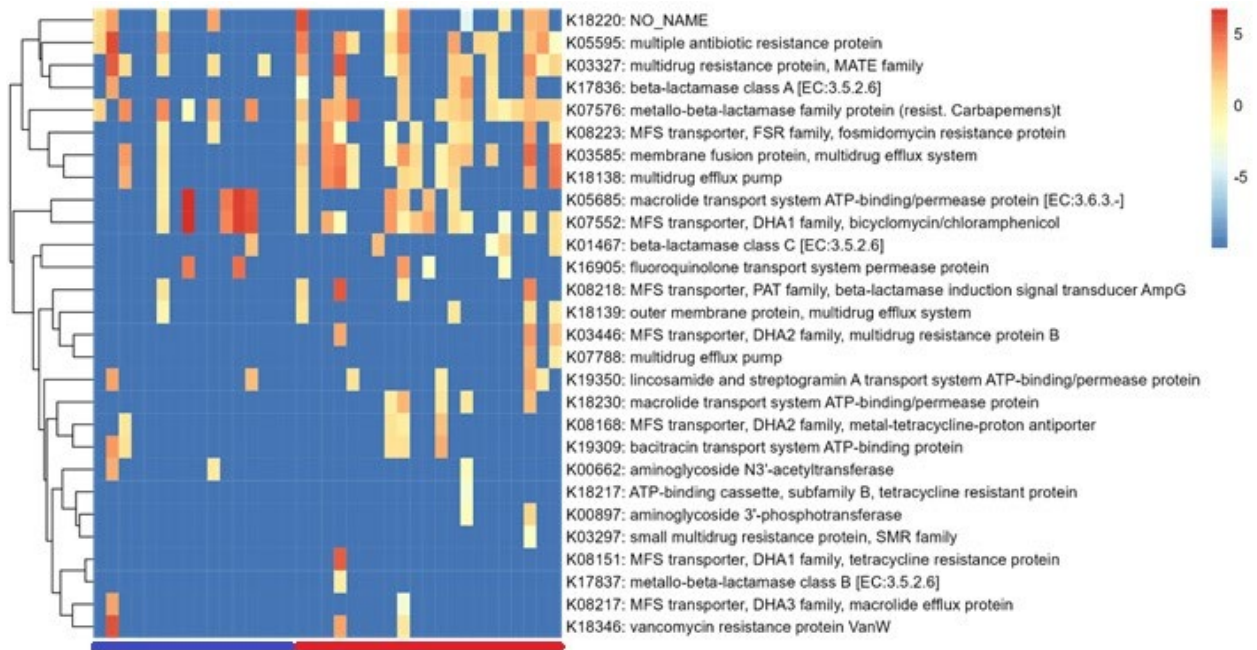
**Table 4.** Summary of sequencing data obtained analyzed with Mann-Whitney U test.  $P > 0.05$ .

