Identification of Antibiotic Resistance Genes in Primary and Persistent Endodontic Infections

1. Introduction and Specific Aims of the Project

It is becoming evident through research on the molecular microbiology of endodontic infections that the diversity of microorganisms in infected root canals is more complex than has ever been appreciated. We also recognize now that the presence of certain species is not as critical in assessing the virulence of a microorganism as the presence of specific virulent strains of this microorganism. Certain genetic variation within the cells of a certain microbial species might render a commensal organism highly pathogenic. Thus, one potentially effective strategy of understanding microbial composition of endodontic infections is to study and analyze the presence of virulence genes.

The intensive use of antibiotics in medicine and dentistry has selected for antibiotic resistant bacteria. When bacteria become resistant to antibiotics, they gain the ability to develop and exchange resistance genes, making them non-susceptible to the antimicrobial substances employed. Studies have shown that although bacteria can be sensitive to antibiotics, resistance genes can still be acquired from other bacteria in vitro (Perreten et al. 2005). A key factor in the development of antibiotic resistance is the ability of infectious organisms to adapt quickly to new environmental conditions. Bacteria are single-celled organisms that, compared with higher life forms, have small numbers of genes. Therefore, even a single random genetic mutation can greatly affect their virulence. And because most microbes reproduce by dividing every few hours, bacteria can evolve rapidly. A mutation that helps a microbe survive exposure to an antibiotic will quickly become dominant throughout the microbial population. Microbes also often acquire genes from each other through plasmid transfer and other mechanisms, including genes that confer resistance (Grohmann et al. 2003). This genetic exchange can occur among organisms of different species, thereby raising the importance of the virulence determinants rather than the species in defining the pathogenicity of a particular microbial profile.

Antimicrobial agents are often categorized according to their principal mechanism of action. Mechanisms include interference with cell wall synthesis (e.g., beta-lactams and glycopeptide agents), inhibition of protein synthesis (macrolides and tetracyclines), interference with nucleic acid synthesis (fluoroquinolones and rifampin), inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole), and disruption of bacterial membrane structure (polymyxins and daptomycin). (Tenover 2006) Bacteria may be intrinsically resistant to one or more classes of antimicrobial agents, or may acquire resistance by de novo mutation or the acquisition of resistance genes from other organisms. Acquired resistance genes may enable a bacterium to produce enzymes that destroy the antibacterial drug, to express efflux systems that prevent the drug from reaching its intracellular target, to modify the drug's target site, or to produce an alternative metabolic pathway that bypasses the action of the drug. Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation, transformation, or transduction, with transposons often facilitating the incorporation of the multiple resistance genes into the host's genome or
plasmids. Use of antibacterial agents creates selective pressure for the emergence of resistant strains. (Tenover 2006)

The hypotheses for this project are: 1) Microorganisms in primary and persistent endodontic infections possess antibiotics resistance virulent genes. 2) Microorganisms in cases with persistent endodontic infections have a higher prevalence of antibiotics resistance genes.

Therefore, the purpose of this project is to use molecular methods to compare the prevalence of antibiotics resistance genes in primary and persistent endodontic infections.

2. Previous Work and Background of the Project

Primary endodontic infections have been found to contain mostly anaerobic bacteria and are polymicrobial in nature (Fouad et al. 2002; Munson et al. 2002). Prevotella spp. found in orofacial and dentalveolar infections, in particular, are reported to be positive for beta-lactamase (Kuriyama et al. 2001). The bacteria associated with persistent endodontic infections were found to be different than those cultured from primary infections. Furthermore, in those cases with persistent disease, gram-positive strains such as enterococci were more prevalent (Fouad et al. 2005; Rocas et al. 2004), and increased the survival rates of other bacteria if present (Moller et al. 2004). Enterococcus faecalis and E. faecium have been shown to possess multiple antibiotic resistance (Dahlen et al. 2002). It has been shown in persistent infections that E. faecalis also demonstrated resistance to antibiotics, such as Erythromycin and Azithromycin, but was susceptible to Amoxicillin and others (Pinheiro et al 2004). Although the previous studies identified particular phenotypes involved in antibiotic resistance, they provided only limited information on the identity of the actual resistance genes harbored by the microbes. In contrast, when specific antibiotic resistant genes are identified, this information can be used to investigate the diversity and the spatial and temporal distribution of resistance genes within and between different host bacteria. The analysis of patient specimens for antibiotic resistance by molecular methods is potentially more efficient and accurate than culture and sensitivity methods.

