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Antibiotic resistance in primary and persistent endodontic infections

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Abstract

Introduction—The presence of antibiotic resistance genes in endodontic microorganisms may render the infection resistant to common antibiotics. The aims of this project were to identify selected antibiotics resistance genes in primary and persistent endodontic infections and determine the effectiveness of contemporary endodontic procedures in eliminating bacteria with these genes.

Methods—In patients undergoing primary endodontic treatment or retreatment, the root canals were aseptically accessed and sampled prior to endodontic procedures as well as following contemporary chemomechanical preparation and medication with calcium hydroxide. Identification of the following antibiotics resistance genes was performed using PCR: $bla_{\text{TEM}-1}$, *cfx*A, *bla*Z, *tet*M, *tet*W, *tet*Q, *van*A, *van*D, and *van*E. Limited phenotypic identification and antibiotic susceptibility verification was also performed.

Results—Overall, there were 45 specimens available for analysis: 30 from primary and 15 from persistent endodontic infections. In preoperative specimens, only $bla_{\text{TEM-1}}$ was significantly more prevalent in primary vs. persistent infections ($p=0.04$). Following contemporary treatment procedures, there was an overall reduction in prevalence of these genes ($p<0.001$). $bla_{\text{TEM-1}}$ and *tet*W were significantly reduced ($p<0.05$), *cfx*A, *bla*Z and *tet*Q were eliminated, but there was no change in *tet*M. No specimens contained *vanA*, *vanD*, or *vanE*. Antibiotic susceptibility testing showed significant differences among the antibiotics ($p<0.001$) and general concordance with the gene findings.

Conclusions—$bla_{TEM\text{-}1}$ was more prevalent in primary than persistent infections. Vancomycin resistance was not present. The genes identified were reduced with treatment except for *tet*M. Genetic testing may be useful as a screening tool for antibiotic resistance.

Introduction

It has become evident through molecular microbiological research of endodontic infections that the diversity of microorganisms in these infections is very complex. It is also very likely

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that although these infections have a diverse microflora, the actual presence of a particular species is not as important as the presence of specific virulent strains of that organism. There are genetic variations within the cell that may render an organism highly pathogenic, and so one potentially effective strategy of understanding microbial compositions of endodontic infections is to analyze and study the presence of genes of virulence factors and/or virulence determinants.

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 exchange this resistance making them non-susceptible to antibiotics prescribed. Bacteria of different species can exchange these genes, which raises the importance of studying these virulence determinants rather than the species when defining pathogenicity (1–3).

Several studies have shown that primary and persistent endodontic infections differ in their microflora. Primary infections are generally composed of mostly anaerobic, Gram-negative organisms, while Gram-positive facultative bacteria comprise persistent infections (4–9). There have also been associations made between specific species in endodontic infections and antibiotic resistance, such as beta-lactamase positive *Prevotella* spp. found in dentoalveolar infections (10, 11) and the multidrug resistance found in *Enterococcus faecalis* associated with persistent endodontic disease (12, 13). Although specific species or strains of these microorganisms have been reported to have antibiotic resistance, there is little data available on antibiotic resistance gene expression in endodontic infections. The identification of antibiotic resistance genes may provide an efficient method of registering resistance to specific agents in clinical specimens, when this is indicated, as molecular tools tend to be much more efficient and sensitive than culture-based technologies. Moreover, with the resurgence in use of antibiotics locally, such as in irrigants or medicaments, the identification of the most effective agents invariably will depend on a better understanding of antibiotic resistance of the flora within the necrotic pulp. In addition, it is important to determine whether contemporary endodontic treatment methods are capable of eliminating microorganisms that harbor antibiotic resistance genes.

Therefore, the purpose of this study was to use molecular methods to identify a group of relevant antibiotic resistance genes in primary and persistent endodontic infections and to determine the effects of contemporary treatment methods on the prevalence of these genes.

