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Detection of antibiotic resistance genes in samples from acute and chronic endodontic infections and after treatment

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ABSTRACT

Objective: The purpose of this study was twofold: survey samples from acute and chronic endodontic infections for the presence of genes encoding resistance to beta-lactams, tetracycline and erythromycin, and evaluate the ability of treatment to eliminate these genes from root canals.

Design: DNA extracts from samples of abscess aspirates ($n = 25$) and root canals of teeth with asymptomatic apical periodontitis ($n = 24$) were used as template for direct detection of the genes *bla*TEM, *cfx*A, *tet*M, *tet*Q, *tet*W, and *erm*C using real-time polymerase chain reaction (PCR). Bacterial presence was determined using PCR with universal bacterial primers. Root canals of the asymptomatic cases were also sampled and evaluated after chemomechanical procedures using NiTi instruments with 2.5% NaOCl irrigation.

Results: All abscess and initial root canal samples were positive for bacteria. At least one of the target resistance genes was found in 36% of the abscess samples and 67% of the asymptomatic cases. The most prevalent genes in abscesses were *bla*TEM (24%) and *erm*C (24%), while *tet*M (42%) and *tet*W (29%) prevailed in asymptomatic cases. The *bla*TEM gene was significantly associated with acute cases ($p = 0.02$). Conversely, *tet*M was significantly more prevalent in asymptomatic cases ($p = 0.008$). Treatment eliminated resistance genes from most cases.

Conclusions: Acute and chronic endodontic infections harboured resistance genes for 3 classes of widely used antibiotics. In most cases, treatment was effective in eliminating these genes, but there were a few cases in which they persisted. The implications of persistence are unknown. Direct detection of resistance genes in abscesses may be a potential method for rapid diagnosis and establishment of proactive antimicrobial therapy.

1. Introduction

The oral microbiota has been suggested to function as a reservoir for several antibiotic resistance genes, including those encoding resistance to commonly used classes of antibiotics, e.g., beta-lactams, tetracyclines, and macrolides.[1–5] This is a matter of concern since these antibiotics have been widely recommended to treat oral infectious conditions, including those of endodontic origin.[6–8]

Antibiotics have been proposed for some specific indications, either for systemic or topical use. Systemic use of antibiotics in endodontics is usually indicated for acute apical abscesses associated with systemic involvement like fever and malaise, spreading infections, localized infections in medically compromised patients, prophylaxis for medically

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compromised patients during routine endodontic therapy, and replantation of avulsed teeth.[7] Topical use of antibiotics in the root canal has been recently recommended as final irrigants[9] or intracanal medication in the so-called ''revascularization'' procedures.[10] Therefore, selection of the most effective antibiotics to be used for systemic or topical use will depend on a better understanding of the patterns of antibiotic resistance in endodontic bacterial communities and their response to treatment.

Inappropriate prescribing and use of antibiotics have been regarded as one of the major causes of emergence and spread of bacterial resistance.[11,12] Studies analysing the antibiotics prescribing habits of endodontists and oral surgeons have revealed both abuse and misuse.[13,14] For instance, antibiotics have been prescribed for infections that can be usually uneventfully treated without antibiotic therapy (e.g., localized abscesses in uncompromised patients), or in cases with no infection (e.g., irreversible pulpitis). These approaches can contribute to the widespread problem of antibiotic resistance.

Several studies have reported on the antibiotic susceptibilities of isolates from endodontic infections.[15–18] These studies have been based on bacteriological culture and antibiotic susceptibility testing of the isolated strains through phenotype-based approaches. While highly reliable and considered the gold-standard, these tests for anaerobic bacteria are usually time-consuming and expensive, in addition to not detecting resistance in difficult-to-grow or uncultivable bacteria. Detection of antibiotic resistance genes in clinical samples by molecular methods has the potential to be an efficient and rapid method of predicting resistance to specific antibiotics. A study surveyed clinical samples directly for the presence of *cfx*A genes in clinical samples (pus and root canal exudates) from dentoalveolar infections and found this gene in 45% of the samples.[19] Moreover, because root canal bacteria may serve as a reservoir for antibiotic resistance genes,[20] it seems important to determine the efficacy of endodontic treatment procedures in eliminating bacteria carrying antibiotic resistance genes.