Tetracycline resistant genes have been shown to occur in oral bacteria (Villedieu et al. 2003). Tetracycline is thought to inhibit the growth of bacteria by entering the bacterial cell, binding to bacterial ribosomes, and stopping protein synthesis (Speer et al. 1992). However, Oliva and Chopra (1992) proposed that tetracyclines be divided into two types based on their modes of action: 1) those that inhibit protein synthesis (e.g., tetracycline) and 2) those that interact with the cytoplasmic membrane (e.g., thiatetacycline). Bacteria can use three strategies to become resistant to tetracycline: 1) limiting the access of tetracycline to ribosomes, 2) altering ribosomes to prevent effective binding of tetracycline, and 3) producing tetracycline-inactivating enzymes (Speer et al. 1992). Villedieu et al (2003) identified tet(M) as the most common tet gene in oral bacteria by confirming its presence in 79% of all isolates. It was followed by tet(W) found in 21% of the isolates and tet(O) and (Q) in 10.5% and 9.5% of the isolates, respectively. All of these genes encoded a ribosomal protection protein. The chromosomal tet(M) gene is frequently transmissible, and transmissible chromosomal
tet(M) determinants are associated with conjugative transposons. The tet(M)-carrying conjugative transposons have a remarkably wide host range and can transfer from gram-positive to gram-negative bacteria as well as to gram-positive bacteria, making it possible to transfer bacterial resistance (Speer et al. 1992). The clinical relevance of tetracycline or tetracycline derivative resistance in endodontics is the use of MTAD. MTAD is a mixture of tetracycline isomer (doxycycline), an acid (citric acid), and a detergent (Tween 80). Although Shabahang and Torebinejad (2003) showed MTAD was effective against *E. faecalis* in vitro, it may not be as effective in vivo, where bacterial species or strains, singly or in combination, may be different. Studies of antibiotic efficacy in vitro using culturing methods are limited by the fact that the antibiotic may be transferred with the specimen to the laboratory, thus conferring a false negative result. Most other non-antibiotic antimicrobials in endodontics have inactivators that can be used to stop their action during in vitro testing.

β-lactams belong to a family of antibiotics characterized by a β-lactam ring. Penicillins, cephalosporins, clavams (or oxapenams), cephemycins and carbapenems are members of this family. The integrity of the β-lactam ring is necessary for the activity which results in the inactivation of a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis. Resistance to β-lactams in clinical isolates is due primarily to the hydrolysis of the antibiotic by a β-lactamase. Mutational events resulting in the modification of penicillin-binding proteins or cellular permeability can also lead to β-lactam resistance. The presence of penicillin-resistant bacteria has been implicated as the cause of clinical failure of treatment in some cases of oral purulent infection (Heimdahl et al. 1980). Dentoalveolar infections usually involve bacteria residing in the oral cavity. In particular, strict anaerobes such as *Peptostreptococci*, *Prevotella*, and *Fusobacterium* spp are reported to be the most common isolates from these infections (Kuriyama et al. 2002). Among them *Prevotella* spp. are penicillin-resistant (Iwahara et al. 2006). The *bla* gene, which encodes the TEM-1 β-lactamase, is the most encountered penicillin resistant marker used in molecular biology (Petrosino et al. 1996). TEM-1 is a widespread plasmidic β-lactamase that attacks narrow-spectrum beta lactam antibiotics and all the anti-gram-negative-bacterium penicillins except temocillin (Petrosino & Palzkill 1996). The TEM-1 enzyme was first reported from an *E. coli* isolate and is now the most common β-lactamase found in enterobacteriaceae (Bradford 2001). Resistance in more than 50% of AmpR *E. coli* clinical isolates is due to TEM-1 (Bradford 2001). Most extended-spectrum β-lactamases derive from TEM-1 by mutations generating 1- to 4-amino-acid sequence substitutions (Bush et al. 1995). Using PCR, Iwahara et al (2006) found that β-lactamase genes *cfxA* and *cfxA2* were present in dentoalveolar infections.