Materials and Methods

Patient selection

All patient-related procedures used in this study conformed to protocols approved by the institutional review board of the University of Maryland. Root canal specimens were aseptically obtained from patients with endodontic infections presenting for treatment. Only one specimen was obtained per patient. Fifty patients who presented with either primary or persistent infections were recruited for this study. All patients had a periapical radiolucency that was at least 2×3 mm in diameter. Patients included in the primary infection category had no previous endodontic treatment and patients included in the persistent infection category had endodontic treatment completed more than 1 year prior to recruitment, but had persistent signs and/or symptoms of disease. Excluded from the study were patients with systemic debilitating diseases such as diabetes mellitus, liver disease, chronic infections, rheumatoid arthritis or any other systemic disease that compromise the immune system, patients on systemic steroids or chemotherapeutic agents, patients who had been on antibiotics in the preceding month or who required prophylactic antibiotic before dental treatment, patients who had active chronic or aggressive marginal periodontitis, women who were pregnant at the time of initial treatment, teeth which were difficult to isolate

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adequately, children less than 18 years of age or teeth with immature apex. The age of patients ranged from 19 to 94 yrs with an average age of 51 yrs.

Endodontic Procedures

Specimens for this study were obtained as follows: following rubber dam isolation, the field was disinfected with 30% H_2O_2, then 5% tincture of iodine and finally with 5% NaOCl. Inactivation of the halides was done with 5% sodium thiosulfate. If caries was present, then it was removed, and the protocol was repeated. This technique was a modification of techniques used in two previous investigations (14, 15), and was intended to assure minimal oral contamination. Access preparation was performed without water coolant, using a sterile bur and if purulence was observed, a specimen was taken with three fine size paper points. If not, sterile saline was introduced into the canals, making sure it did not overflow. A size 15–30 size file (depending on canal size) was used to negotiate the canal to the estimated length and disrupt the bacterial biofilm. This instrument was then aseptically separated and collected in the sampling vial. If the canal was calcified, Gates Glidden burs size 2 or 3 were used to provide straight line access to the files and papers points for optimal depth penetration. If the tooth was multicanaled, the file was activated in all canals and a paper point was used in each canal to obtain a sample. Therefore, for each specimen one file and three fine paper points were collected (16), leaving the last paper point in the canal (the largest canal in multi-canaled teeth) for 30 seconds. In persistent cases, the old root filling material was removed using hand and rotary instrumentation without solvents. The paper points and the file were placed in sterile, DNA- and RNA-free vials containing 1.5mL of filter sterilized 10mM Tris-HCl, (pH 8.0), and 0.5 g of sterile glass beads. The vials had been pre-reduced in an anaerobic chamber. The specimens were frozen at −70°C until used.

The root canals were then instrumented in the following manner: following working length determination, straight-line access was performed using Gates Glidden burs #2–4 (if not done previously), rotary instrumentation was performed used a combination of Profile GT and Light Speed/LSX instruments. The latter was used until about 10–12 pecks were needed to bring the instrument to the working length. Irrigation throughout the instrumentation procedure was with 2.5% sodium hypochlorite used after every other instrument. At the end of the instrumentation phase, 17% EDTA was used alternating twice with the NaOCl to remove the smear layer, making sure that the last irrigant of the two was EDTA. Finally the canals were dried with paper points and a final irrigation with 1 ml of 2% Chlorhexidine was done (17).

The canals were then medicated with Ca(OH)2 aqueous paste (D.T. Temporary Dressing, Global Dental Products, New York, NY) placed with lentulo spirals of appropriate size, for 1–3 weeks and the tooth sealed with a combination of Cavit and Fuji IX temporary filling with no cotton pellets. At the second appointment, the medicament was removed using files and the canals were irrigated with NaOCl and 17% EDTA, which were then dried. Final irrigation was with 2% Chlorhexidine. The canals were then dried and a pre-obturation specimen was obtained using 1 file and three paper points, and placed into vials that had the Tris-HCl, as noted before. In order to inactivate the chlorhexidine, 0.75 g (0.3%) L-α-phosphatidylcholine (L-α-lecithin) dissolved in 3 mL (3%) of polyoxyethylenesorbitan monooleate (Tween 80) and added to 97 mL of 0.43% sterile saline solution (18) was added to the Tris-HCL of the pre-obturation specimens. This was done rather than irrigate the canal with the inactivation agent as it was assumed that this inactivation would be instantaneous, and to prevent the inactivation agent from reducing the substantivity of the chlorhexidine in the canals.

