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LUDMILA COUTINHO MORAES

PRESENÇA DE GENES DE RESISTÊNCIA A AGENTES ANTIMICROBIANOS EM SALIVA, BIOFILME SUPRAGENGIVAL E CANAIS RADICULARES INFECTADOS

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Mestre em Odontologia, área de concentração em Clínica Odontológica/ Endodontia. Linha de pesquisa: Epidemiologia, Etiopatogenia e Repercussão das Doenças da Cavidade Bucal e Estruturas Anexas.

Orientador: Prof. Dr. Marcus Vinícius Reis Só

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Dedico este trabalho…

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“C'est le temps que tu as perdu pour ta rose qui fait ta rose si importante”

Antoine de Saint-Exupéry

LISTA DE FIGURAS

Artigo 1

Artigo 2

LISTA DE TABELAS

Artigo 1

Artigo 2

RESUMO

Embora diferentes estudos indiquem que há um aumento da resistência dos microrganismos aos agentes antimicrobianos prescritos, pouco se sabe a respeito da sua distribuição na cavidade oral. A presente dissertação está dividida em dois capítulos, representados por dois diferentes manuscritos. No manuscrito I, uma revisão sistemática foi realizada e teve como objetivo determinar quais genes de resistência a antimicrobianos foram pesquisados em saliva (S), no biofilme supragengival (SB) e canal radicular (RC). Os termos foram usados em várias combinações nas seguintes bases de dados eletrônicas (MEDLINE, EMBASE, ISI, SCOPUS e OPENGREY). Após seleção dos títulos e análise dos resumos, o texto integral de cada estudo foi obtido. Foram estabelecidos critérios de inclusão e exclusão. Dados epidemiológicos, características metodológicas e os resultados foram obtidos a partir dos estudos. Devido à falta de padronização da metodologia entre os trabalhos, não foi possível realizar uma meta-análise. Realizou-se análise descritiva dos dados. Um total de 151 títulos foram identificados para análise preliminar. Quarenta e nove resumos foram selecionados e 22 textos completos foram obtidos. Sete artigos contemplavam aos critérios de inclusão (S = 2; SB = 0 , e RC = 5). Vinte e seis diferentes genes-alvo foram avaliados. Os genes de resistência mais frequentemente descritos foram os relacionados às tetraciclinas e lactâmicos (5/7). Observa-se que poucos artigos foram selecionados pela estratégia de busca adotada para esta revisão sistemática. Este fato demonstra a falta de padronização nas metodologias dos estudos que utilizam técnicas moleculares para a detecção de genes de resistência bacteriana, tanto em condições de saúde ou doença. No manuscrito II, um estudo de observação clínica e laboratorial foi desenvolvido para detectar a presença de espécies-alvo de *Prevotella* e o gene *cfxA/cfxA2* em amostras de saliva (S), biofilme supragengival (SB) e canais radiculares infectados (RC). Amostras pareadas de S, SB e RC foram coletadas de 42 indivíduos. Os pacientes foram divididos em três grupos: Grupo I - sem infecção aguda/crônica primária do canal radicular (n = 15), Grupo II - presença de infecção aguda primária no canal radicular (n = 12) e Grupo III - presença de infecção crônica no canal radicular (n = 15). Foram coletadas amostras de RC apenas para os Grupos II e III. Após o isolamento do DNA, a presença de *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella tannerae* e do gene *cfxA/cfxA2* foi detectada através de PCR. Comparou-se a frequência das espécies e do gene de

resistência, considerando diferentes ambientes e condições clínicas. A análise estatística foi realizada. Todas as amostras de S, SB e RC foram positivos para a detecção de DNA bacteriano. As taxas de detecção para espécies de *Prevotella* foram: S= 53,97%; SB=47,62%, e RC=34,56%. O gene *cfxA* não foi detectado simultaneamente em três ambientes do mesmo paciente. A presença ou ausência de sintomas espontâneos não influenciou as taxas de detecção para as espécies-alvo e para o gene *cfxA/A2* em amostras de RC (teste exato de Fisher , P> 0,05). Espécies de *Prevotella* e o gene *cfxA/cfxA2* foram frequentemente detectados na saliva, placa dental e amostras de canais radiculares.

Palavras-chave: boca; saliva; placa dental; cavidade pulpar; resistência a medicamentos; Endodontia; PCR.

ABSTRACT

Although different studies indicate that there is an increased resistance of microorganisms to antimicrobial agents prescribed in Endodontics, little is known about their distribution in the oral cavity. The present dissertation was divided into two chapters, represented by two different manuscripts. In manuscript I, a systematic review was conducted and aimed to determine which antimicrobial resistance genes were investigated in saliva (S), the supragingival biofilm (SB) and root canal (RC). The terms were used in various combinations in the following electronic databases (MEDLINE, EMBASE, ISI, SCOPUS and OPENGREY). After title screening and abstract analysis, the full text of each study was obtained. The relevance of each study to the question of interest was determined through inclusion and exclusion criteria. Epidemiologic data, methodological characteristics and results were collected from the studies. Due to lack of methodology standardization among the papers, it was not possible to perform a meta-analysis. Descriptive data analysis was performed. A total of 151 titles were identified for preliminary analysis. Forty-nine abstracts were selected, and full texts of 22 studies were obtained. Seven articles matched the inclusion criteria (S=2; SB= 0; and, RC=5). Twenty-six different targeted genes have been evaluated. The most frequently investigated groups of resistance genes were related to tetracycline and lactamics (5/7). Few articles in the literature fit in the search strategy adopted by this systematic review, demonstrating the lack of standardization in the methodologies of studies using molecular techniques for the detection of bacterial resistance genes to antibiotics in oral cavity, either in health or disease conditions. In the manuscript II, an observational clinical and laboratorial study was developed to identify the presence of targeted Prevotella species and the cfxA/cfxA2 gene in samples from saliva (S), supragingival biofilm (SB) and infected root canals (RC). Paired samples of S, SB and RC were collected from 42 subjects. Patients were divided into three groups: Group I - no acute or chronic root canal infection (n = 15); Group II - presence of acute root canal infection (n = 12), and Group III - presence of chronic root canal infection (n=15). RC samples were collected for Groups II and III. After DNA isolation, the presence of *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella tannerae* and the *cfxA/cfxA2* gene was detected through gene-specific PCR. The frequency of the species and of the resistance was compared, considering different environments and clinical conditions. Statistical analysis was carried out. All

samples from S, SB and RC were positive for the presence of bacteria. The overall detection rates for *Prevotella* species were: S = 53.97%; SB = 47.62%; and, RC = 34.56%. The *cfxA* gene was not detected simultaneously in the three environments from the same patient. The presence of absence of spontaneous symptoms had not influenced the detection rates for the targeted species and for the cfxA/A2 gene in RC samples (Fisher Exact Test, P>.05). *Prevotella* species and the gene *cfxA/cfxA2* were frequently detected in saliva, dental plaque and root canal samples.

Keywords: mouth, saliva, dental plaque, dental pulp cavity, drug resistance, Endodontics, PCR.

SUMÁRIO

INTRODUÇÃO

Nos últimos anos, estudos epidemiológicos têm demonstrado, em todo o mundo, aumento nas taxas de resistência microbiana aos agentes terapêuticos empregados (Owens Jr, 2008). A emergência de resistência microbiana não respeita limites geográficos, e o trânsito populacional entre as diversas regiões do planeta tornam este um problema global (Hawkey, 2008). Dessa forma, os antibióticos representam uma das poucas classes de fármacos que têm o potencial de afetar populações, além dos efeitos sobre o paciente que está sendo tratado (Handal & Olsen, 2000). A pressão seletiva gerada por este grupo de medicamentos em comunidades microbianas de um indivíduo favorece o desenvolvimento de resistência, sendo esta relacionada principalmente a exposições prévias e repetidas a antimicrobianos (Costelloe *et al*., 2010).

O tratamento das infecções endodônticas primárias agudas no interior dos canais radiculares e tecidos apicais tem como base a abordagem local, associadas ou não à complementação com agentes antimicrobianos de uso sistêmico (Montagner, 2010). Em Endodontia, o uso de agentes antimicrobianos está indicado para o tratamento dos abscessos apicais agudos acompanhados de dor severa e celulite, linfadenite, trismo, febre, taquicardia, falta de apetite e mal-estar geral, indicando para o profissional que os sistemas de defesa do paciente não estão conseguindo controlar o processo infeccioso (Wynn & Bergman, 1994; Andrade & Souza-Filho, 2006). Os grupos de antibióticos frequentemente empregados em Endodontia como adjuvantes à terapêutica local são as penicilinas, as cefalosporinas, as tetraciclinas, as lincosamidas e os macrolídeos (Groppo *et al.,* 2006).

Para a seleção adequada de um antibiótico no tratamento de uma infecção é necessário que se tenha um conhecimento de fatores relativos aos agentes infectantes (microbiota mais comum das infecções, padrão habitual de sensibilidade dos microrganismos aos antibióticos), à natureza da infecção que se vai tratar, às características do hospedeiro que vai receber o antibiótico e também aos aspectos básicos da farmacologia do antibiótico a ser utilizado (Rocha, 2002).

