

Saliva, supragingival biofilm and root canals can harbor gene associated with resistance to lactamic agents

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Abstract: This study aimed to determine the presence of *Prevotella* strains and genes associated with resistance to lactamics in different oral niches from patients with/without primary endodontic infections. Saliva (S) and supragingival biofilm (SB) were collected from three patient groups: Group I – no endodontic infection (n = 15); Group II – acute endodontic infection (n = 12); and Group III – chronic endodontic infection (n = 15). Root canal (RC) samples were collected from Groups II and III. The presence of *P. intermedia*, *P. nigrescens*, *P. tannerae* and *cfxA/cfxA2* gene was assessed by PCR. The *cfxA/cfxA2* gene was not detected in all environments within the same patient. The *cfxA/cfxA2* gene was present in 23.81% of S samples, 28.57% of SB samples, and 7.41% of RC samples. *Prevotella* species were detected in 53.97%, 47.62% and 34.56% of the S, SB, and RC samples, respectively. *P. intermedia* had a high frequency in saliva samples from Group 3. Saliva samples from Group 1 had higher detection rates of *P. nigrescens* than did Groups 2 and 3. Patients without endodontic disease had high frequencies of *P. nigrescens* in the SB samples. The presence or absence of spontaneous symptoms was not related to the detection rates for resistance genes in the RC samples. Saliva, supragingival biofilm and root canals can harbor resistant bacteria. The presence of symptomatology did not increase the presence of the *cfxA/cfxA2* gene in the supragingival biofilm and inside root canals.

Keywords: Drug Resistance; Mouth; Endodontics; Molecular Biology.

Introduction

In endodontics, antimicrobial agents are used as a complementary measure for the control of acute infections. Among these agents, beta-lactam drugs are safer, but certain classes have specific adverse effects.¹ The over- and misuse of antibiotics can promote the selection of resistant pathogens and allow their emergence and dissemination.² The most efficient mechanism of resistance to beta-lactam antibiotics is enzyme production.³ The isolation of beta-lactamase-producing strains has rapidly increased due to intra- and inter-species genetic transmission.⁴ The production of beta-lactamase enzymes is encoded by the chromosomal genes *cfIIA* and *cfxA/cfxA2*. The *cfxA/cfxA2* gene was cloned from a strain of *Prevotella intermedia* that had been isolated from the oral cavity and exhibited 98% similarity to the sequence originally

described for the *cfxA* gene in *Bacteroides vulgatus*.⁴ The *cfxA/cfxA2* gene is associated with the degradation of beta-lactamics, especially by black-pigmented bacilli, such as *Prevotella* spp.⁵

Recent studies have investigated the presence of genes exhibiting bacterial resistance to antibiotics, including the *cfxA/cfxA2* gene, in clinical samples from endodontic infections.^{6,7,8} However, no report has investigated the presence of these genes in oral cavity sites, such as the supragingival plaque, which is an important source of microorganisms for endodontic infections. This study aimed 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.

Methodology

The present research was approved by the Ethics Committee in Research (protocol number, CAE 08375712.1.0000.5347) from the *Universidade Federal do Rio Grande do Sul* – UFRGS (Porto Alegre, RS, Brazil). Forty-two subjects (> 18 years old) attending the Urgency Service at the Dental Clinics (Dental School, UFRGS, Porto Alegre, Brazil) were selected for the present study. Before participating, patients were asked to provide informed consent. None of the patients were excluded if they had received antibiotic treatment during the preceding 3 months. Patients with systemic disease or teeth with periodontal probing depth greater than 4 mm were also not included. No included patients wore total or partial dentures.

These patients were divided into three groups according to the following criteria: Group I – absence of 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 all patients. Root canal samples (RC) were collected for Groups II and III. Sample size was determined based on previous results reported by Gomes *et al.*⁹ (frequency of the species) and according to Jungermann *et al.*⁶ (frequency of detecting the *cfxA/cfxA2* gene).