In nine cases (seven persistent and two primary) the treatment was completed in three visits. In these cases, an additional visit was necessary because of: complex root canal anatomy (2

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cases), inability to remove a post (1 case), Thermafil obturator (1 case) or the entire old gutta percha fillings (3 cases) at the first appointment, a flare-up necessitating an unscheduled appointment (1 case) or non-resolving sinus tract (1 case). In all cases, preoperative specimen was obtained as soon as working length was reached with no prior disinfectants used in the canal system, the pre-obturation specimen was obtained following at least 1 week of calcium hydroxide medication, in the same appointment as the obturation.

DNA extraction

DNA extraction was performed using techniques shown to be effective in prior studies (5, 7). The vials with paper point specimens were vortexed for 2 minutes to disperse microbial cellular material into suspension. The suspension was removed from the original vial and transferred to 2-mL sterile vials, which were then centrifuged at 7,500 rpm (Eppendorf, Westbury, NY centrifuge) for 10 minutes, and the supernatant was again removed. DNAs were extracted from the cellular pellet by the enzymatic extraction method, according to the protocol described for the QIAamp DNA mini kit (Qiagen, Valencia, CA). The pellet was suspended in 180μL of enzyme solution (20mg/mL of lysozyme, 20 mM Tris HCl ph8.0, 2 mM EDTA, 1.2% Triton) and incubated for 30 minutes at 37°C. Proteinase K (20 μL) and RNAseA (4 μL at 100 mg/mL) was added, and the specimen was incubated for 2 min at room temperature. Buffer AL (200 μL) was added, vortexed and incubated at 56°C for 30 minutes and then for 15 minutes at 95°C. Ethanol (200 μL at 96 to 100%) was added, mixed followed by brief centrifugation. The mixture was then added to a QIAamp spin column and centrifuged at 8,000 rpm for 1 minute. The column was then placed in a clean 2-mL collection tube, 500 μL of AW1 was added, and the mixture centrifuged at 8,000 rpm for 1 minute. The column was again placed in a clean 2-mL collection tube, and 500 μL of buffer AW2 was added, and then centrifuged at 14,000 rpm for 3 minutes. Then, buffer AE (200 μL) was added then centrifuged at 8,000 rpm for 1 min. The elutions were combined for a total yield of 400 μL, which was aliquoted in sterile DNA- and RNA-free vials and frozen at −20°C until used.

Antibiotic resistance gene selection

The root canal specimens were analyzed for the presence of nine common antibiotic resistance genes that have been associated with a number of different microorganisms in oral bacteria, dento-alveolar infections, and persistent endodontic disease (Table 1). The resistance genes studied were for three groups of antibiotics: β-lactams, tetracyclines and vancomycin.

PCR amplification

PCR amplification was performed based on previous protocols established in prior studies referenced in Table 1. The primer pairs selected for specific PCR amplification of the resistance genes are listed in Table 1. At least duplicate experiments were run for each specimen. In addition to the antibiotic resistance genes, PCR with broad range 16SrRNA gene primers was done to determine bacterial presence (Table 1). PCR amplification were performed in a thermal cycler (PE9700 or PE2400; Perkin-Elmer Applied Biosystems, Foster City, Calif.). It was 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 1), 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 consisted of sterile ultrapure water. The PCR conditions were generally be as follows: the initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94–96°C for15–60 sec, annealing at a temperature that depended on the primer (Table 1), and extension at 72°C for 30–90 s. The final extension was 72°C for 5–10 min, and then the

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products were cooled to 4°C until they were removed. The amplification products were 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.) was set at 110 mA for 2 h or 95 V for 1 h. The gels were stained with 0.5 μg of ethidium bromide per ml for 30 min and then destained with water for 20 min. The PCR products were visualized under UV light with an Alpha Imager (Alpha Innotech Corp., San Leandro, Calif.). PCR controls were obtained from groups who have investigated these genes previously with proven results. Positive controls were freeze-dried strains obtained that possess the desired gene (e.g. *tet*(M) positive strains). In the case of $bla_{\text{TEM-1}}$, a PUC vector was used from Invitrogen containing the $bla_{\text{TEM-1}}$ gene. DNA was extracted from these strains and then amplified. A positive result at the appropriate size was indicative of a true positive control. The negative control will be ultrapure water.