Embora diferentes estudos indiquem que há um aumento da resistência dos microrganismos aos agentes antimicrobianos prescritos em Endodontia, pouco se sabe sobre a sua distribuição na cavidade oral. O presente estudo está dividido em capítulos, representados

por dois manuscritos. No primeiro capítulo, realizou-se uma revisão sistemática buscando a compilação de dados científicos que detectavam a presença de genes de resistência bacteriana a antibióticos na cavidade oral. No segundo capítulo, correspondente a estudo clínico-laboratorial transversal, buscou-se identificar a presença de microrganismos e de fatores de virulência associados à resistência microbiana em amostras de diferentes nichos da cavidade oral. Avaliou-se a influência da presença da patologia endodôntica aguda e crônica na frequência de espécies de *Prevotella* e do gene *cfxA/cfxA2*.

MANUSCRITO I

Distribution of genes related to antimicrobial resistance in different oral environments – a systematic review.

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ABSTRACT

Objective – Mechanisms of bacterial resistance to antibiotics have been attributed to the resistance genes. Different oral cavity environments can harbor specific bacterial strains and resistance genes, acting as a reservoir. This systematic review evaluated distribution of genes of bacterial resistance to antibiotics in different sites of the oral cavity.

Methods – A systematic review was performed to check all clinical studies that detected genes of bacterial resistance to antibiotics in saliva (S), supragingival biofilm (SB) and root canal (RC). The terms were used in various combinations in the electronic databases (MEDLINE, EMBASE, ISI, SCOPUS and OPENGREY). No language restriction was applied to the search. After title screening and abstract analysis, the full text of each study was obtained. The relevance of each study to the question of interest was determined through inclusion and exclusion criteria. Data regarding research group, geographic location, sample source, number of subjects included in the study, methods for sample analysis including the targeted gene groups and their detection rates were collected from the studies. Due to lack of methodology standardization among the papers, it was not possible to perform a meta-analysis. Descriptive data analysis was performed.

Results – A total of 151 titles were identified for preliminary analysis. Forty-nine abstracts, when available, were reviewed, and full texts of 22 studies were obtained. Seven articles matched the inclusion criteria (S=2; SB= 0; and, RC=5). Twenty-six different targeted genes have been evaluated. The most frequently investigated groups of resistance genes were for tetracycline and lactamics (5/7). The most tested gene was *tet*M for tetracycline resistance and it was reported in 5 papers (S=1; RC=4), followed by the genes *tet*Q. *tetW* and *cfx*A genes were searched in 4 papers each (tetracycline: S=1, RC=3; lactamics: RC=4).

Conclusion – Few articles in the literature fit in the search strategy adopted by this systematic review, demonstrating the lack of standardization in the methodologies of studies using molecular techniques for the detection of bacterial resistance genes to antibiotics in oral cavity, either in health or disease conditions.

KEY-WORDS: saliva, dental plaque, dental pulp cavity, drug resistance.

INTRODUCTION

The discovery of antibiotics in the early 20th century was not only a turning point in medicine, but in human history as well. Although antibiotics are generally considered adjunctive therapy (Flynn, 2000), their value should not be underestimated, especially when drainage cannot be achieved or the infection shows signs of local extension or systemic involvement (Stefanopoulus *et al.,* 2004). Beta-lactams, tetracyclines and macrolides have been used in Endodontics especially for the treatment of acute apical abscesses associated with systemic involvement, spreading infections, abscesses in medically compromised patients who are at increased risk of a nonoral secondary infection following bacteremia, prophylaxis for medically compromised patients during routine endodontic therapy, and replantation of avulsed teeth (Siqueira, 2011).

Antibiotic resistance is a phenomenon of crucial importance in the treatment of diseases caused by pathogenic microorganisms (Vester & Long, 2009). Bacterial resistance to antibiotics is multifactorial. Antibiotic resistance occurs by both intrinsic defenses (Wise *et al*., 1998) and by genetic mutation in bacteria. The mutations happen both spontaneously and as a result of horizontal gene transfer. Planktonic bacteria typically acquire resistance in 1 of 4 ways: (1) alteration of a drug's target site, (2) inability of a drug to reach target site, (3) inactivation of an antimicrobial agent, or (4) active elimination of an antibiotic from the cell. Gene transfer via plasmids results in a change of the microbe's DNA, and the resistant genes are passed to subsequent generations. (Harvard & Ray, 2011). It can be also attributed to long-term, indiscriminate and repetitive use of broad-spectrum antibiotics. The nontherapeutic use in agriculture, and careless disposal of waste by the pharmaceutical industry can contribute to the problem (Harvard & Ray, 2011).

Mechanisms of bacterial resistance to antibiotics have been attributed to the resistance genes. Specific groups of genes have been found in the oral cavity, which can operate as a reservoir for antibiotic resistance genes (Sommer *et al*. 2009). The objective of this systematic review was to evaluate the distribution of genes of bacterial resistance to antibiotics in different sites of the oral cavity. To build a search strategy was formulated a question to be answered in this review [in PICO (Population, Intervention, Comparison, Outcome) format],

defining inclusion and exclusion criteria. The question may be frame as follows: in different human oral sites (saliva, supragingival biofilm or root canals), how are distributed genes associated with bacterial resistance to antibiotics frequently prescribed in Dentistry?

MATERIALS AND METHODS

A systematic review was performed to check all clinical studies that detected genes of bacterial resistance to antibiotics in the oral cavity (in three sites of oral cavity: saliva; supragingival biofilm only and root canal with primary endodontic infection). The terms were used in various combinations in the electronic databases as follows: MEDLINE (PUBMED); EMBASE; ISI; SCOPUS and OPENGREY. No language restriction was applied to the search. **Figure 1** describes the search strategy adopted in the study.

Figure 1. The search strategy adopted for the study, presenting the MeSH keywords and search terms for the presence of resistance genes to antimicrobial agents in the oral environment.

Terms: ("mouth"[MeSH Terms] OR "mouth"[All Fields] OR ("oral"[All Fields] AND "cavity"[All Fields]) OR "oral cavity"[All Fields]) AND ("drug resistance, microbial"[MeSH Terms] OR ("drug"[All Fields] AND "resistance"[All Fields] AND "microbial"[All Fields]) OR "microbial drug resistance"[All Fields] OR ("antibiotic"[All Fields] AND "resistance"[All Fields]) OR "antibiotic resistance"[All Fields]) AND ("genes"[MeSH Terms] OR "genes"[All Fields] OR "gene"[All Fields])

After title screening and abstract analysis, the full text of each study was obtained. The relevance of each study to the question of interest was determined through inclusion and exclusion criteria. The full texts of the articles were reviewed by 2 reviewers (F.M. and L.C.M) based on the following inclusion criteria: (1) clinical studies in healthy or with oral disease adults (cross-section or longitudinal studies); (2) studies that detected bacterial resistance genes of antibiotics by molecular techniques; and (3) studies with samples collected from oral cavity (saliva; supragingival biofilm or root canal with primary endodontic infection). Reviews, *in vitro* studies, and

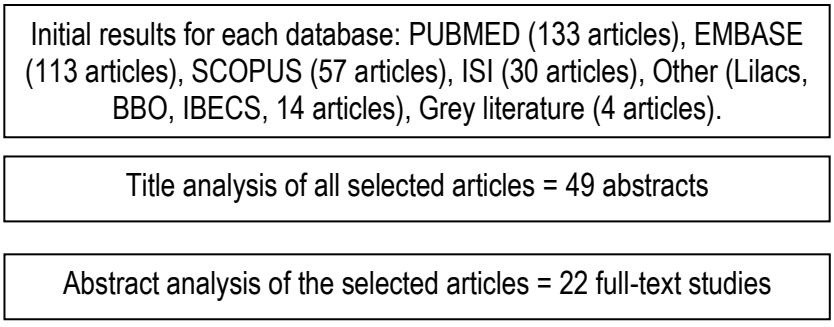
studies that mixed oral fluids with fluids from other sites of the body were not included in this review. Exclusion criteria comprised: (1) clinical studies in children; (2) Literature reviews; (3) *in vitro* studies; (4) studies that mixed oral fluids with fluids from other sites of the body; (5) studies that detected genes in other sites of the oral cavity besides those cited in the inclusion criteria; (6) studies that did not use molecular methods for detection of the presence of the resistance genes; and (7) lack of measurable data.

Data regarding research group, geographic location, sample source, number of subjects included in the study, methods for sample analysis including the targeted gene groups and their detection rates were collected from the studies. Due to lack of methodology standardization among the papers, it was not possible to perform a meta-analysis. Descriptive data analysis was performed.

RESULTS

A total of 151 titles were identified for preliminary analysis for all the databases. After title screening, 49 abstracts, when available, were reviewed, and full texts of 22 studies were obtained (**Figure 2**). All the selected articles are listed in **Table 1** and, if present, the reasons for its exclusion were also shown.

Figure 2. Results after the search strategy developed to find studies related to the presence of resistance genes to antimicrobial agents in different oral environments.