*E. faecalis* has been shown to possess multiple antibiotic resistance, including resistance to vancomycin and macrolides (Martothe et al. 2005). Glycopeptide-resistant enterococci are classified genotypically into five main groups. The *van*(A)-type strains display high level resistance to the glycopeptides vancomycin and teicoplanin, whereas the *van*(B)-type strains show variable levels of resistance to vancomycin (Miele et al. 1995). *Van(D)* is found in strains resistant to various levels of vancomycin and teicoplanin (Woodford 2001). Several genes have been identified on this bacteria as responsible for this resistance including the *van*(B), *van*(E) and *van*(G) genes. (Cassone et al. 2006; Perreteen et al. 2005)
Several antibiotic-resistant genes have been elucidated through studies mentioned above, including the tetracycline resistant genes $tet(M)$, $tet(W)$, $tet(O)$ and $tet(Q)$, the beta-lactamase ($bla$) genes $bla_{TEM-1}$, $blaz$, $bla1$, $bla2$, and $cfxA/A2$. Vancomycin resistant genes have been associated with multiple bacterial strains as in $van(D)$ and $van(A)$, and have been found to be associated with $E. faecalis$, as in $van(B)$, $van(E)$, $van(G)$ (Call et al. 2003; Depardieu et al. 2004; Fines et al. 1999; Iwahara et al. 2006; Miele et al. 1995; Perreten et al. 2005; Villedieu et al. 2003). Because there have been an extensive array of bacterial strains isolated from both primary and persistent infections of endodontic origin, the probability exists that antibiotic-resistant-genes can be identified in the endodontic microbiome. The identification of these resistance genes in endodontic infections will enable us to properly use antibiotics in treatment modalities and better predict ways to combat these infections. This includes the design and implementation of local strategies, such as the use of MTAD, or the treatment of patients with spreading infections by systemic antibiotics. Much of the research in this area has relied on culturing techniques. Molecular methods are much more powerful and efficient than culturing methods and open an opportunity for wide scale efficient clinical testing for antibiotic resistance.

3. Significance of Project with respect to AAE Priorities:

This project directly addresses AAE priority #C3, which encompasses the identification, clarification and use of genetic or genomic factors to manage endodontic disease. It is currently not known whether root canal microflora contain antibiotics resistance genes. If particular types of resistance genes are present, this would be a significant clinical problem, because the root canal of a pulpless tooth is isolated from host defense mechanisms, and can act as a site for potentially harmful organisms to survive. Antibiotic resistance research has been and still is confined primarily to the study of cultivable bacterial isolates of mostly clinical origin. However, the cultivable isolates may represent only a fraction of the actual microbiota where the antibiotic resistance reside and certainly no antibiotic resistance profile can be determined if cultivation fails. Another important issue with antibiotic resistance is the fact that the wide use of antibiotics not only selects for drug-resistant pathogenic bacteria but also exerts selective pressure on the normal commensal microbiota. In light of the ubiquitously demonstrated phenomenon of horizontal antibiotic resistance gene transfer in the microbial world, the presence of such reservoirs may explain the rapid dissemination of antibiotic resistance from commensal organisms to the pathogenic microbiota. However, information regarding the antibiotic resistance pool in the endodontic microbiome does not exist. Therefore, development of genotyping tools for detection and tracking of antibiotic resistance genes in a variety of commensal and pathogenic bacteria using molecular methods is essential for understanding the ecology of antibiotic resistance. PCR assays are relatively rapid and can be used with a variety of sample types, such as individual bacterial colonies. Multiplex PCR assays have been devised for detection of a large number of genes, and this approach could be used for different classes of antibiotic resistance genes in endodontic infections. DNA microarrays offer an alternative method for screening for the presence of a wide diversity and large number of genes. In this format, probes specific to each gene are deposited onto a solid substrate (usually glass) in
a lattice pattern. DNA is then labeled and hybridized to the array, and specific target-probe duplexes are detected with a reporter molecule and bacteria is then screened for these genes. The future possibility exists that a microarray can be designed based on our PCR results, specifically for endodontic infections, thus employing a completely biological, patient specific approach to fighting infection. This biological approach will allow for the proper prescription use of antibiotics and may lead to the development of rapid chairside antibiotic resistance testing.

4. Experimental Method

Patient selection. We have based our sample size calculation on the following statistical power calculation: with an N of 29 per group, a \( p \leq 0.05 \) and an expectation of a ratio of 2:1 (persistent:primary), power will be 0.80. If the assumption is not met that the expected value is \( > 5 \) in 80% of the cells, than Fisher’s exact test will be used.