Sequencing

PCR products in positive reactions from preoperative specimens were sequenced to assure that the PCR product was indeed the anticipated one. This was performed as follows: original PCR products were reamplified using 1μl of product and previously stated primers and conditions. They were purified using Rapid PCR purification system (Marligen Biosciences, Ijamsville, MD). Sequencing reaction mixes were prepared for all antibiotic resistant gene primers. The purified DNA was sequenced in the University of Maryland-Baltimore School of Medicine Biopolymer and Genome core facility (ABI Prism 3100 genetic analyzer, Applied Biosystems). The results for the forward and reverse primers were aligned, compared, and a consensus sequence obtained that has the highest purity and accuracy. The resulting sequences were used to search the databases available through the National Center for Biotechnology Information (NCBI), using the BLAST algorithm.

Phenotypic Analysis of Antibiotic Resistance

Recognizing that the presence of antibiotic resistance genes does not necessarily indicate antibiotic resistance of the specimens, we conducted a limited antibiotic resistance analysis in a group of 16 preoperative specimens from the 50 patients included in this study, using the same specimens that were collected in prereduced Tris HCl buffer, for the molecular study. A sample of 100 mL, from each specimen was cultured aerobically (three days on BHI plates) and another sample anaerobically (eight days on CDC blood agar plates). Bacterial colonies were sampled and their antibiotic resistance was evaluated using the E-test (bioMérieux, Inc., Durham, NC), against 8 antibiotics: amoxicillin, amoxicillin + clavulanic acid (Augmentin), amifloxacin (a second generation fluoroquinolone under investigation), doxycycline, tetracycline, metronidazole, clindamycin and tigecycline (a novel glycylcycline antibiotic). Antibiotic resistance thresholds were determined from the twenty first informational supplement of the Performance Standards for Antimicrobial Susceptibility Testing (M100-S21), produced by the Clinical and Laboratory Standards Institute (CLSI) in January 2011.

Data Analysis

The prevalence of different antibiotics resistance genes in primary and persistent infections, as well as in preoperative and pre-obturation specimens were compared using Chi square or Fishers Exact test ($p \leq 0.05$), with McNemar test used for data that is related such as preoperative and preobturation in the same specimens. The presence of the antibiotic resistance genes in different specimens was related to the presence of resistance to respective actual antibiotics in the same specimen. Finally, the susceptibilities of bacterial colonies to various antibiotics were compared using Chi-square analysis ($p \leq 0.05$).

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Results

Three patients were excluded because their preoperative and preobturation specimens were negative with broad range 16S r RNA gene PCR. Two other patients' specimens were excluded from respective analyses, one because the patient did not return for obturation appointment, and one case was started as a primary treatment case, and then a cotton pellet was discovered in the chamber under the permanent restoration, and so the case could not be classified as primary or persistent. The final sample for gene analysis included 45 specimens, with 30 from primary, 15 from persistent cases.

There were no positive results in any of the samples for the vancomycin genes: *van*(A), *van*(D), or *van*(E). The results for all other genes are summarized in Table 2. In preoperative specimens, only $bla_{\text{TEM-1}}$ was significantly more prevalent in primary than persistent infections (Fisher's Exact test; p=0.04), moreover, the other relatively prevalent penicillin resistance gene, *cfx*A, was only present in primary infections. The *tet*M and *tet*W genes were almost equal in prevalence in preoperative specimens, and were more prevalent than *tet*Q. However, in preobturation specimens, while *tet*W and *tet*Q were almost totally eliminated, *tet*M overall prevalence was not changed. In these specimens there was also a tendency for more reduction in prevalence of *tet*M in persistent (7%) compared to primary (30%) infections (p=0.077).