Tables 2 and **3** showed information about the selected studies. They were carried out in different periods of time, and evaluated samples collected from different geographical location. Seven articles matched the inclusion criteria, despite the oral environment. There were 2 articles for saliva samples and 5 studies on primary endodontic infections. According to the including criteria, there was no paper that assessed the presence of resistance genes in supragingival plaque samples. A total of 26 different genes have been evaluated in these 7 articles, as follows:

- Beta-lactam: *blaTEM*; *blaZ*; *cfxA*; *cfxA2*; *blaCMY2*; *ampC*; *mecA* (methicilin); *pbp1A*; *pbp2X*; *pbp2B*;
- Macrolide: *ermA*; *ermB*; *ermC*; *ermE*; *ermV*;
- Tetracyclin: *tetM*; *tetO*; *tetQ*; *tetS*; *tetW*;
- Vancomicin: *vanA*; *vanB*; *vanC1*; *vanC2/3*; *vanD*; *vanE*;

The most frequently investigated groups of resistance genes were for tetracycline and lactamics (5 out of 7 articles). The most tested gene was *tet*M for tetracycline resistance and it was reported in 5 papers (1 in saliva and 4 in root canal samples), followed by the genes *tet*Q. *tetW* and *cfx*A genes were searched in 4 papers each (tetracycline: 1 on saliva, 3 on root canal samples; beta-lactamics: 4 studies on root canal samples).

The frequency for the detection of the *tet*M gene ranged from 5% to 100% (5%-60% on four root canal samples and 100% in 1 saliva samples study). The tetracycline resistance genes *tetQ* and *tetW* frequencies were detected in 0 to 10% and 20 to 29% of the root canal samples, respectively. One study that evaluated the *tetQ* and *tetW* genes in saliva did not provide measurable data, regarding its frequency in this specific environment.

The presence of genes associated with the resistance of lactamic agents was assessed in the oral environment. The gene *cfxA/cfxA2* was searched in 4 studies and its detection rate ranged from 0% to 40% in root canal samples. *bla*TEM was searched also in root canals (3 articles), ranging from 17% to 43% of the samples. The genes *pbp1a*, *pbp2x*, and *pbp2b* were observed in saliva samples, with an overall positive detection in 52% of the samples.

Macrolide resistance genes were surveyed in root canal (3 articles) and saliva samples (1 article). The *erm*C gene was detected in 10 to 24% of endodontic infections. Both *erm*A and *erm*B genes were not found in root canal samples. However, *ermB* was found in high frequency in saliva.

Vancomycin resistance genes were searched in root canal samples, but no positive results were observed.

DISCUSSION

The methods employed in current studies to depict the structure and virulence factors associated with microbial communities are labor-intensive and expensive. Therefore, these studies have been embracing a small number of samples obtained from a restrict population source. Systematic reviews have been suggested as a tool for a critical analysis of the literature. They can be associated with statistical methods such as meta-analysis to a summation of the compiled results from studies with small sample sizes, serving as a guide for evidence-based practice (Spangberg, 2007). This systematic review aimed to identify, evaluate and synthesize all clinical studies that met the pre-specified eligibility criteria to answer the research question: "How genes of bacterial resistance are distributed in different oral environment (saliva, supragingival biofilm and root canals infected)?".

In the present study, broad topics were used in the electronic databases to reduce the possibility of overlooking a study. Additional databases and the grey literature sources were also employed, followed by a careful hand search to avoid the risk of ignore studies. Seven articles matched the inclusion criteria: 2 articles for saliva samples, no study for supragingival plaque, and 5 studies on primary endodontic infections. Of the five selected articles that evaluated the presence of resistance genes in root canals, three were directly examined from pooled samples of acute endodontic infections (Jungermann *et al.* 2011; Roças & Siqueira, 2012; Roças & Siqueira, 2013), while two tested bacterial strains isolates from those infections (Iwahara *et al.* 2006; Lins *et al.* 2013). There was any gene that has been tested in all studies. For example, the genes related to resistance to beta-lactams were tested in four out of the five root canal papers. The critical evaluation of the selected studies showed few factors connecting them, such as diversity of patient characteristics, lack of standardization for sampling the same environment, the diversity of methods for gene detection, and the samples size was too small. Several studies were also excluded because did not provide full data in the result section. Therefore, it was very hard to

conduct a systematic review and the meta-analysis.

During the literature search for clinical studies that evaluated the distribution of bacterial resistance genes to antibiotics in the oral cavity, there was no consensus regarding the patient criteria for inclusion, sampling and the methods for sample evaluation. Studies that evaluated saliva samples comprised only subjects without endodontic diseases. Seville *et al.* (2009) compared the presence of resistance genes in different European countries, not confining the evaluation in a specific geographic area. Nakayama & Takao (2003) investigated the percentage of beta-lactams resistance genes in isolates of *Streptococcus* spp. Jungermann *et al.* (2011) reported the also the presence of resistance genes in samples collected after chemo mechanical preparation, however only the results obtained before the treatment were considered. Considering the selected articles that evaluated root canal samples, three were conducted in Brazil, one in Japan and one in the USA. The most detected genes were: *bla*-TEM; *cfx*A; *tet*W; *tet*M and *ermC*, with a high range for their detection values.

Few articles in the literature fit in the search strategy adopted by this systematic review, demonstrating the lack of standardization in the methodologies of studies using molecular techniques for the detection of bacterial resistance genes to antibiotics in oral cavity, either in health or disease. Conducting systematic reviews, using rigid methodology, in order to minimize bias, aims to produce reliable results that can be used for decision making, especially when it is possible to perform a meta-analysis.

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Table 1. Full-text articles analysis and determination of exclusion criteria.

Author/year	Country	Title	Oral Site	Criteria
Andres *et al.* 1998	Spain	Antimicrobial Susceptibilities of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* spp. isolated in Spain.	Dental plaque	4
Arseze *et al*. 2000	Italy	Detection of tetQ and ermF antibiotic resistance genes in *Prevotella* and *Porphyromonas* isolates from clinical specimens and resident microbiota of humans.	Gingival biofilm	4
Díaz-Mejía *et al.* 2002	Mexico	Antibiotic resistance in oral commensal streptococci from healthy Mexicans and Cubans: resistance prevalence does not mirror antibiotic usage.	Oral samples	7
Diaz-Torres *et al.* 2006	UK	Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach.	Saliva and dental plaque	5
Furuya *et al*. 2007	Japan	Antimicrobial resistance in clinical isolates of Neisseria subflava from the oral cavities of a Japanese population.	Oral cavity samples	5
Garcia *et al.* 2008	Spain	Genetic determinants for cfxA expression in *Bacteroides* strains isolated from human infections.	Humans infections	4, 5
Ioannidis *et al.* 2009	Greece	Prevalence of tetM, tetQ, nim and bla(TEM) genes in the oral cavities of Greek subjects: a pilot study.	Dental plaque and tongue samples	5
Kim *et al.* 2011	USA	Characterization of antibiotic resistance determinants in oral biofilm.	Dental plaque	5
Penas *et al*. 2013	Brazil	Analysis of Genetic Lineages and Their Correlation with Virulence Genes in Enterococcus faecalis clinical Isolates from Root Canal and Systemic Infections.	Root canal	5
Reynaud *et al*. 2006	Sweden	Antimicrobial susceptibility and molecular analysis of Enterococcus faecalis originating from endodontic infections in Finland and Lithuania.	Root canal	5, 6
Tribble *et al*. 2010	USA	Genetic analysis of mobile tetQ elements in oral *Prevotella* species.	Oral *Prevotella* species	5
Villedieu *et al* 2004	UK	Genetic Basis of Erythromycin Resistance in Oral Bacteria.	Saliva and dental plaque	5
Villedieu *et al*. 2003	UK	Prevalence of tetracycline resistance genes in oral bacteria.	Saliva and dental plaque	5
Warburton *et al*. 2009	UK	Characterization of tet(32) genes from the oral metagenome.	Oral cavity samples	6
Zhu *et al.* 2010	China	Prevalence, phenotype, and genotype of Enterococcus faecalis isolated from saliva and root canals in patients with persistent apical periodontitis.	Saliva and root canal	5, 6

(1) clinical studies in children; (2) Literature reviews; (3) *in vitro* studies; (4) studies that mixed oral fluids with fluids from other sites of the body; (5) studies that detected genes in other sites of the oral cavity besides those cited in the inclusion criteria; (6) studies that did not use molecular methods for detection of the presence of the resistance genes; (7) lack of measurable data.

Table 2. Selected articles for literature review for the presence of resistance genes in saliva samples, according to the inclusion criteria.

Author/year	Country	Sample source (n. of patients)	Sample characteristics	Method	Genes (% of detection)	Additional information
Nakayama *et al*. 2003	Japan	Healthy subjects (52 patients)	Non-stimulated saliva	E-Test/PCR	▪ Betalactamics: pbp1a, pbp2x, pbp2b (52%)	All three genes tested together.
Seville *et al.* 2009	UK, France, Italy, Finland, Norway, Scotland	Healthy subjects (20 from each country, 120 total)	Stimulated saliva	PCR	▪ Tetraciclines: tetM, tetW, tetQ, tetO ▪ Macrolides: ermB, ermV, ermE (values were not expressed in percentage)	tetM was found in all samples analyzed. tetQ second most commonly detected tet gene. tetO was only present in the Norwegian and Finnish metagenomes at very low levels. The most abundant Erm genes were ermB and ermV. No erm genes were detected in the English saliva sample.

Table 3. Selected articles for literature review for the presence of resistance genes in endodontic samples, according to the inclusion criteria.