**Mann Whitney test**

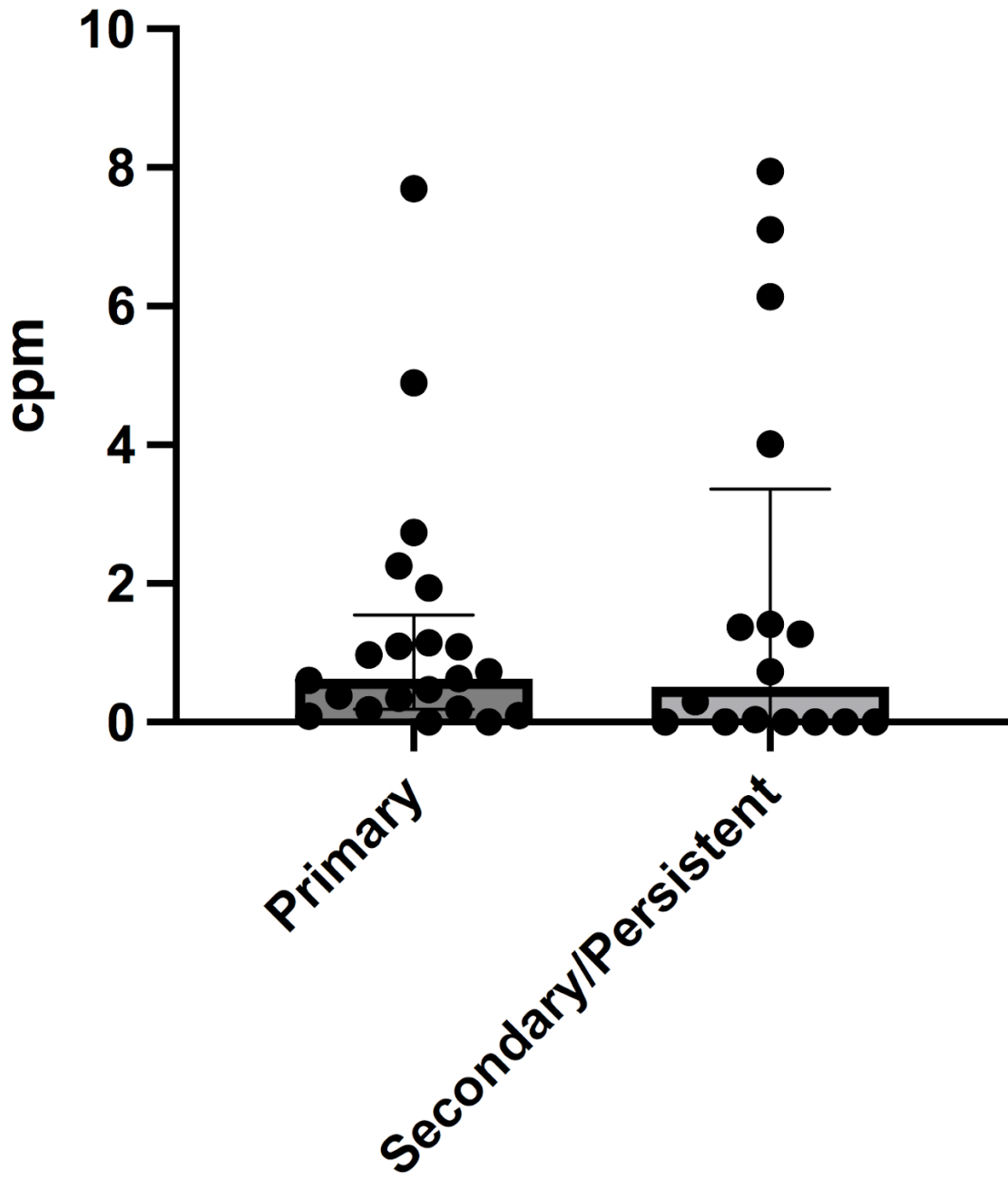
<b>P value</b>	<b>0.6532</b>
Exact or approximate P value?	Exact
P value summary	ns
Significantly different ( $P < 0.05$ )?	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	414 , 289
Mann-Whitney U	153
Difference between medians	
<b>Median of column A</b>	<b>0.6313, n=21</b>
<b>Median of column B</b>	<b>0.5131, n=16</b>
Difference: Actual	-0.1182
Difference: Hodges-Lehmann	-0.08742
	-0.6313 to
95.29% CI of difference	0.8979
Exact or approximate CI?	Exact



**Figure 1.** Heatmap of the log<sub>2</sub> abundance for antibiotic resistant genes (ARGs) present in bacteria that present ARGs in primary and secondary endodontic infections.



**Figure 2.** Heatmap of the  $\log_2$  abundance for the top 28 antibiotic resistant genes in primary and secondary endodontic infections. Primary endodontic infection samples (red), secondary endodontic infection samples (blue).



**Figure 3.** Relative abundance in count per million (cpm) found in primary and secondary endodontic infections. Mann-Whitney U test  $P > 0.05$ .