There was an overall reduction in prevalence of the antibiotics resistance genes detected from the preoperative to the preobturation specimens (cumulative prevalence was reduced from 15.2% to 5.6% --McNemar test; p<0.001). The presence of *cfx*A, *bla*Z and *tet*Q was eliminated, and the prevalence of $bla_{\text{TEM-1}}$ (p=0.01) and *tet*W (p=0.04) were significantly reduced following instrumentation and medication. However, there was no change in the overall prevalence of *tet*M in preoperative and preobturation specimens. Several of the negative preoperative specimens became positive following instrumentation and medication, for *tet*M in particular, but in a few other instances.

Twenty four colonies were cultured from 16 preoperative specimens, of which four were aerobes and 20 anaerobes (Table 3). Antibiotic susceptibility was tested with 8 common antibiotics, with overlapping categories with the resistance genes tested. Vancomycin was not included because of the lack of vancomycin resistance genes in the specimens. The antibiotic susceptibility of the 24 colonies tested was significantly different among the antibiotics (chi-square; p<0.001). None of the colonies were resistant to augmentin or tigecycline, whereas the highest resistance was to metronidazole and clindamycin (Table 3).

A comparison of selected antibiotic resistance genes presence and the identification of colonies with bacterial resistance to the respective β-lactam or tetracycline antibiotics is presented in Table 4. There were three specimens of the 16 analyzed with resistant colonies β-lactams or tetracyclines and corresponding positive gene identification of *cfx*A or *tet*M. Five other specimens were positive for resistance genes. Three of these (specimens 6, 12 and 14) had no bacterial growth. One specimen (#15) was positive for *tet*M and had a *Prevotella intermedia* colony with relatively high MIC values for the doxycycline (3 mg) and tetracycline (12 mg) that did not reach the levels of resistance indicated for tetracyclines in the CLSI publication.

Discussion

This study presented novel information about the pattern of presence of common antibiotic resistance genes in primary and persistent endodontic infections, their status following contemporary treatment methods, and their correlation with the antibiotic resistance of readily cultivable bacteria from the root canal environment.

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Among the antibiotic resistance genes studied, *bla*$_{\text{TEM-1}}$ was the most prevalent in endodontic infections, and was significantly more prevalent in primary compared with persistent endodontic infections. *Cfx*A was only present in primary infections. Bacteria harboring both genes were significantly reduced or eliminated following instrumentation and medication. Beta-lactamase related resistance in endodontic infections has been frequently related to Gram negative anaerobic bacteria, mainly members of the *Prevotella* spp. (11, 19, 20). It is likely that this may be the reason there were more *bla*$_{\text{TEM-1}}$ positive specimens in primary infections, and that this and *cfx*A-positive bacteria were eliminated following contemporary treatment procedures. Gram negative anaerobes are readily eliminated with hypochlorite and exposure to oxygen. The presence of penicillin-resistant bacteria in general has been implicated as the cause of clinical failure of treatment in some cases of oral purulent infection (19). The *bla* gene encoding the TEM-1 β-lactamase is the most encountered penicillin resistant marker used in molecular biology (21). The β-lactamase genes *cfxA* and *cfxA2* were reported to be present in all β-lactamase positive isolates of *Prevotella* spp. obtained from dentoalveolar infections, which constituted 37% of all isolates (10). It is interesting to note, that not only were none of the other isolates in this study β-lactamase positive, but also that 14% of the culture negative specimens in the same study had these genes. This shows the improved sensitivity of molecular methods compared with culturing in identifying this particular antibiotic resistance.