Author/year	Country	Sample source	Sample characteristics	Method	Genes (% of detection)
Iwahara *et al.* 2006	Japan	Root canal Acute apical abscess	Root canal (15 samples) Acute apical abscess (72 samples)	Nitrocefin and Real-time PCR	▪ 87 samples: ▪ Betalactamics: *cfx*A; *cfxA2* (44,82%)* percentage calculated from data reported by the author
Jungermann *et al.* 2011	USA	Root canal	30 subjects with primary endodontic infection (S1 - preoperative sample, and S2 – preobturation sample) 15 subjects with secondary endodontic infection (S1 - preoperative sample, and S2 – preobturation sample)	E-test and PCR	▪ Primary infection (preoperative) ▪ Betalactamics: *blaTEM*-1 (43%); *cfxA* (17%); , *blaZ* (0) ▪ Tetraciclines: *tetM* (17%), *tetW* (20%); *tetQ* (10%) ▪ Vancomycins: *vanA*; *vanD*; *vanE* (not detected)
Lins *et al.* 2013	Brazil	Root canal	20 isolates of E. faecalis recovered from 43 patients with primary endodontic infections	E-test and PCR	▪ *ermA*(0); *ermB*(0); *tetM* (60%); *tetL*(20%); *vanA*(0), *vanB*(0); *vanC1*(0); *vanC2*/3(0)
Rôças & Siqueira 2012	Brazil	Root canal	41 strains, isolated from 26 subjects	Real-time PCR	▪ Betalactamics: *blaCMY*-2; *blaTEM* (17%); *blaZ*(0), *ampC*(0), *cfxA* (2%), *mecA*(0) ▪ Macrolides: *erma*(0), *ermB*(0), *ermC* (10%) ▪ Tetraciclines: *tetM* (5%), *tetO*(0), *tetS* (2%), *tetQ*(0), *tetW* (10%)
Rôças & Siqueira 2013	Brazil	Root canal	25 abscess (acute) 24 asymptomatic apical periodontitis	Real-time PCR	▪ Acute: *blaTEM* (24%); *ermC* (24%); *cfxA*(0); *tetQ*(0) ▪ Asymptomatic: *tetM* (42%); *tetW* (29%) *cfxA(*0); *tetQ(*0)

MANUSCRITO II

Frequency of targeted *Prevotella* species and the gene for the resistance to lactamic agents in oral environments and infected root canals

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ABSTRACT

Aim – There is an increased resistance of microorganisms to antimicrobial agents prescribed in Endodontics. The *cfxA/cfxA2* gene associated with the resistance to lactamic agents was detected in *Prevotella* strains, however little is known about their distribution in the oral cavity. The aim of this study was to identify the presence of targeted *Prevotella* species and the *cfxA/cfxA2* gene in samples from saliva (S), supragingival biofilm (SB) and infected root canals (RC).

Methods – Paired samples of S, SB and RC collected from 42 subjects. Patients were divided into three groups: Group I - no acute or chronic root canal infection (n = 15); Group II - presence of acute root canal infection (n = 12), and Group III - presence of chronic root canal infection (n=15). RC samples were collected for Groups II and III. After DNA isolation, the presence of *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella tannerae* and the *cfxA/cfxA2* gene was detected through gene-specific PCR. The frequency of the species and of the resistance was compared, considering different environments and clinical conditions. Statistical analysis was carried out.

Results – All samples from S, SB and RC were positive for the presence of bacteria. The overall detection rates for *Prevotella* species were: S = 53.97%; SB = 47.62%; and, RC = 34.56%. The *cfxA* gene was not detected simultaneously in the three environments from the same patient, however it was present in: S = 23.81%; SB = 28.57%; and, RC = 7.41%. *P. intermedia* had a high frequency in saliva samples from G3 patients (Pearson chi-square test, P=.011). S samples from G1 had higher detection rates than for G3 and G2 (Pearson Chi-square test, P=.003) for *P. nigrescens*. For the SB, *P. nigrescens* was detected at high levels in G1 (Pearson Chi-square Test, *P*=.000). The presence of absence of spontaneous symptoms had not influenced the detection rates for the targeted species and for the *cfxA/A2* gene in RC samples (Fisher Exact Test, P>.05).

Conclusion – *Prevotella* species and the gene *cfxA/cfxA2* were frequently detected in saliva, dental plaque and root canal samples. The presence of an acute primary endodontic infection did not seem to promote high values for the detection of the *cfxA/cfxA2* in the supragingival biofilm and also inside root canals.

Keywords: Drug resistance, Mouth, Endodontics, Molecular biology.

INTRODUCTION

The endodontic infection is heterogeneous and caused by microorganisms that invade and colonize the necrotic pulp tissue at first, and mainly consists of strict anaerobes, some being difficult to grow, previously unidentified or unculturable species (Rôças *et al*., 2002; Sakamoto *et al.* 2006; Montagner *et al.* 2010a). Studies show that species belonging to the genera *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., and *Treponema* spp. are frequently detected in these infections (Montagner *et al.,* 2010b; Montagner *et al*., 2012).

In Endodontics, antimicrobial agents can be used as a complementary measure for the control of acute infections. Among them, the beta-lactam drugs are safer, but certain classes have adverse effects such as induction of hypersensitivity reactions (Owens, 2008). Bacteria can become resistant to an antimicrobial agent when the drug cannot reach its target, the drug is inactivated or the target is changed (Chambers & Sander, 1996). The most efficient mechanism of resistance to beta-lactam antibiotics is the production of enzymes (Chambers & Sander, 1996). In recent years, the isolation of bacterial strains capable of producing beta-lactamases rapidly increased due to genetic transmission intra-and inter-species (Geddes *et al.* 2007). The production of beta-lactamase enzymes is encoded by chromosomal genes strain, *cfiIA* and *cfxA*. Recently, *cfxA2* gene was cloned from a strain of *Prevotella intermedia* isolated from the oral cavity and showed 98% similarity to the sequence originally described for *cfxA* gene in *Bacteroides vulgatus* (Madinier *et al.* 2001). It was also observed that the gene *cfxA2* is associated with the degradation of beta-lactams especially by black-pigmented bacilli such as *Prevotella* spp. (Madinier *et al*. 2001). Recent studies have surveyed the presence of genes of bacterial resistance to antibiotics, including *cfxA* gene, in clinical samples from endodontic infections (Jungermann *et al.* 2011; Rôças & Siqueira 2012; Rôças & Siqueira 2013). However, none of these works relate the presence of these genes in other sites of the oral cavity in the same patient.

Although different studies indicate that there is an increased resistance of microorganisms to antimicrobial agents prescribed in Endodontics (Gomes *et al*., 2011), little is known about their distribution in the oral cavity. The aim of this study was to identify the presence

of microorganisms and virulence factors associated with antimicrobial resistance in samples from different niches of the oral cavity in different clinical situations.

MATERIAL AND METHODS

The present research was approved by the Ethic Committee in Research from the Federal University of Rio Grande do Sul (Porto Alegre, RS, Brazil). Forty-two subjects (older than 18 yrs-old) that attended the Urgency Service at the Dental Clinics (Dental School, Federal University of Rio Grande do Sul, Porto Alegre, Brazil) were selected for the present study. Before joining the study, patients were asked to sign an informed consent. None of the patients had received antibiotic treatment during the preceding 3 months. Subjects with systemic disease or teeth with periodontal probing depth greater than 4mm were not included in this study. No the patients included in the study could wear total or partial dentures.

These patients were divided into three groups according to the criteria described as follows: Group I - no acute or chronic root canal infection (n = 15); Group II - presence of acute root canal infection (n = 12), and Group III - presence of chronic root canal infection (n=15). Saliva samples (S) and supragingival biofilm (SB) were collected from patients belonging to Groups I, II and III. Root canal samples (RC) were collected for Groups II and III.

Sampling Procedure

Saliva

The non-stimulated saliva was collected in a sterile Eppendorf tube for 1 minute (Papapostolou *et al.*, 2011). Codes were assigned to the samples and these were stored in a freezer at -20 ° C.

Supragingival biofilm

The sites selected for the collection were isolated with cotton rolls and gently dried with air jets. The supragingival biofilm was collected randomly in 15 sites, using sterile microbrushes (DENTSPLY Caulk, Milford, DE, USA), which were immediately placed in sterile Eppendorf tubes. Codes were assigned to the samples and were stored in a freezer at -20 ° C. (Papapostolou *et al*., 2011)

Root canal

Samples were collected as described by Gomes *et al.* (2004). Briefly, the region of the involved tooth was anesthetized locally. All contaminants such as coronary caries and defective restorations were removed. The involved tooth received coronary polishing. It was isolated with rubber dam. Samples were taken from both external tooth surface and operative field after disinfection to guarantee the absence of contaminants.

Aseptic techniques were performed with sterile swabs moistened initially with 30% hydrogen peroxide, and then with a solution of 5.25% sodium hypochlorite for 30 seconds each. The inhibition of these compounds was performed with a solution of sterile 5% sodium thiosulfate. The canals were exposed under manual irrigation with sterile saline solution using sterile burs.

The samples of root canals were collected using three sterile paper points. In cases of multirooted tooth, samples were collected from the largest canal.

The paper points were introduced near the total length of the root canal, according to the preoperative radiographs. They were kept in position for 1 minute. If the canals were dry, they were moistened with sterile saline, in order to ensure a viable sample. Then, the paper points were introduced sequentially into Eppendorf tubes. Codes were assigned to the samples and were stored in a freezer at -20 °C.

Detection of *P. intermedia*, *P. nigrescens*, *P. tannerae*, and *cfxA/cfxA2* gene by using PCR

The DNA was isolated with QIAamp DNA Minikit (Qiagen, Valencia, CA) according to manufacturer's instructions. The DNA from all saliva, supragingival biofilm and root canal samples was first amplified with universal prokaryotic ribosomal 16S primer as described previously by Nadkarni *et al.* (2002).