The present study surveyed acute apical abscess aspirates and root canal samples from teeth with asymptomatic apical periodontitis for the presence of genes encoding resistance to beta-lactams (*bla*TEM and *cfx*A), tetracycline (*tet*M, *tet*Q and *tet*W) and erythromycin (*ermC*). Moreover, elimination of bacteria carrying these genes was evaluated after chemomechanical procedures. The choice for the 6 antibiotic resistance genes targeted in this study was based on a previous study showing that these genes have already been detected in bacterial isolates from primary endodontic infections.[21]

2. Materials and methods

2.1. Subjects, sample taking and treatment procedures

Samples were taken from 50 patients who were seeking treatment in the Department of Endodontics, Estácio de Sá University, Rio de Janeiro. Only single-rooted teeth from adult patients (ages ranging from 19 to 64 years), all of them having carious lesions, necrotic pulps and radiographic evidence of periradicular bone loss were included in this study. In general, samples of primary endodontic infections were distributed as follows: 25 cases diagnosed as asymptomatic apical periodontitis and 25 cases diagnosed as acute apical abscesses. Diagnosis of acute apical abscess was based on the presence of spontaneous pain, exacerbated by mastication, and localized or diffuse swelling, along with fever, lymphadenopathy, or malaise. No fistula connecting the abscess to the oral cavity or skin surface was observed. Patients included in the study have not made use of antibiotics within the previous 3 months. All teeth showed no periodontal pockets deeper than 4 mm. The study protocol was approved by the Ethics Committee of the Estácio de Sá University.

All patients were asked to rinse the oral cavity for 1 min with 0.12% chlorhexidine before sampling procedures. Abscesses were sampled by aspiration of the purulent exudate from the swollen mucosa over each abscess. The overlying mucosa was disinfected with 2% chlorhexidine solution, and a sterile disposable syringe was used to aspirate the purulent exudate, which was immediately injected into cryotubes containing Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) and frozen at −20 °C. In cases of asymptomatic apical periodontitis, samples were obtained from the root canals under strict aseptic conditions, which included rubber dam isolation and a two-step disinfection protocol of the operative field with 2.5% NaOCl, as previously described.[22] Paper points used for sampling the root canals were transferred to cryotubes containing TE buffer and immediately frozen at −20 °C. Sterility control samples taken from the tooth crown were tested by using polymerase chain reaction (PCR) with universal primers for the bacterial 16S rRNA gene. Accordingly, one case was excluded because of a positive result.

Root canal samples from the teeth with asymptomatic apical periodontitis were also taken after chemomechanical procedures in order to evaluate the effects of treatment on endodontic bacterial communities that were positive for antibiotic resistance genes. Root canals were instrumented with NiTi hand or rotary instruments at a working length (WL) established 1 mm short of the apical foramen with the aid of an electronic apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel) and confirmed by radiographs. Patency of the apical foramen was confirmed with a small file throughout the procedures and under control with the apex locator. The size of apical preparation ranged from #40 to #55. For irrigation, 2.5% NaOCl was used in all canals, 2 ml after each file size, and delivered by disposable syringes and NaviTip needles (Ultradent, South Jordan, UT) inserted up to 4 mm short of the WL.

After preparation, smear layer was removed by rinsing the canal with 17% EDTA and 2.5% NaOCl. The canal was dried using sterile paper points and then flushed with 5 ml of 5% sodium thiosulfate to inactivate NaOCl. Next, a postpreparation (S2) sample was taken from the canals as for the initial sample.

2.2. Real-time PCR for antibiotic resistance genes

DNA was extracted from all samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer. The presence of bacteria

Table 1 – Primers used for detection of antibiotic resistance genes.

Antibiotic	Primer	Sequence	Ta	Size (bp)	Reference
Beta-lactam	*bla*TEM	5′-CCA ATG CTT AAT CAG TGA GG-3′ 5′-ATG AGT ATT CAA CAT TTC CG-3′	60	858	32
Beta-lactam	*cfx*A	5′-GCG CAA ATC CTC CTT TAA CAA-3′ 5′-ACC GCC ACA CCA ATT TCG-3′	60	802	19
Macrolide	*erm*C	5′-AAT CGG CTC AGG AAA AGG-3′ 5′-ATC GTC AAT TCC TGC ATG-3′	54	562	33
Tetracycline	*tet*M	5′-GTG GAC AAA GGT ACA ACG AG-3′ 5′-CGG TAA AGT TCG TCA CAC AC-3′	55	406	34
Tetracycline	*tet*Q	5′-TTA TAC TTC CTC CGG CAT CG-3′ 5′-ATC GGT TCG AGA ATG TCC AC-3′	55	904	34
Tetracycline	*tet*W	5′-GAG AGC CTG CTA TAT GCC AGC-3′ 5′-GGG CGT ATC CAC AAT GTT AAC-3′	64	168	35

in clinical samples was determined by using PCR with universal primers for the bacterial 16S rRNA gene as described previously.[23]