Sampling procedure

The non-stimulated saliva was collected in a sterile Eppendorf tube.¹⁰ The supragingival biofilm was collected randomly at 15 sites using sterile microbrushes (Dentsply Caulk, Milford, USA). The tip of each brush was cut, and the biofilms were immediately placed in sterile Eppendorf tubes.⁹ Root canal samples were collected as described by Montagner *et al.*^{11,12} Coronal caries and defective restorations were removed. The involved tooth was isolated. Control samples were taken from both the external tooth surface and operative field after disinfection. The operative field disinfection was performed with 30% hydrogen peroxide and 5.25% sodium hypochlorite, followed by neutralization with 5% sodium thiosulfate. The canals were exposed under manual irrigation (sterile saline solution) using sterile burs. The paper points were introduced near the total length of the root canal according to the preoperative radiographs. The joints were kept in position for 1 min. If the canals were dry, they were moistened with sterile saline. The paper points were then introduced into tubes. All samples were stored at -20°C.

Detection of the *Prevotella* species and *cfxA/cfxA2* gene using PCR

The DNA was isolated with a QIAamp DNA Minikit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The DNA from all saliva, supragingival biofilm and root canal samples was first amplified with universal prokaryotic ribosomal 16S primer¹³ (forward: TCCTACGGGAGGCAGCAG, and reverse: GGACTACCAGGGTATCTAATCCTGTT).

The PCR amplification was used to detect the species *Prevotella intermedia*, *P. nigrescens* and *P. tanneriae*. Species-specific primers were used that were targeted to the region of the 16S rRNA gene in bacterial DNA. The sequences of the oligonucleotides (5'-3') were obtained from the literature: *P. intermedia*:¹⁴ forward: TTTGTTGGGGAGTAAAGCGGG, and reverse: TCAACATCTCTGTATCCTGCGT; *P. nigrescens*:¹⁵ forward: ATGAAACAAAGTTTTCCGGTAAG, and reverse: CCCACGTCCTGTGGGCTGCGA; and *P. Tanneriae*:¹⁶ forward: CTTAGCTTGCTAAGTATGCCG, and reverse: CAGCTGACTTATACTCCCG.

PCR reactions were processed in 10 µl for each sample: 8 µl containing *Taq* PCR Master Mix Kit (Qiagen, Valencia, USA), 0.5 µl of a solution of 10 µM forward primer (Invitrogen, Sao Paulo, Brazil), 0.5 µl of a 10 µM reverse primer solution (Invitrogen, Sao 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 s), annealing (temperature specific for each primer set, 1 min) and extension (72°C, 2 min), followed by a final extension (72°C, 10 min).

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

PCR was also used to detect the presence of the *cfxA/cfxA2* gene according to the protocol described by Giraud-Morin *et al.*¹⁷ As a positive control, the *Bacteroides vulgatus* CLA 341 strain was used. PCR reactions were processed in 10 µl for each sample: 8.6 µl of *Taq* PCR Master Mix Kit (Qiagen, Valencia, USA), 0.2 µl of each *cfxA/cfxA2* primer (10 µM): forward GAAAAAACAGAAAAACAAATC, reverse: TTAAGATTTTACTGAAGTTTG, and 1 µl of microbial DNA.

The PCR reaction steps were as follows: initial denaturation (94°C; 5 min); 25 cycles of denaturation (94°C, 1 min); annealing (58°C, 1 min); extension (72°C, 30 s); and a final extension (72°C, 10 min).

The presence of specific amplicons in each sample was verified by gel electrophoresis in 1% agarose (Invitrogen, Sao Paulo, Brazil) and staining (Sybr Green, Applied Biosystems, Sao Paulo, Brazil). Positive reactions were determined by the presence of bands with the proper length visualized under ultraviolet transillumination.

Data Analysis

The data (clinical features and gene detected) were entered into a spreadsheet and analyzed (SPSS for Windows, SPSS Inc., Chicago, USA). The Pearson Chi-square test was used to assess differences in the detection of specific genes in the same oral environment (S, SB or RC) and in different groups.

The Cochran's Q Test was used to assess differences in the detection of a specific gene in the same group but in different oral environments (S, SB or RC). Significance was defined as 5%.