The PCR amplification was used to detect the species *Prevotella intermedia*, *Prevotella nigrescens* and *Prevotella tannerae*, in samples from saliva, supragingival biofilm and root canal. Species-specific primers were used, targeted to the region of the 16S rRNA in bacterial DNA. The sequences of the oligonucleotides were obtained from literature references.

PCR reactions were processed in total amount of 10µL for each sample: 8µL containing *Taq* PCR Master Mix Kit (Qiagen, Valencia, California), 0.5 µL of a solution of 10 mM Foward Primer (Invitrogen, San Paulo, São Paulo, Brazil), 0.5 µL of a 10 mM solution of Reverse Primer (Invitrogen, São Paulo, São Paulo, Brazil), and 1 µL of DNA extracted from clinical samples.

The steps of the PCR cycle comprised an initial denaturation (95 °C, 2 min), 33-36 cycles of denaturation (94 °C, 30 sec), annealing (temperature specific for each primer set, 1 min) and extension (72 °C, 2 min) followed by a final extension (72 °C, 10 minutes). The annealing temperatures were determined from the use of specific primers and bacterial DNA from standard ATCC strains, which are subject to a temperature gradient in thermal cycler (TC-312 Thermal Cycler PCR, Techne®, Bibby Scientific Limited, Staffordshire, UK) and also based support literature.

Aliquots of bacterial DNA extracted from ATCC strains (*Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 33563, *Prevotella tannerae* ATCC 51259) and also aliquots of ultrapure water were used as positive and negative controls, respectively.

PCR was also used to detect the presence of the gene *cfxA/cfxA2* in the microbial strain, according to the protocol described by Giraud-Morin *et al.* (2003). As a positive control, the strain *Bacteroides vulgatus* CLA 341 was used. The *Escherichia coli* producing TEM-1 and SHV-1 was used as negative control. PCR reactions were processed in total amount of 10µl for each

sample: 8.6µl of *Taq* PCR Master Mix Kit (Qiagen, Valencia, California), 0.2 ml of each primer (10µM) and 1µl of microbial DNA.

The cycle of the PCR reaction involved an initial denaturation at 94 °C for 5 minutes followed by 25 cycles comprising denaturation (94 °C for 1 minute), annealing (58 °C, 1 minute) and extension (72 °C, 30 seconds). A final extension was carried out for 10 minutes at 72 °C.

The sequences of nucleotides, the specific annealing temperatures, the length of the amplified fragment and references for each primer are listed in **Table 1**.

The presence of specific amplicons in each sample to the sequences of the species studied was verified by gel electrophoresis in 1% agarose (Invitrogen, São Paulo, SP, Brazil) in buffer Trisborato 10x diluted HCl (pH 8.0) and stained with Sybr Green (Applied Biosystems, São Paulo, SP, Brazil). Positive reactions were determined by the presence of bands with the proper length visualized under ultraviolet transillumination.

Table 1. Sequences, annealing temperature, fragment length and reference to the nucleotides used in the PCR reactions.

Primer	Sequence	Anneling temp (°C)	Amplicon size (bp)	Reference
Universal 16SrRNA	5' TCC TAC GGG AGG CAG CAG T 3' 5' GGA CTA CCA GGG TAT CTA ATC CTG TT 3'	60	466	Nadkarni *et al.* (2002)
P. intermedia	5' TTT GTT GGG GAG TAA AGC GGG 3' 5' TCA ACA TCT CTG TAT CCT GCG T 3'	58	575	Slots *et al*. (1995)
P. nigrescens	5' ATG AAA CAA AGG TTT TCC GGT AAG 3' 5' CCC ACG TCT CTG TGG GCT GCG A 3'	58	804	Bogen & Slots (1999)
P. tannerae	5' CTT AGC TTG CTA AGT ATG CCG 3' 5' CAG CTG ACT TAT ACT CCC G 3'	55	550	Xia *et al.* (2000)
cfxA/cfxA2	5' GAAAAAAACAGAAAAAAACAAATC-3' 5' TTAAGATTTTACTGAAGTTTG-3'	58	966	Giraud-Morin *et al.* (2003)

Data Analysis

The data collected for each group (clinical features and gene detected) were entered into a spreadsheet and statistically analyzed using SPSS for Windows (SPSS Inc, Chicago, IL). The Pearson chi-square test was chosen to test the null hypothesis that there was no difference for the detection of a specific gene, in the same oral environment (S, SB or RC), in different groups. The Cochran's Q Test was used to test the null hypothesis that there was no difference for the detection of a specific gene, in the same group but in different oral environments (S, SB or RC). The significance level was set at 5%.

RESULTS

The mean age of the subjects was 38.7 years (range, 19-69 years). Fifty-two percent of the patients were female. All subjects from Group II reported a history of spontaneous pain and, all Group II subjects had asymptomatic apical lesions. RCs of 17 incisors, 1 canine, 3 premolars, and 6 molars were sampled. These teeth were caries free (4/27), had caries (7/27), or had restorations (14/27). Gingivitis was associated with 16/27 teeth. Thirty-three percent of the teeth belonging to Group II had mobility. Thirty-three percent of the teeth from Group III had fistula. Radiographic examination showed widening of the apical periodontal ligament space for 25% and 13%, or apical radiolucency for 58% and 86% in Groups II and III, respectively.

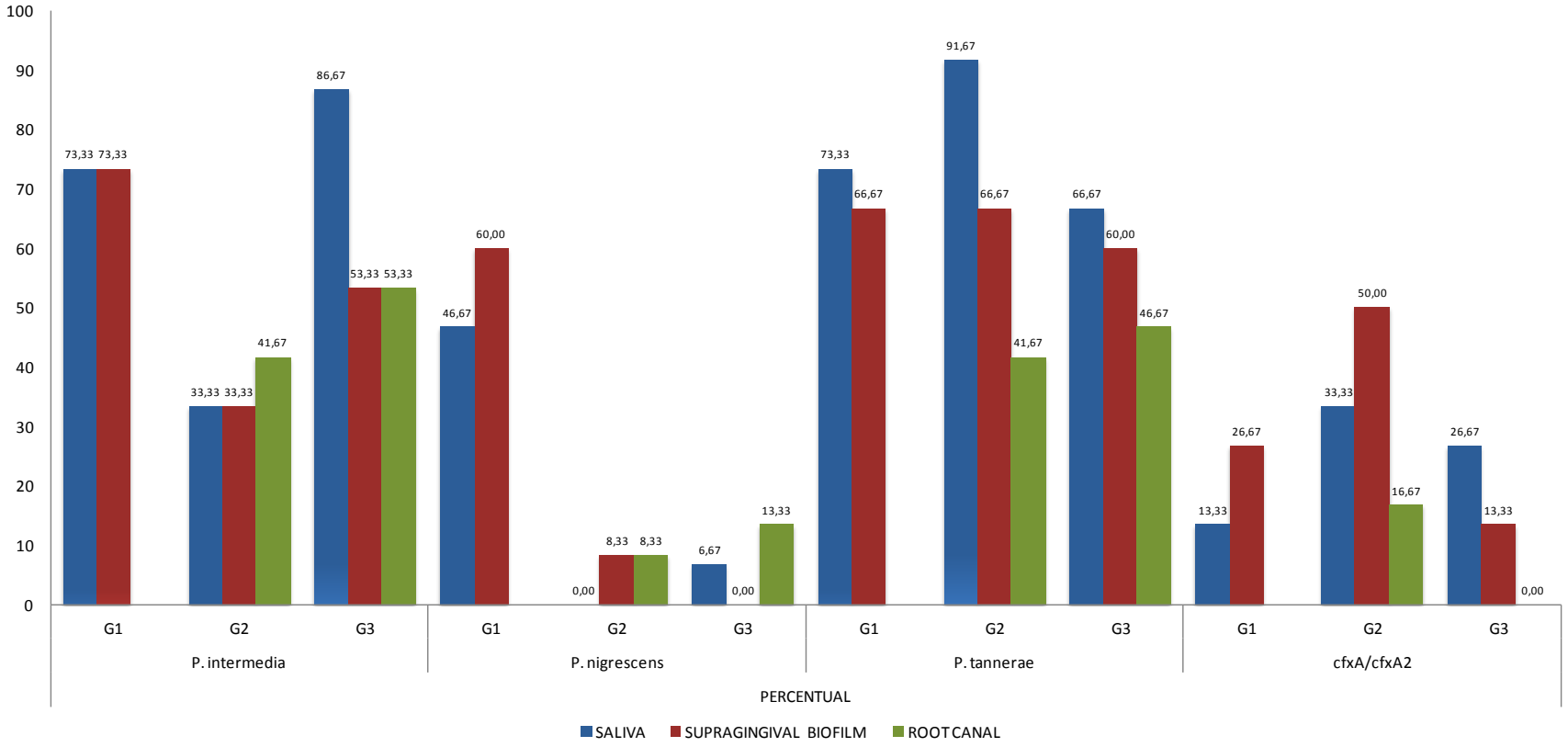
All samples from saliva, supragingival biofilm and root canal had positive results for the presence of bacteria as determined by universal 16S rRNA gene-base PCR. No positive results were obtained for the presence of bacterial DNA in control samples.