Clinical samples were analysed for the presence of 6 target antibiotic resistance genes (Table 1). Real-time PCR amplification was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μl. The annealing temperatures for each primer pair were based on previous protocols established in previous studies (Table 1). Primers in a concentration of 0.5 μM each and extract DNA volume of 2 μl were added to the PCR master mix in MicroAmp Optical 96-well reaction plates. Plates were sealed, centrifuged and then subjected to amplification. Cycling conditions for the qPCR included: 95 °C/10 min; 40 repeats of the following steps: 95 °C/1 min, annealing for 1 min (specific temperatures shown in Table 1), and 72 °C/1 min. All the tests were run in duplicate. Triplicates of appropriate negative controls containing no template DNA were subjected to the same procedures. Positive controls included strains or samples that yielded positive results for these genes with results previously confirmed by amplicon sequencing. Following amplification, melting curve analysis was performed to determine the specificity of the amplified products. Melting curve was obtained from 60 °C to 95 °C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed using the ABI 7500 software v2.0.4 (Applied Biosystems). To confirm positive results, PCR products were subjected to electrophoresis in agarose gels and representative amplicons were sequenced.

Data for the prevalence of the target resistance genes in samples from acute abscesses and asymptomatic apical periodontitis were compared by using the Fisher's exact test. The same test was used to evaluate the ability of chemomechanical preparation in reducing the incidence of cases positive for the target resistance genes. The level of significance was set at 5% ($p < 0.05$).

3. Results

All samples from abscess aspirates and the initial samples from root canals of teeth with asymptomatic apical periodontitis gave positive results for the presence of bacteria as determined by universal 16S rRNA gene-based PCR. Nine of the 25 (36%) abscess samples were positive for at least one of 4 antibiotic resistance genes (Table 2). The most prevalent resistance genes in samples from acute abscesses were in decreasing order *bla*TEM (6/25, 24%), *erm*C (6/25, 24%), *tet*W (3/25, 12%) and *tet*M (2/25, 8%). The genes *cfx*A and *tet*Q were not detected. Two cases were positive for 3 target genes and 4 other cases yielded 2 genes.

Of the 24 root canals of teeth with asymptomatic apical periodontitis, 16 (67%) were positive for at least one target resistance gene (Table 2). The most prevalent resistance genes were in decreasing order *tet*M (10/24, 42%), *tet*W (7/24, 29%) and *erm*C (6/24, 25%). No asymptomatic case yielded the other 3 target genes. One case was positive for 3 target genes and 2 genes were concomitantly detected in 5 other cases.

Statistical analysis revealed that the *bla*TEM gene was significantly more found in acute than in asymptomatic cases, in which it was not found ($p = 0.02$). Conversely, the *tet*M gene was significantly more prevalent in asymptomatic cases than in acute abscesses ($p = 0.008$). No significant differences were observed for the other genes.

Samples were also taken from the root canals of teeth with asymptomatic apical periodontitis after chemomechanical

Table 2 – Samples positive for 16S rRNA and antibiotic resistance genes.

Sample	Target genes						
	16S rRNA	*bla*TEM	*cfx*A	*erm*C	*tet*M	*tet*Q	*tet*W
Acute apical abscess	25/25[a]	6/25	0/25	6/25	2/25	0/25	3/25
Asymptomatic apical periodontitis	24/24	0/24	0/24	6/24	10/24	0/24	7/24
Post-instrumentation	14/24	0/24	0/24	2/24	3/24	0/24	2/24

[a] Number of cases with positive result/number of cases examined.

preparation using 2.5% NaOCl as the irrigant. Of the 24 initially infected canals, 14 (58%) remained positive for bacterial presence as determined by universal 16S rRNA-gene based PCR. As for the target antibiotic resistance genes, most cases that were positive before treatment became negative after chemomechanical debridement. Five (31%) of the 16 cases positive for at least one resistance gene were still positive after chemomechanical procedures (Table 2). Of the genes persisting after instrumentation, *tet*M occurred in 3 S2 samples (eliminated from 7 cases), *tet*W in 2 (eliminated from 5 cases) and *erm*C in 2 (eliminated from 4 cases).

4. Discussion

The purpose of this clinical study was twofold. First, the prevalence of 6 antibiotic resistance genes was directly examined in samples from acute and chronic endodontic infections, all of which were positive for the presence of bacteria as determined by PCR using universal bacterial primers. The genes targeted in this study encode resistance to beta-lactams, macrolides and tetracyclines, and were selected on the basis that they have been previously detected in samples from the oral cavity, including root canals.[3,5,20]

Endodontic abscesses rarely cause life-threatening diseases and, as a consequence, rapid microbiologic identification results are not usually necessary. Culture and antibiotic susceptibility testing of anaerobic bacteria provide results in about 7–14 days, which is usually too late. Antibiotics are therefore prescribed based on the empiric knowledge of endodontic infections. However, situations like abscesses rapidly disseminating to facial and/or neck anatomic spaces may require rapid diagnosis for the benefit of both the patient and the clinician. Rapid molecular diagnosis targeting antibiotic resistance genes has the potential to allow clinicians to manage infectious diseases proactively.[24] Although the presence of a resistance gene in a sample does not necessarily imply phenotypic resistance, its absence does imply a lack of resistance through that particular genetic mechanism.[25]