Results

The mean age of the patients was 38.7 years (range, 19-69 years). Fifty-two percent of the patients were female. All patients in Group II reported a history of spontaneous pain, and Group III patients had asymptomatic apical lesions without the presence of sinus tracts. RCs of 17 incisors, 1 canine, 3 premolars, and 6 molars were sampled. 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% and apical radiolucency for 58% and 86% of patients in Groups II and III, respectively.

All S, SB and RC samples were positive results for the presence of bacteria as determined by universal 16S rRNA gene-base PCR. No control samples were positive presence of bacterial DNA. All three species of *Prevotella* were present in 53.97% of S samples, in 47.62% of SB and 34.56% of the RC samples. The overall rate detection of the *cfxA/cfxA2* gene was 23.81% in the saliva samples; 28.57% in the supragingival biofilm samples, and 7.41% in the root canal. The *cfxA/cfxA2* gene was not detected in the S, SB and RC of the same patient. However, the *cfxA/cfxA2* gene was present in the saliva and supragingival samples of five patients. Only one patient (1/27) harbored the *cfxA/A2* gene in their SB and RC samples. Table 1 shows the detection rates for each *Prevotella* species and the *cfxA/A2* gene in the groups considering the different oral environments (saliva, supragingival biofilm and root canal samples).

In Group I, the selected patients did not have acute or chronic primary endodontic infections at the time of collection. These patients presented to the Urgency Service for other reasons, such as dental caries, dental trauma or 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%;

Table 1. Frequency and percentage of each *Prevotella* species and *cfxA/A2* gene in the collected samples.

	Saliva			Supra-gingival Biofilm			Root Canal		
	G1 (n = 15)	G2 (n = 12)	G3 (n = 15)	G1 (n = 15)	G2 (n = 12)	G3 (n = 15)	G1 (n = 15)	G2 (n = 12)	G3 (n = 15)
	Absence EI	Acute EI	Chronic EI	Absence EI	Acute EI	Chronic EI	Absence EI	Acute EI	Chronic EI
<i>P. intermedia</i>	11 (73.3%) ^a	4 (33.3%) ^{a,A}	13 (86.6%) ^a	11 (73.3%)	4 (33.3%) ^A	8 (53.3%)	-	5 (41.6%) ^A	8 (53.3%)
<i>P. nigrescens</i>	7 (46.6%) ^b	0 (0%) ^b	1 (6,6%) ^b	9 (60%) ^c	1 (8.3%) ^c	0 (0%) ^c	-	1 (8.3%)	2 (13.3%)
<i>P. tanneriae</i>	11 (73.3%)	11 (91.6%)	10 (66.6%)	10 (66.6%)	8 (66.6%)	9 (60%)	-	5 (41.6%)	7 (46.6%)
<i>cfxA/cfxA2</i> gene	2 (13.3%)	4 (33.3)	4 (26.6%)	4 (26.6%)	6 (50%)	2 (26.6%)	-	2 (16.6%)	0 (0%)

* EI = Endodontic Infection; Small letters indicate statistical significance difference for the presence of the same species, in the same environment, for different test groups (Pearson Chi-square test, $p < 0.05$); Capital letters indicate statistical significance difference for the presence of the same species, in the same group, for different environments (Cochran Q test, $p < 0.05$)

SB = 73.33%; *P. nigrescens*: S = 46.67%, SB = 60%; and *P. tanneriae* S = 73.33%; SB = 66.67%). *cfxA/cfxA2* gene was detected in 13.33% of saliva samples and 26.67% of supragingival biofilm. No significant differences in the detection of the same species or the resistance gene were observed in the saliva and supragingival biofilm from Group I participants (Cochran's Q Test, $p > .05$)

In the samples collected from patients with acute primary endodontic infections (Group II), *P. tanneriae* was frequently detected in S samples (91.67%), whereas *P. nigrescens* was not detected in any sample from this environment. Significant differences were observed in the detection of *P. intermedia* among S, SB and RC samples (Cochran's Q Test, $p = .048$). In the supragingival biofilm, the *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 the presence of *P. nigrescens* or the *cfxA/cfxA2* gene in SB and RC samples, respectively. *P. intermedia* and *P. tanneriae* were simultaneously present in the S, SB and RC samples of 3/15 patients. No significant differences in the detection of the same species or the resistance gene were observed among the saliva, supragingival biofilm and root canal Group III participants (Cochran's Q Test, $p > .05$).