Among the tetracycline resistance genes studied, *tet*M and *tet*W were found to be more common than *tet*Q, with no significant difference between primary and persistent infections. *Tet*M was less prevalent in our specimens (18%) compared with a previous study of its prevalence in oral specimens (79%) (22). However, *tet*W and tetQ were comparable in prevalence in our specimens (18% and 9% respectively), to oral specimens in that study (21% and 10% respectively. T*et*M has been identified in tetracycline-resistant *Enterococcus faecalis* found in endodontic infections (23, 24). These studies found that eight of fifteen tetracycline-resistant bacteria isolated possessed the *tet*M gene and were resistant to tetracycline irrigation in an in vitro tooth model. Four of these eight genes contained the conjugative transposon Tn916 which is a unique region linked to the *tet*M gene, and could confer the ability to transfer resistance among different bacterial species. These findings are consistent with our finding that *tet*M was the only gene studied whose prevalence did not change following treatment procedures. Tetracycline was not used during the treatment in this study, however, it is likely that organisms like *E. faecalis*, which typically resist treatment methods, survived and harbored this gene. The clinical relevance of tetracycline resistance in endodontics is with the use of BioPureMTAD™. Although MTAD has been shown to be effective against *E. faecalis in vitro* (25), other studies have shown it may not be effective (26, 27). As far as we are aware, this was the first study to report the presence of *tet*W and *tet*Q in endodontic infections.

E. faecalis has been shown to possess multiple antibiotic resistance, including resistance to vancomycin and macrolides (28, 29). Six types of glycopeptide resistance genes have been described in enterococci that can be distinguished on the basis of the sequence of the structural gene for the resistance ligase (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*) (30). None of the vancomycin resistant genes investigated in this study were found in any specimens. Although we are not aware of other studies of these resistance genes in endodontic infections, several other studies have shown *E. faecalis* from endodontic infections to be susceptible to vancomycin (12, 13, 31). Therefore, our findings support these earlier studies, at least with respect to the genes studied. However, our group has previously published an analysis of the microbial proteins identified from seven of the specimens used in this study (32). Proteomic analysis revealed that 3 specimens contained Van proteins, including one that contained Van E. The reason that this specimen was positive for the expressed Van E protein, but not to the gene is not clear, but it may be

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related to the different sensitivities of the assays, less preferential amplification of the PCR reaction used or reduced amount of overall bacterial DNA present in the specimen.

Although the present study identified the presence of several antibiotic resistance genes, this information does not indicate that these bacteria are functionally resistant to antibiotics. The regulation of gene expression in a cell begins at the level of transcription of DNA into mRNA. Although subsequent processes such as differential degradation of mRNA in the cytoplasm and differential translation also regulate the expression of genes, it would be of great interest to estimate the relative quantities of mRNA in populations of bacterial cells. The circumstances under which a particular gene is up-regulated or down-regulated provide important clues about gene function. The simultaneous expression profiles of many genes can provide additional insights into disease processes that are mediated by the coordinated action of sets of genes.

In order to provide some information on the actual antibiotic resistance of these specimens, and to determine the relative merit of pursuing this line of research, we undertook a limited antibiotic resistance experiment of a number of readily cultured bacterial colonies. The cultured microorganisms represented normally identified endodontic pathogens including several *Prevotella* spp. The exception was *Moraxella* spp. *M catarrhalis* is commonly a commensal organism in the nasopharynx, but it may be involved in acute otitis media, and some mucosal disease. Antibiotic analysis showed that most, if not all, bacteria identified were sensitive to amoxicillin, augmentin, doxycycline, tetracycline and tigecycline. The superiority of the β-lactam antibiotics, particularly augmentin, and the reduced relative efficacy of clindamycin the agent of choice for patients allergic to penicillin, are all consistent with previous studies (33–35). However, more relevant to this study was the finding that there were three specimens of the 16 analyzed with resistant colonies to β-lactams or tetracyclines and corresponding positive gene identification of *cfx*A or *tet*M. Among the other specimens positive for resistance genes, three had no bacterial growth and one had some resistance but not to the accepted E-test threshold. None of these colonies had antibiotic resistance that was not identified in this limited set of genes analyzed. Therefore, in future, the possibility exists that a molecular method, such as a microarray-based device, could be designed based on PCR results such as those presented here, specifically for endodontic infections, to determine the expression of resistance genes thus employing a completely biological, patient-specific approach to fighting infection. This approach may lead to the development of rapid chair-side antibiotic resistance testing tool to assist with the prompt and proper prescription of antibiotics.