The three species of *Prevotella* tested were present simultaneously in 53.97% of S samples, in 47.62% of SB and 34.56% of the RC samples. The overall rate detection for *cfxA* gene was 23.81% of the saliva samples; 28.57% in supragingival biofilm samples, and 7.41% of the root canal. The *cfxA/cfxA2* gene was not detected simultaneously in S, SB and RC from the same

patient. However, in 5 patients it was present in saliva and supragingival samples at the same time. Only 3,7% (1/27) of the SB and RC samples from the same patient harbor the *cfxA/A2* gene simultaneously.

Figure 1 shows the detection rates for each *Prevotella* species and *cfxA/A2* gene in the groups, considering the different oral environments (saliva, supragingival biofilm and root canal samples).

Figure 1. Distribution of each *Prevotella* species and *cfxA/A2* gene in the collected samples (in percentage).



In Group I, the fifteen patients did not have acute or chronic primary endodontic infections at the time of collection. They were at the Urgency Service for other reasons such as dental caries, dental trauma, and pulpitis. Only S and SB samples were analyzed. *Prevotella* species were frequently detected in both the saliva and supragingival biofilm samples (*P.intermedia*: S = 73.33%; SB = 73.33%; *P. nigrescens* : S = 46.67%, SB = 60%; and *P. tannerae* S = 73.33%; SB = 66.67%). *cfxA/cfxA2* gene was detected in 13.33% of saliva samples and 26.67% of supragingival biofilm. There was no statistical difference for the detection of the same species or the resistance gene in the saliva and supragingival biofilm from patients belonging to Group I (Cochran's Q Test, *P*>.05)

In the samples collected from patients with acute primary endodontic infections (Group II), *P. tannerae* was frequently detected in S samples (91.67%), while *P. nigrescens* was not detected in any sample from this environment. Significant statistical difference was observed for the detection of *P. intermedia* in S, SB and RC samples (Cochran's Q Test, *P*=.048) In the supragingival biofilm, *cfxA/cfxA2* gene was detected in 50% of the samples.

In the samples collected from patient with chronic primary endodontic infections, *P. intermedia* was present in 13/15 saliva samples. No sample was positive for presence of *P. nigrescens* and *cfxA/cfxA2* gene on SB and RC samples respectively. *P. intermedia* and *P. tannerae* were present simultaneously in S, SB and RC samples in 3/15 patients. There was no statistical difference for the detection of the same species or the resistance gene in the saliva, supragingival biofilm and root canals from patients belonging to Group III (Cochran's Q Test, *P*>.05).

There was a high frequency of *P. intermedia* in saliva samples from patients presenting chronic primary endodontic infections than in patients with acute endodontic infections or in the control group (Pearson chi-square test, *P*=.011).

Positive association was observed for the presence of *P. nigrescens* in saliva, considering the presence of an acute or chronic endodontic infection (Pearson Chi-square test, *P*=.003). The presence of *P. nigrescens* was higher for the control group, followed by the saliva samples collected from patients with chronic and then acute primary endodontic infections.

There was no statistical difference among the frequencies for *P. tannerae* and the *cfxA/cfxA2* genes in saliva samples collected from patients belonging to the control group, or to the acute and chronic primary endodontic infections (Pearson Chi-square test, *P*>.05).

When the frequency of detection of *P. intermedia*, *P. tannerae* in and cfxA/cfxA2 gene in supragingival biofilm was compared, there was no statistical significant difference among the tested groups (Pearson Chi-square Test, *P*>.05). However, *P. nigrescens* was most frequently detected in the supragingival biofilm from patients without primary endodontic disease than in patients with acute or chronic root canal infections (Pearson Chi-square Test, *P*=.000).

The presence or absence of spontaneous symptoms had not influenced the detection rates for the targeted species and for the *cfxA/A2* gene in RC samples (Fisher Exact Test, *P*>.05). There was no statistical difference for the detection of *P. intermedia*, *P. nigrescens*, *P. tannerae* and the *cfxA/A2* gene in root canal samples collected from Group II and Group III patients.

DISCUSSION

Although it is known that infected root canals can harbor resistance genes against antimicrobial agents (Jungermann *et al.,* 2011; Rôças & Siqueira, 2013), there is little evidence on their distribution in the oral environments associated with these infections. Using gene-directed PCR allowed comparisons to be made of the prevalence of some *Prevotella* species and the cfxA/cfxA2 gene related to resistance to lactamics in saliva, supragingival biofilm and root canal samples from patients with chronic and acute primary endodontic infections.

The oral cavity is a major gateway to the human body. Microorganisms colonizing one area of the oral cavity have a significant probability of spreading on contiguous epithelial surfaces to neighboring sites (Dewhirst *et al.,* 2010). Zaura *et al*. (2009) evaluated samples from dental surfaces, saliva and other intraoral niches and observed that the major proportion of oral microbiomes was common across unrelated healthy individuals, supporting the concept of a core-microbiome at health. Similar results may not be associated with oral diseased sites. Montagner *et al.* (2010a) and Montagner *et al.* (2012) reported that root canal samples and acute apical abscess samples from the same patient harbored unique microbial communities, with a low clustering behavior. The root canal environment and the apical tissues are more restricted environments than the healthy oral cavity, because the microbiota diversity was the highest in oral samples followed by the acute abscess samples then by the root canal samples (Hsiao *et al.,* 2012). Hsiao *et al*.

(2012) also highlighted that there is a lack of establishment of a baseline oral microbiome for patients sampled, as well for the examination of specific microbial taxa without consideration of the virulence genes and proteins that may be expressed. Obtaining samples from matched sites in researches that aim to evaluate root canal microbial communities allowed to understand the species and resistance gene's distribution in the healthy and diseased sites.

The present study assessed the prevalence of *Prevotella intermedia*, *Prevotella nigrescens* and *Prevotella tannerae* in samples from saliva, supragingival biofilm and root canals from patients with acute and chronic primary infections. *Prevotella* spp was the largest genus (approximately 50 species) found in an analysis of *1*6S rRNA gene sequences from 36,043 clones and over 1,000 isolates from both clone- and culture-based studies from a wide range of oral health and disease studies, including periodontitis, caries, endodontic infections, and noma, as reported by Dewhirst *et a*l. (2010). The overall rate detection for *P. intermedia*, *P. nigrescens* and *P. tannerae* were 66%, 19% and 76% of the saliva samples, respectively. In the supragingival biofilm samples, *P. intermedia*, *P. nigrescens* and *P. tannerae* were found in 54%; 23.81%, and 64% of the samples, respectively. He *et al.* (2012) evaluated matched samples from saliva and supragingival and biofilms in Chinese adults without periodontal disease and observed that 83.3% and 70.8% of the saliva and supragingival samples, respectively, harbored *P. intermedia*. Microbial profiles can vary in different oral sites, even from the same bacterial species. Simón-Soro *et al.* (2013) reported that several bacterial genera, including *Prevotella*, considerably detected in saliva are not represented in other sites such as teeth and gingivae. In the present study, there was no statistical difference between the detection rates for the targeted *Prevotella* spp in the sampled sites from G I patients. Zaura *et al.* (2009) reported that the healthy patients had a core microbiome in several oral environments for patients with neither acute nor chronic endodontic infections. However, the detection rates for the targeted *Prevotella* strains were different in matched samples of saliva, supragingival biofilm and root canal samples only for patients with acute endodontic infection. Higher levels of *P. intermedia* and *P. nigrescens* were detected in both RC and SB than in S samples from Group II. Diversity in the composition of acute endodontic infections can be explained by the presence of a highly virulent microbial community. Acute infections are related to bacterial cells in a planktonic state, with some tissue invasion ability and at high counts (Siqueira & Rôças, 2009). Furthermore, directed-species PCR protocols have a cell

detection limit threshold (Siqueira & Rôças, 2005) that might be favored by the increased number of bacterial cells in acute infections, as compared to chronic infections.

The presence or absence of an infection in the root canal system has not affected the detection of some species in saliva or supragingival biofilm. Similar frequencies of *P. tannerae* were observed in saliva samples for all groups of patient. *P. intermedia*, *P. tannerae* and *cfxA/cfxA2* detection rates in the supragingival biofilm were not affected by the presence/abscense of an endodontic infection. A unique behavior was observed for the *P. nigrescens*. Comparing the detection rates of *P. nigrescens* among the groups of patients, this microorganism was most frequently detected in saliva and supragingival biofilms from subjects without acute/chronic primary endodontic infections. Results reported by Teles *et al. (2012*) suggested that *Prevotella nigrescens* had an increase for its detection rates in supragingival biofilms collected from healthy patients with no periodontitis, especially after a 7 day-period.