In the present study, 36% of the abscess samples were positive for at least one of the target antibiotic resistance genes. The most prevalent ones were *bla*TEM, *erm*C, *tet*W and *tet*M, representing the 3 classes of antibiotics evaluated. It was curious that in many cases more than one resistance gene was simultaneously detected. Samples from untreated canals of teeth with asymptomatic apical periodontitis were also evaluated and a higher percentage of cases carrying at least one target gene was observed when compared to acute cases; two-thirds of the asymptomatic cases were positive. The most prevalent resistance genes were *tet*M, *tet*W and *erm*C and many cases were also positive for more than one target gene.

An intriguing finding was that the *bla*TEM gene was only found in acute cases and as one of the most prevalent resistance genes. TEM beta-lactamases are widespread in Gram-negative bacteria and are known to attack several beta-lactamic antibiotics.[26,27] TEM confers resistance to penicillins and early cephalosporins and has shown an astonishing functional plasticity in response to the introduction of novel derivatives of these antibiotics.[28] The gene *bla*TEM has been reported to be widely distributed among periodontal biofilm samples, regardless of the disease state.[3,5] Jungermann et al.[20] found that *bla*TEM was the most prevalent antibiotic resistance gene in samples from primary and persistent/secondary root canal infections, but there are no reports on the association with symptoms. The reasons why this gene was found only in symptomatic cases are not clear, but the possibility exists that patients with abscesses may have experienced previous acute episodes and made use of beta-lactam antibiotics (before the 3-month period exclusion criterion), which may have promoted a selection of resistant strains. Also, because some species may be more associated with symptomatic infections,[29] and if hypothetically the *bla*TEM gene occurs more frequently in these same species, it would be possible to speculate that the high prevalence of *bla*TEM in abscesses is coincidental. Further studies are required to clarify this issue.

Noteworthy was also that the *tet*M gene was significantly more prevalent in asymptomatic cases. The mechanism of antibiotic resistance encoded by *tet*M gene is ribosomal protection and this gene has been very prevalent in oral samples.[2–5] Similar to this study, the genes *tet*M and *tet*W were also commonly found in root canal infections in a previous study.[20] The high prevalence not only of *tet*M but also of *tet*W calls into question the use of tetracyclines as irrigants during root canal treatment. Theoretically, not only should the efficacy of these antibiotics be reduced, but they might select for resistant strains. Clinical implications of these phenomena require further elucidation.

Resistance to erythromycin has been widely shown for endodontic isolates.[15–17] It is most commonly due to the acquisition of *erm* genes which codes for rRNA methylases. In the present study, the *erm*C gene was found in one-fourth of both acute and asymptomatic cases. After root canal instrumentation of the asymptomatic cases, two samples were still positive for this gene, while it was eliminated from 4 other cases.

The second purpose of this study was to examine the ability of chemomechanical preparation to reduce the number of cases positive for the target resistance genes. After root canal instrumentation with NiTi instruments using NaOCl as the irrigant, about 60% of the cases still had detectable bacteria. Most cases positive for antibiotic resistance genes were rendered negative after chemomechanical debridement. This confirms that endodontic treatment is effective in eliminating a possible reservoir of antibiotic resistance gene in the majority of cases. However, in about 30% of the previously positive cases, resistance genes were still detected. It is not clear from our experiment whether these genes remained inside the owner bacterial cell that survived treatment or remained free in the environment. The results from PCR using universal bacterial primers suggest that both conditions may have occurred, since not only cases that were positive for universal PCR also yielded positive results for resistance genes; instead, two negative cases for 16S rRNA gene were positive for resistance genes. Further interappointment medication and obturation are expected to contribute still more to elimination of bacteria carrying these genes. This requires further investigation.

In conclusion, acute and chronic endodontic infections were shown to harbour species carrying resistance genes for 3

classes of widely used antibiotics. These infections are characterized by multispecies bacterial biofilms and cells within biofilms are in close contact with one another. This makes cells within biofilms be very conducive to gene transfer,[30,31] which may favour the spread of resistance genes to other species. Therefore, it is important that root canal treatment eliminates these biofilms and the cells carrying resistance genes. In most cases, treatment was effective in this regard, but there were a few canals in which these genes persisted. The implications of such persistence are unknown but are expected to be minimal, if any, following further intracanal medication, root canal filling and coronal restoration. Direct detection of resistance genes in abscesses is possible and may be a potential method for rapid diagnosis and proactive therapy. Further studies evaluating the outcome of antibiotic therapy dictated by the results of antibiotic resistance gene detection should be of great value.

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Competing interests

None declared.

Ethical approval

The study protocol was approved by the Ethics Committee of the Estácio de Sá University, under the reference number 106-03.

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