All patient-related procedures used in this study will conform to protocols approved by the Institutional Review Board of the University of Maryland-Baltimore College of Dental Surgery and have been used by our group in prior studies to obtain samples (Fouad et al. 2002; Fouad et al. 2003; Fouad et al. 2005; Kaufman et al. 2005). The purpose and scope of this study will be explained to patients presenting for endodontic treatment for a tooth with either a primary or persistent (previously endodontically treated) endodontic infection.

Inclusion criteria. Inclusion criteria for this study will be the following:
- The patient will be 18 years of age or older
- The tooth from which the sample will be obtained will have a periradicular lesion at least 2 mm in diameter
- For the tooth to be included in the primary infection group, there will have been no previous endodontic treatment on that tooth
- The pulp necrosis will be a result of caries and its sequelae or traumatic injuries (primary treatment cases)
- For retreatment cases (persistent infection), the tooth to be sampled will have seemingly adequate endodontic treatment and a restoration over two years before that is not healing an for which non-surgical retreatment is indicated.

Exclusion criteria. Exclusion criteria for this study will be as follows:
- Any systemic debilitating disease, such as diabetes mellitus, liver disease, chronic infections, rheumatoid arthritis or any other systemic disease that compromise the immune system
- Women who are pregnant at the time of initial treatment
- Anyone who has been taking antibiotics in the preceding month or require prophylactic antibiotic before dental treatment
- Anyone who is using chronic systemic steroids or chemotherapeutic agents
- Patients will be excluded, if, upon periodontal examination, they are found to have chronic or aggressive periodontitis, advanced periodontitis on the tooth involved, interproximal clinical attachment loss of more than 6 mm, or probing of more than 5 mm on the tooth involved
- Teeth that will be difficult to adequately isolate
Sample collection. Samples used for this study will include specimens taken by the following method: isolation of tooth with a rubber dam, disinfection of the field with 30% H2O2 and then 5% tincture of iodine, removal of all caries, then wiping of the cavity with a sterile cotton pellet moistened with NaOCl, taking care not to allow entrance of the NaOCl into the canal. Inactivation of the disinfectants will have been done with 5% sodium thiosulfate. The pulp chamber will be accessed with a sterile bur and if purulence is observed, three samples will be taken with three fine size paper points. If not, sterile saline will be introduced to the canals, making sure it does not overflow. A size 15 to 30 size file (depending on canal size) will be used to negotiate the canal to the estimated length and if the canal is calcified, Gates Glidden burs size 2 or 3 will be used so the paper point can penetrate to the approximate length of the canal. Three fine paper points will then be used to obtain the sample, leaving the last paper point in the canal for 30 seconds. If the tooth is multicanalled, a paper point will be used in each canal to obtain a sample. A file is also used to disrupt the biofilm on canal walls, and will be aseptically sectioned into the collection vial. The paper points and the file will then be placed in sterile, DNA- and RNA-free vials containing 1.5ml of filter sterilized 10mM Tris-HCl, (pH8.0), and 0.5 g of sterile glass beads. The vials will be frozen at -70 °C until used.

DNA extraction. Our group has performed DNA extraction techniques in prior studies (Fouad et al. 2002; Fouad et al. 2003; Fouad et al. 2005; Kaufman et al. 2005). The vials with paper point specimens will be vortexed for 2 min to disperse microbial cellular material into suspension. The suspension will be removed from the original vial and transferred to 2-ml sterile vials, which will then be centrifuged at 7,500 rpm (Eppendorf [Westbury, N.Y.] microcentrifuge) for 10 min, and the supernatant will again be removed. DNAs will be extracted from the cellular pellet by the enzymatic extraction method, according to the protocol described for the QIAamp DNA mini kit (Qiagen, Valencia, Calif.). The pellet will be suspended in 180 µl of enzyme solution (20 mg/mL of lysozyme , 20 mM Tris HCl [pH 8.0], 2 mM EDTA,1.2% Triton) and incubated for 30 min at 37°C. Proteinase K (20 µl) and RNaseA (4 µl at 100 mg/ml) will be added, and the specimen will be incubated for 2 min at room temperature. Buffer AL (200 µl) will be added, vortexed and incubated at 56°C for 30 min and then for 15 min at 95°C. Ethanol (200µl at 96 to 100%) will be added, mixed followed by brief centrifugation. The mixture will then be added to a QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The column will then be placed in a clean 2-ml collection tube, 500 µl of buffer AW1 will be added, and the mixture will be centrifuged at 8,000 rpm for 1 min. The column will again be placed in a clean 2-ml collection tube, and 500 µl of buffer AW2 will be added, followed by centrifugation at 14,000 rpm for 3 min. Then, buffer AE (200 µl) will be added, followed by centrifugation at 8,000 rpm for 1 min. The elutions will be combined for a total yield of 400 µl, which will be aliquoted in sterile, DNA- and RNA-free vials and frozen at −20°C until use.