In summary, this study has determined that beta-lactam resistance genes, and specifically $bla_{TEM\text{-}1}$, were more prevalent in primary than persistent infections, and were significantly reduced or eliminated following treatment. Tetracycline resistance genes were identified in a smaller percentage of cases, and the bacteria that harbored *tet*M in particular were resistant to endodontic treatment methods. Vancomycin resistance genes were not identified. Correlative experiments showed that genetic testing may be useful as a screening tool for antibiotic resistance in endodontic infections.

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Table 1

Oligonucleotide primer pairs and the PCR conditions used

Gene	Primer Sequence (5' to 3')*	Annealing Temperature	Size (bp)	Antibiotic resistance	Reference
*tet*M	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	55°C	406	Tetracycline	(36)
*tet*W	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	64°C	168	Tetracycline	(37)
*tet*Q	TTATACTTCCTCCGGCATCG ATCGGTTCGAGAATGTCCAC	55°C	904	Tetracycline	(36)
$bla_{\text{TEM-1}}$	CCAATGCTTAATCAGTGAGG ATGAGTATTCAACATTTCCG	55°C	858	Penicillin	(38)**
*bla*Z	CAGTTCACATGCCAAAGAG TACACTCTTGGCGGTTTC	54°C	846	Penicillin	(2)
*cfx*A	GCAAGTGCAGTTTAAGATT GCTTTAGTTTGCATTTTCATC	58°C	802	Penicillin	(39)
van(A)	ATGAATAGAATAAAAGTTGCAATAC CCCCTTTAACGCTAATACGAT	62°C	1029	Vancomycin	(40)
van(D)	TGTGGGATGCGATATTTCAA TCGACGCAAGTATCCGGTAA	54°C	500	Vancomycin	(30)
van(E)	TGTGGTATCGGAGCTGCAG GTCGATTCTCGCTAATCC	52°C	513	Vancomycin	(41)
16S rRNA gene	AGA GTT TGA TCC TGG CTC AG ACG GCT ACC TTG TTA CGA CTT	56 °C	1500	All bacteria	(5)

* The top primer is the sense primer, and the bottom is the antisense primer.

** With the exception of the annealing temperature which was modified.

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Table 2

Antibiotic resistance genes identified in specimens from primary and persistent cases.

Resistance Gene	Primary Infection		Persistent Infection		Overall Prevalence	
	Preoperative N=30	Preobturation N=30	Preoperative N=15	Preobturation N=15	Preoperative N=45	Preobturation N=45
bla$_{\text{TEM-1}}$	13 (43%)	3 (10%)	2 (13%)	1 (7%)	15 (33%)	4 (9%)
*cfx*A	5 (17%)	0	0	0	5 (11%)	0
*bla*Z	0	0	1 (7%)	0	1 (2%)	0
*tet*M	5 (17%)	9 (30%)	3 (20%)	1 (7%)	8 (18%)	10 (22%)
*tet*W	6 (20%)	0	2 (13%)	1 (7%)	8 (18%)	1 (2%)
*tet*Q	3 (10%)	0	1 (7%)	0	4 (9%)	0

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Table 3

Bacterial colonies identified and their antibiotic resistance minimum inhibitory concentration (MIC) mg