A 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 frequency

of *P. nigrescens* presence was higher in the control group, followed by the saliva samples collected from participants with chronic and then acute primary endodontic infections.

P. nigrescens was most frequently detected in the supragingival biofilm from participants without primary endodontic disease compared with participants with acute or chronic root canal infections (Pearson Chi-square Test, $p = .000$).

The presence or absence of spontaneous symptoms did not influence the detection rates for the targeted species and the *cfxA/A2* gene in the RC samples (Fisher's exact test, $p > .05$). No significant differences were observed in the detection of *P. intermedia*, *P. nigrescens*, *P. tanneriae* and the *cfxA/A2* gene in root canal samples collected from Group II and Group III participants.

Discussion

The present study assessed the prevalence of *P. intermedia*, *P. nigrescens* and *P. tanneriae* in saliva, supragingival biofilm and root canals samples from patients with acute and chronic primary infections. The overall detection rates for *P. intermedia*, *P. nigrescens* and *P. tanneriae* were 66%, 19% and 76% of the saliva samples, respectively. In the supragingival biofilm samples, *P. intermedia*, *P. nigrescens* and *P. tanneriae* were found in 54%, 23.81%, and 64% of the samples, respectively. He *et al.*¹⁸ 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*.

The presence or absence of an infection in the root canal system did not affect the detection of some species in saliva or supragingival biofilm. Similar frequencies of *P. tanneriae* were observed in saliva samples for all patient groups. *P. intermedia*, *P. tanneriae* and *cfxA/cfxA2* detection rates in the supragingival biofilm were not affected by the presence/absence of an endodontic infection. Unique behavior was observed for the *P. nigrescens*. Comparing the detection rates of *P. nigrescens* among the patient groups, this microorganism was most frequently detected in saliva and supragingival biofilms from subjects without acute/chronic primary endodontic infections. Teles *et al.*¹⁹ suggested that *Prevotella nigrescens* had increased detection rates in supragingival biofilms collected from healthy patients with no periodontitis, especially after a 7 day period without oral hygiene.

According to Fosse *et al.*,²⁰ *Prevotella* are the main beta-lactamase-producing species in odontogenic infections. Beta-lactamase production is encoded by chromosomal genes, such as *cfxA/cfxA2*, *cblA*, *cepA*, and *cfiA*. *P. intermedia* carry *cfxA/cfxA2* genes, and their presence is primarily related to the origin of the strains.¹⁹ Handal *et al.*²¹ demonstrated the occurrence of *cfxA/cfxA2* genes in 100% of beta-lactam-positive *Prevotella* strains from American and Norwegian patients with periodontal diseases.

The oral environment appeared to be a reservoir for *cfxA/cfxA2*-positive bacteria. In the present study, 18/42 (42.86%) of the patients harbored the *cfxA/cfxA2* gene. However, it should be emphasized that it is important to not only describe the presence of the resistance gene but also determine whether the bacteria are functionally resistant to antibiotics. No patient exhibited positive simultaneous detection of the *cfxA/cfxA2* in his or her saliva, supragingival plaque and root canal samples. In

the present study, the targeted gene was detected in both the saliva and supragingival biofilm samples from all the groups. There are no reports 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%.^{5,6,7} 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 samples, as observed in the samples obtained from patients with teeth presenting acute primary endodontic infections.

Oral environments have heterogeneous characteristics (nutrients, oxygen availability, pH) and may exert selective pressure,²² producing different bacterial community profiles and species selection, influencing also the presence of specific virulence factors, such as resistance genes.

Conclusion

Different oral niches can be a source of beta-lactamase resistant bacteria. Local conditions, such as the presence of symptomatology, were not related to an increased presence of the *cfxA* gene in the supragingival biofilm and inside root canals.

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