Antibiotic resistance gene selection. We will evaluate the root canal specimens for the presence of 11 common antibiotic resistance genes that have been associated with a number of different microorganisms in oral bacteria, dentoalveolar infections, and persistent endodontic disease (Table1). Our selection of the resistance genes was based
on the following criteria: resistance genes that have been found in oral bacteria (tetracycline-resistant genes) (Aminov et al. 2001; Villedieu et al. 2003), resistance genes that have been found in orofacial and dentoalveolar infections, (beta-lactamases) (Heimdahl et al. 1980; Iwahara et al. 2006; Kuriyama et al. 2001), and resistance genes known to be present in bacteria associated with persistent endodontic infections, such as \textit{E. faecalis}, which has multiple antibiotic resistance (Vancomycin-resistant genes)(Cassone et al. 2006; Marothi et al. 2005; Miele et al. 1995; Perreten et al. 2005).

Table 1. Antibiotic-resistance genes to be examined

<table>
<thead>
<tr>
<th>Gene Primer</th>
<th>Sequence (5' to 3')(^a)</th>
<th>Size (bp)</th>
<th>Annealing temp (°C)</th>
<th>Reference</th>
<th>Antibiotic resistance</th>
</tr>
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<tr>
<td>\textit{tet}(M)</td>
<td>GTGGCAAAAGGTACACACGAG GGTTAAATGGTCACACAC</td>
<td>406</td>
<td>55</td>
<td>Villedieu et al 2003</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>\textit{tet}(W)</td>
<td>GAGACCTGCTATATGCAACGGCGGCATCCACAAATG</td>
<td>168</td>
<td>64</td>
<td>Villedieu et al 2003</td>
<td>Tetracycline</td>
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<tr>
<td>\textit{tet}(O)</td>
<td>AACTTGGCATTCTGCTACATCCCCAATGCTAACGAT</td>
<td>515</td>
<td>55</td>
<td>Villedieu et al 2003</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>\textit{tet}(Q)</td>
<td>TTATCTTTCCTGCGCACTGATCGGTTCGAGAATGTC</td>
<td>904</td>
<td>55</td>
<td>Villedieu et al 2003</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>\textit{bla}_{TEM-1}</td>
<td>CCAATGTCTTAATGATAGGTGAATGGATGTTCGTTCCCTATATGC</td>
<td>858</td>
<td>60</td>
<td>Call et al 2003</td>
<td>Penicillin</td>
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<tr>
<td>\textit{cfxA2}</td>
<td>CGTAGTTTTGAGTATAGCCTTATAGGTGAATGGATGTTCG</td>
<td>802</td>
<td>58</td>
<td>Giraud-Morin et al 2003</td>
<td>Penicillin</td>
</tr>
<tr>
<td>\textit{cfxA}</td>
<td>GCCAAATCTTCCCTTAACAAACCACCAACATTTTGT</td>
<td>802</td>
<td>60</td>
<td>Iwahara et al 2006</td>
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<td>\textit{bla}_{Z}</td>
<td>TTGGTTATCTTCAAGGCCAATCTGAACCTTTTTTATCTAAAGGCCAATCTGAACCT</td>
<td>846</td>
<td>54</td>
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<td>\textit{van}(A)</td>
<td>ATGAATAGAATAAAGGATGTTGAATCGAT</td>
<td>1029</td>
<td>62</td>
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<tr>
<td>\textit{van}(B)</td>
<td>GGCATCTTCTTCCCTGCAAAACCC</td>
<td>457</td>
<td>59</td>
<td>Miele et al 1995</td>
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<tr>
<td>\textit{van}(D)</td>
<td>TGGGTTTCTAGTGGCATATTTC</td>
<td>500</td>
<td>54</td>
<td>Depardieu et al 2004</td>
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<tr>
<td>\textit{van}(E)</td>
<td>TGTGGTATCGGACGTGCAGTGCATCTCAGAATTTCCA</td>
<td>513</td>
<td>52</td>
<td>Fines et al 1999</td>
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<tr>
<td>\textit{van}(G)</td>
<td>GCCCATCCTGCTTGTATGGA GGGTAAAGGCAATGTCGTTGCC</td>
<td>941</td>
<td>54</td>
<td>Depardieu et al 2004</td>
<td>Vancomycin</td>
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\(^a\) The top primer is the sense primer, the bottom primer is the antisense primer