Sample	Bacterial culture	O2 Req	Amoxicillin	Augmentin	Amifloxacin*	Doxycycline**	Tetracycline	Metronidazole	Clinda-mycin	Tige-cycline
1	*Propionibacterium acnes*	An	0.032	0.047	0.19	0.19	0.5	>256	0.032	0.094
2	alpha *Streptococcus* spp. (*viridans* gp)	Ae	≤ 0.016	≤ 0.016	0.032	≤ 0.016	0.19	>256	≤ 0.016	≤ 0.016
2	*Moraxella* spp.	Ae	0.5	0.5	0.047	0.25	0.25	>256	24	0.38
2	*Parvimonas micra*	An	≤ 0.016	≤ 0.016	>32	0.75	0.5	0.064	0.25	≤ 0.016
2	*Prevotella corporis*	An	0.094	0.032	0.25	2	64	>256	0.032	0.032
2	*Prevotella intermedia*	An	3	≤ 0.016	0.25	4	6	0.032	>256	0.094
4	*Prevotella denticolla*	An	0.032	≤ 0.016	0.25	0.064	0.19	0.19	≤ 0.016	0.125
4	*Prevotella intermedia*	An	3	0.064	0.25	3	12	0.064	≤ 0.016	0.064
4	*Prevotella buccae*	An	16	0.094	0.25	1.5	6	0.125	≤ 0.016	0.094
7	alpha *Streptococcus* spp. (viridans gp)	Ae	0.19	0.125	0.125	48	48	>256	>256	≤ 0.016
8	*Fusobacterium* spp.	An	≤ 0.016	≤ 0.016	0.047	≤ 0.016	0.064	0.032	≤ 0.016	≤ 0.016
8	*Peptostreptococcus* spp.	An	0.032	≤ 0.016	0.047	≤ 0.016	0.19	0.125	≤ 0.016	0.047
9	*Fusobacterium* spp.	An	≤ 0.016	≤ 0.016	0.032	≤ 0.016	0.064	≤ 0.016	≤ 0.016	0.023
9	*Peptostreptococcus* spp.	An	≤ 0.016	0.125	0.064	0.25	1	≤ 0.016	32	0.125
9	*Prevotella buccae*	An	8	0.064	0.25	0.047	0.19	0.19	>256	0.25
10	*Fusobacterium* spp.	An	≤ 0.016	≤ 0.016	0.023	0.023	0.047	≤ 0.016	≤ 0.016	≤ 0.016
10	*Peptostreptococcus* spp.	An	0.047	≤ 0.016	≤ 0.016	0.19	0.25	≤ 0.016	≤ 0.016	0.064
11	*Propionibacterium* acnes	An	0.094	≤ 0.016	0.125	0.064	0.19	>256	≤ 0.016	≤ 0.016
13	*Lactobacillus* spp.	An	0.047	0.032	>32	0.75	1.5	>256	4	0.032
13	*Lactobacillus* spp.	An	0.023	0.047	>32	0.5	1.5	>256	4	0.047
15	*Peptostreptococcus* spp.	An	≤ 0.016	≤ 0.016	>32	≤ 0.016	0.064	≤ 0.016	≤ 0.016	≤ 0.016
15	*Peptostreptococcus* spp.	An	1	0.125	0.125	0.25	0.75	≤ 0.016	0.25	0.75
15	*Prevotella intermedia*	An	0.5	≤ 0.016	0.5	3	12	0.125	≤ 0.016	0.032
16	alpha *Streptococcus* spp. (*viridans* gp)	Ae	0.5	0.5	0.25	0.125	0.38	>256	48	0.064
	Resistant		1	0	4	1	2	9	6	0
	Susceptible		23	24	20	23	21	15	18	24
	% susceptibility		95.8	100.0	83.3	95.8	87.5	62.5	75.0	100.0

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Ae: Aerobic, An: anaerobic; gray boxes: resistant to antibiotic.

* Other fluoroquinolones resistance >2–8 mg

** Used threshold for tetracycline

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Table 4

Correlation of antibiotic resistance and presence of respective antibiotic resistance gene in specimens 1–16.

Sample	Type	Growth	Antibiotics		Genes*		Antibiotics		Genes*	
			amoxicillin	augmentin	*bla*$_{TEM-1}$	*cfx*A	tetracycline	doxycycline	*tet*M	tetQ
2	Primary	Growth					R		X	
4	Primary	Growth	R			X				
6	Primary	No Growth								X
7	Primary	Growth					R	R	X	
8	Primary	Growth								
9	Primary	Growth								
10	Primary	Growth								
11	Primary	Growth								
15	Primary	Growth							X	
1	Persistent	Growth								
3	Persistent	No Growth								
5	Persistent	No Growth								
12	Persistent	No Growth		X						
13	Persistent	Growth								
14	Persistent	No Growth							X	
16	Persistent	Growth		X						

X: gene detected; R: antibiotic resistance in cultured colony

*blaZ and *tet*W were not found in any of these samples.