Several studies have been reported that there is a significantly higher diversity of bacteria in root canals associated with acute endodontic infections when compared to asymptomatic chronic infections (Santos *et al*., 2011; Saito *et al*., 2009; Jacinto *et al*., 2003). In the present study, there was no statistical difference between the detection rates for *P. intermedia*, *P. nigrescens*, and *P. tannerae* in root canal samples from acute or chronic endodontic infections samples (G II and G III). Siqueira *et al.* (2001) found 7.4% of positive samples for *P. nigrescens*, and 5.6% for *P. intermedia* from 54 root canal samples. Fouad *et al*. (2002) observed a lower detection frequency for *P. intermedia* than for *P. nigrescens*. Gomes *et al.* (2005) found rates of 56% and 32% of *P. intermedia* and *P. nigrescens* in 50 cases of primary endodontic infection. Seol *et al.* (2006) reported a low frequency of *P. tanneare* in root canal samples when compared to other *Prevotella*, such as *P. intermedia* and *P. nigrescens*. In the present study, *P. tannerae* was frequently detected in samples from supragingival biofilm and root canal samples. Xia *et al*. (2000) reported that 60% of the samples from endodontic infections were positive for the presence of *P. tannerae*. Baumgartner *et al.* (2004) observed that there was a low prevalence of *P. tannerae* in samples collected from acute apical abscesses in patients from Brazil and United States (61% and 13%, respectively). Montagner *et al.* (2012) have no positive detection to *P. tannerae* in samples isolated from root canals with primary acute infections. Therefore, such variation in the results might be associated with patient selection, sampling methods, DNA isolation and PCR protocols.

Geographical location seems to influence the bacterial prevalence on endodontic infections (Baumgartner *et al*., 2004), even for samples collected from the same country.

Gomes *et al.* (2011) reported that *P. intermedia/nigrescens* isolates had been demonstrating high susceptibility to lactamic agents such as penicillin V, amoxicillin and amoxicillin + clavulanic acid over a 9 year-period. When compared to other anaerobic organisms, *Prevotella* species remain the most prevalent anaerobes producing beta-lactamase (Hecht, 2006). According to Fosse *et al.* (1999), *Prevotella* are the main beta-lactamase-producing species in odontogenic infections. beta-lactamasis production is encoded by cromossomal genes such as cfxA/cfxA2, cblA, cepA, and cfiA. *P. intermedia* carry *cfxA* genes and their presence is mostly related to the origin of the strains (Giraud-Morin *et al.* 2003). Handal *et al.* (2005) demonstrated the occurrence of *cfxA* and *cfxA2* genes in 100% of beta-lactam positive *Prevotella* strains from American and Norwegian patients with periodontal diseases.

The oral environment seemed to be a reservoir for *cfxA/cfxA2* positive bacteria. It was also observed that 18/42 (42.86%) of the patients harbored the *cfxA/cfxA2* gene. However, it should be emphasized that it is important not only to describe the presence of the resistance gene but also to determine if the bacteria are functionally resistant to antibiotics. There was no patient with simultaneous detection of the *cfxA/cfxA2* in the saliva, supragingival plaque and root canal matched samples. In the present study, the targeted gene was detected in both saliva and supragingival biofilm samples from all the groups. There are no reports in the literature regarding the frequency of *cfxA/cfxA2* only in saliva samples. In the present study, the overall detection rate for the targeted gene was 23.81%. According to the literature, the detection rates of *cfxA/cfxA2* in root canals samples from teeth with acute primary infections range from 0% to 17% (Rôças & Siqueira, 2013; Rôças & Siqueira, 2012; Jungermann *et al*., 2011). In the present study, only root canal samples from acute endodontic infections had the *cfxA/cfxA2* gene (16.7% of the samples). No root canal samples from chronic primary endodontic infections had the resistance gene. Supragingival biofilm had a higher number of *cfxA/cfxA2* positive samples than saliva and root canal, as observed in samples obtained from patients with teeth presenting acute primary endodontic infections.

The oral environments could be heterogeneous in their characteristics (nutrients, oxygen availability, pH) and can exert selective pressure (Simón-Soro *et al*., 2013), producing

different bacterial community profiles, influencing also the presence of specific virulence factors, such as resistance genes. In conclusion, *Prevotella* species and the gene *cfxA/cfxA2* were frequently detected in saliva, dental plaque and root canal samples. The presence of an acute primary endodontic infection seemed to promote high values for the detection of the *cfxA/cfxA2* in the supragingival biofilm and also inside root canals.

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CONSIDERAÇÕES FINAIS

Baseado nos achados destes dois estudos pode-se concluir que:

1. Poucos artigos se ajustaram nos critérios de busca propostos, o que demonstrou a falta de padronização nas metodologias de estudos utilizando técnicas moleculares para a detecção de genes de resistência bacteriana a antibióticos em cavidade oral, seja na saúde ou na doença, impossibilitando, assim, a realização de uma meta-analise dos dados. Todavia, a descrição dos dados encontrados nos permite um entendimento prévio de como genes de resistência bacteriana a antibióticos se distribuem na cavidade oral.

2. As espécies de *Prevotella* e o gene *cfxA/cfxA2* foram frequentemente detectados na saliva, placa dental e amostras de canais radiculares, quando se utilizou o método de PCR gene-específico. A presença de uma infecção endodôntica primária aguda pareceu não influenciar na frequência de detecção da *cfxA/cfxA2* no biofilme supragengival e também no interior dos canais radiculares.

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ANEXO I – Documento de aprovação do CEP-UFRGS.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Alteração no Projeto "DETECÇÃO DE Prevotella spp., Porphyromonas spp. E DO GENE ASSOCIADO À PRODUÇÃO DE LACTAMASES EM INFECÇÕES ENDODÔNTICAS AGUDAS"

Pesquisador: FRANCISCO MONTAGNER

Área Temática:

Versão: 1

CAAE: 08375712.1.0000.5347

Instituição Proponente: Universidade Federal do Rio Grande do Sul/ Pró-Reitoria de Pesquisa -

DADOS DO PARECER

Número do Parecer: 137.263

Data da Relatoria: 11/10/2012

Apresentação do Projeto:

O projeto de pesquisa foi aprovado pelo CEP UFRGS em 25/01/2012. Porém, os pesquisadores solicitam a inclusão de um grupo adicional de pacientes no projeto de pesquisa aprovado. Assim, ajustes foram feitos no texto do projeto, não para modificar o que foi aprovado, mas para incluir o novo grupo a ser avaliado no estudo.

Objetivo da Pesquisa:

Com a inclusão de um novo grupo de pacientes houve ajustes no texto dos objetivos específicos. Foi adicionado o seguinte item ao texto: "Detectar a presença dos microrganismos Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Prevotella tannerae em amostras de saliva e biofilme microbiano supragengival em pacientes que não apresentam patologia periapical. Ainda, nos objetivos específicos foi adicionada a palavra "crônico", pois o estudo avaliará a presença dos microorganismos descritos anteriormente e do gene cfxA/cfxA2 também em pacientes acometidos por patologia periapical crônica.

Avaliação dos Riscos e Benefícios:

Os riscos e benefícios estão devidamente mostrados no texto do projeto de pesquisa.

Comentários e Considerações sobre a Pesquisa:

O projeto de pesquisa foi adequadamente ajustado com a inclusão do novo grupo de pacientes a ser estudado.

Endereço: Av. Paulo Gama, 110 - 2º andar do Prédio da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

ANEXO I – Documento de aprovação do CEP-UFRGS (Continuação).

Considerações sobre os Termos de apresentação obrigatória:

Os pesquisadores anexaram carta solicitando a aprovação das mudanças e destacando as alterações feitas no projeto de pesquisa.

Recomendações:

Recomenda-se a aprovação da nova versão do projeto de pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Não há sugestão de mudanças na nova versão do projeto de pesquisa. Assim, recomenda-se aprovação.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Parecer adequado, encaminhe-se.

PORTO ALEGRE, 01 de Novembro de 2012

Assinador por:
José Artur Bogo Chies
(Coordenador)

Endereço: Av. Paulo Gama, 110 - 2º andar do Prédio da Reitoria - Campus Centro
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APENDICE I - Termo de Consentimento Livre e Esclarecido fornecido ao paciente, como forme de convite para participação e autorização de sua inclusão na pesquisa clínica, conforme solicitado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, para a aprovação do projeto.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO PARA PESQUISA CLÍNICA

As informações contidas neste termo foram fornecidas pelo orientador Prof. Dr. Francisco Montagner e/ou pela aluna de pós-graduação Ludmila Coutinho Moraes, com o objetivo de convidar o voluntário a participar, ter conhecimento do tratamento de urgência a ser realizado, e de que maneira poderá contribuir para o trabalho de pesquisa. Estando ciente disso, o voluntário autoriza a sua participação, tendo concordado sem qualquer coação.

I. TÍTULO DA PESQUISA

"Detecção de Prevotella spp., Porphyromonas spp. e do gene associado à produção de lactamases em infecções endodônticas agudas"

II. JUSTIFICATIVA

Este estudo permite que se conheçam quais são os microrganismos presentes nestes casos e permitem estudar a sua sensibilidade aos antibióticos empregados na Odontologia.

II. OBJETIVOS

Este trabalho tem o objetivo de estudar as bactérias encontradas nos dentes que precisam de tratamento de canal quando a pessoa está com dor e edema na face. Descobrindo estas bactérias iremos:

a) Relacionar essas bactérias com a dor;

b) Fazer testes para ver qual antibiótico é melhor contra estas bactérias;

Isto poderá ajudar a entendermos a causa da dor. O estudo de bactérias que continuam no canal radicular com dor e no edema, permite a realização de um teste que orientará a escolha correta de um antibiótico, e se for necessário irá complementar o seu tratamento ajudando o organismo a combater a infecção.

III. PROCEDIMENTOS

A coleta de amostra de bactérias é simples e faz parte do tratamento de canal. Ela consiste em colocar um cone de papel absorvente dentro do canal no comprimento do dente que foi observado na radiografia. Este cone de papel é normalmente usado para secar o canal. Depois de secar o canal, o cone de papel será colocado num meio de cultura, que permite a conservação e o crescimento das bactérias. Neste estudo não há grupos placebo ou controle. O tratamento para a dor de origem do canal e do edema é o mesmo que os voluntários receberiam se não aceitassem em participar do trabalho, não havendo nenhum método alternativo de tratamento da dor.