**PCR amplification.** PCR amplification has been performed by our group in previous studies (Fouad et al. 2002; Fouad et al. 2003; Fouad et al. 2005; Kaufman et al. 2005). The primer pairs selected for specific PCR amplification of the resistance genes are listed in Table 1. At least duplicate experiments will be run for each specimen. PCR amplification will be performed in a thermal cycler (PE9700 or PE2400; Perkin-Elmer Applied Biosystems, Foster City, Calif.). It will be carried out in a volume of 50 µl containing 10 µl of extracted sample DNA or 5 µl of positive control selected for each resistance gene (Table 2), 5 µl of 10X PCR buffer, 0.5 µl (2.5U) of HotStar Taq DNA Polymerase (Qiagen), 1.5 mM MgCl\(_2\), 0.2 mM concentrations of each of the four deoxynucleoside triphosphates (Takara, Otsu, Shiga, Japan), and a 0.5 µM concentration (500 ng) of each (sense and antisense) primer; the balance will consist of sterile ultrapure water. The PCR conditions will generally be as follows: the initial denaturation will at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at a...
temperature that depends on the primer (Table 1) for 15 s, and extension at 72°C for 45 s. The final extension will be at 72°C for 5 min. Then the products will be cooled to 4°C until they are removed. The amplification products will be analyzed by 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]). The Power Pac 1000 apparatus (Bio-Rad, Hercules, Calif.) will be set at 110 mA for 2 h or 95 V for 1 h. The gels will be stained with 0.5 µg of ethidium bromide per ml for 30 min and then destained with water for 20 min. The PCR products will be visualized under UV light with an Alpha Imager (Alpha Innotech Corp., San Leandro, Calif.). PCR controls will be obtained from the ATCC Global Bioresource Center. Positive controls will be freeze-dried strains obtained that possess the desired gene (e.g. tet(A) positive strains). DNA will be extracted from these strains and then amplified. A positive result will be indicative of a true positive control. The negative control will be ultrapure water.

**Sequencing.** PCR products will be sequenced as our group has done in previous studies. The purpose of the sequencing will be to assure that the PCR product is indeed the anticipated one. This will be performed as we have done previously (Fouad et al. 2002; Fouad et al. 2003; Fouad et al. 2005) as follows: original PCR products will be reamplified using 1µl of product and previously stated primers and conditions. They will be purified using Rapid PCR purification system (Marligen Biosciences, Ijamsville, MD). Sequencing reaction mixes will be prepared for all antibiotic resistant gene primers. The purified DNA will be sequenced in the University of Maryland-Baltimore School of Medicine Biopolymer and Genomiscore facility (ABI Prism 3100 genetic analyzer, Applied Biosystems). The results for the forward and reverse primers will be aligned, compared, and a consensus sequence will be obtained that has the highest purity and accuracy. The resulting sequences will be used to search the databases available through the National Center for Biotechnology Information (NCBI), using the BLAST algorithm.

5. **Data analysis:**

The prevalence of different antibiotics resistance genes will be compared to determine a hierarchy of genes pertinent to primary and persistent infections. The prevalence of each of the antibiotics resistance genes will be compared in primary and persistent cases using Chi-square analysis ($p \leq 0.05$).

6. **Resources and Environment:**

All samples will be obtained from patients presenting for endodontic treatment at the University of Maryland-Baltimore College of Dentistry. All experiments will be performed in the microbiology lab of co-author Dr. Ashraf Fouad, Director of the Advanced Education Program in Endodontics and Chairman of the Department of Endodontics, Periodontics and Prosthodontics at the University of Maryland-Baltimore College of Dentistry.
### 7. Time Schedule for Research:

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### References