IV. DESCONFORTOS OU RICOS ESPERADOS

A coleta da amostra é indolor, pois a área que receberá tratamento e o dente envolvido estarão sob efeito de anestesia local. Se você sentir dor, esta não será por causa da coleta de amostra e sim pela realização da anestesia, limpeza do dente ou mesmo causados pela infecção no canal radicular e região do edema. Este desconforto poderia ocorrer independentemente de ser realizada a coleta. Após o tratamento, se a dor e o edema persistirem, você poderá ser atendido no Plantão de Emergência da FO-UFRGS, que funciona normalmente de segunda à sexta-feira, de 8:00 às 12:00 h e de 13:30 às 17:30 h. Caso não seja possível ser atendido no Plantão, você poderá procurar os Pesquisadores responsáveis (Francisco Montagner e Ludmila Coutinho Moraes). Os telefones para contato são: 51 3308-5023 (Faculdade) ou 51 8185-3800.

V. GARANTIA DE ESCLARECIMENTOS

Você tem toda a liberdade de pedir esclarecimentos sobre a metodologia antes e durante a pesquisa, podendo ou não concordar em participar.

VI. GARANTIA DE RECUSA À PARTICIPAÇÃO OU SAÍDA DO ESTUDO

Caso você não queira participar do estudo, não haverá prejuízo ao seu tratamento, o qual será prosseguido normalmente. Se você se recusar a participar ou se você quiser desistir, em qualquer fase da pesquisa, não será penalizado e não haverá prejuízo ao seu tratamento, o qual continuará normalmente.

VII. GARANTIA DE SIGILO

Apesar dos resultados clínicos e microbiológicos serem divulgados publicamente para fins acadêmicos e científicos, será preservada a sua privacidade (seu nome não será revelado) quanto aos dados confidenciais que possam ser envolvidos na pesquisa.

VIII. GARANTIA DE RESSARCIMENTO

A pesquisa não lhe acarretará nenhum gasto previsível.

IX. GARANTIA DE INDENIZAÇÃO OU REPARAÇÃO DE DANOS

Não há riscos previsíveis associados à pesquisa, e portanto não há previsão de indenização ou reparação de danos.

Eu, _____
certifico que tenho lido as informações acima e suficientemente esclarecido(a) de todos os ítens pela Prof. Dr. Francisco Montagner e/ou Ludmila Coutinho Moraes, estou plenamente de acordo com a realização do experimento. Assim, eu autorizo a execução da pesquisa, exposta acima, em mim.

Deste termo de consentimento livre esclarecido, serão geradas duas vias, sendo uma direcionada ao voluntário e a demais para o pesquisador.

Porto Alegre, ____ de _____ de 201__.

Nome:_____
RG:_____

Assinatura: _____

ATENÇÃO: A sua participação em qualquer tipo de pesquisa é voluntária. Em caso de dúvida quanto aos seus direitos, escreva para o Comitê de Ética em Pesquisa da FO – UFRGS, endereçado a Av. Paulo Gama, 110 - 7° andar - Porto Alegre/RS - CEP: 90040-060 - Fone: (51) 3308.4085. e-mail: pro-reitoria@propesq.ufrgs.br

APÊNDICE II – Isolamento do DNA microbiano

Os procedimentos de isolamento foram realizados utilizando-se o conjunto QIAmp mini Kit. (QIAGEN, Valencia, Califórnia, EUA, Ref. 51306 – 250 reações)

. Remover 300μL da amostra e adicionar a um eppendorf de 1,5mL.

. Adicionar 180 μL de ATL e 20 μL de Proteinase K.

. Agitar e incubar a 56°C por 2 horas.

. Adicionar 200 μL de AL.

. Agitar e incubar a 70°C por 10 min em banho seco.

. Adicionar 200 μL de etanol puro.

. Agitar e transferir para os tubos com filtros/colunas.

. Centrifugar a 8000 rpm por 1 min.

. Transferir o filtro para o outro tubo vazio do kit.

. Adicionar 500 μL de AW1.

. Centrifugar a 8000 rpm por 1 min.

. Transferir a coluna para outro tubo.

. Adicionar 500 μL do AW2.

. Centrifugar a 13000 rpm por 3 min.

. Transferir o filtro para um eppendorf normal de 1,5mL com tampa.

. Adicionar 100 μL de AE e aguardar 3 minutos.

. Centrifugar a 8000 rpm por 1 minuto.

. Armazenar o DNA extraído a -20°C.

APÊNDICE III – Métodos para realização das Técnicas Moleculares de PCR Simples.

PREPARO DOS PRIMERS

Quando receber os primers, fazer a reidratação dos primers e preparar uma solução de trabalho.

1. Centrifugar o tubo com o primer liofilizado por 1 minuto, 10.000 rpm a 4°C.

2. Adicionar 1mL de água Milli-Q ou Tampão TE e verificar na bula do primer a molaridade do primer em nmoles. No final, você terá uma solução com primer na concentarção em M.

3. Esperar o primer hidratar por 2 minutos.

4. Homogeneizar no vórtex por 15 segundos.

5. Preparar solução de trabalho na concentração desejada.

IMPORTANTE

M	mM	M	nM	pM	fM
1M	10^{-3} M	10^{-6} M	10^{-9} M	10^{-12} M	10^{-16} M

A MOLARIDADE expressa a concentração de uma solução em Molar. 1 Molar = 1 mol de soluto em 1L de solução. Por sua vez,
1 mol de soluto = massa molecular em gramas do soluto. E, a Massa molecular do soluto = soma das massas atômicas que compõe o soluto.
Por isso, quando misturamos o primer (pó) que está na concentração em nmol com 1mL de água, vamos obter a concentração da mistura em M.
1 n mol / 1 mL = 1.10^{-9} mol / 1.10^{-3} L = 1.10^{-6} mol/L = 1.10^{-6} M = 1M

PREPARO DA MISTURA PARA A REAÇÃO DE PCR

Componentes para a reação de PCR:

1. PRIMER 1
2. PRIMER 2
3. *Taq* PCR Master Mix Kit
4. DNA microbiano isolado

A *Taq* PCR Master Mix Kit (Qiagen) inclui *Taq* DNA polimerase em um formato pré-misturado. Esta solução pronta para uso, também inclui o tampão de PCR, MgCl2, dNTPs e agua ultrapura em concentrações optimizadas. Apenas os *primers* e amostra do DNA necessitam de ser adicionados para configurar PCR. Devido ao formato conveniente do master mix, erros de pipetagem são minimizados, garantindo resultados de PCR altamente reprodutível.
Esta solução vai para o termociclador programado com os protocolos de reação da PCR para cada gene pesquisado, conforme descrito nos artigos de referência.

PREPARO DA MISTURA PARA A SOLUÇÃO DE PCR

COMPONENTES	**Espécies bacterianas**	**Gene resistência**
PRIMER (F)	0,5 μL	0,2 μL
PRIMER (R)	0,5 μL	0,2 μL
Taq PCR MasterMix	8 μL	8,6 μL
DNA amostra	1μL	1μL
Total	**10 μL**	**10 μL**

Em um Eppendorf de 200 μL, adicionar 9 μL do Mix + 1L da solução que contém o DNA bacteriano isolado.
Colocar as amostras no termociclador.

PREPARO DA AGAROSE

Misturar os seguintes componentes, para obter TBE 1x:
100mL de TBE 10x + 900mL de água destilada
Armazenar em um frasco para meio de cultura.
Esta solução será empregada para preparo da agarose e para preencher a cuba de eletroforese.

PREPARO DO GEL DE AGAROSE CONCENTRAÇÃO 1%
Gel de agarose (Ultra Pure TM Agarose – Invitrogen #16500-100) 100 g
Bandeja Grande (100mL)

1. 120mL de TBE 1%

2. 0,8 g de agarose.

Colocar no microondas por 1 minuto. Ver se ficou transparente. Caso necessário colocar mais alguns segundos.

Esperar esfriar um pouco.

Colocar o Sybr Green (4 µL)

Verter na bandeja.

Colocar o pente e esperar esfriar.

CUBA DE ELETROFORESE

1. A cuba está com TBE 1x.
2. Verificar a direção de corrida (NEGATIVO PARA POSITIVO)
3. Colocar o gel na cuba com os casulos voltados para o lado NEGATIVO.
4. Verificar se ele está coberto pela solução de TBE 1x.

PREENCHIMENTO DOS CASULOS COM A REAÇÃO DE PCR

1. SELEÇÃO DO MARCADOR
A seleção do marcador depende do tamanho da banda que você vai ter. Marcador: 100bp DNA Ladder (Invitrogen cat no 15628-019) 50 g
Misturar 3uL do Ladder com 2uL do x6 Loading Dye Solution em uma lâmina de vidro. Colocar esta mistura no primeiro casulo e no casulo intermediário para que possam servir como controle.

2. COLOCAÇÃO DAS AMOSTRAS NA AGAROSE
Misturar em uma lâmina de vidro 1uL de Loading Dye Solution e 5uL da reação de PCR.

3. FONTE DE ELETROFORESE
Programar a corrida para: 80 V e 120mA, por 1 hora.