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Correlation of periodontal pathogens in concurrent endodontic-periodontal diseases

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Abstract

Objectives: This study investigated the correlation between *Tannerella forsythia*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans* at dual sites in concurrent endodontic-periodontal diseases.

Material and methods: Samples were collected from *endodontium* and *periodontium* in cases of concurrent endodontic-periodontal diseases from thirty participants. The sensitivity and specificity of SYBR Green real-time PCR was used to identify the targeted species. Absolute number of targeted genome copies in tested samples were extrapolated from respective calibration curve.

Results: No statistical difference was found in the number of detected endodontic-periodontal pathogens between the *endodontium* and *periodontium*. The Pearson test detected significant correlation (P<0.001) between targeted bacteria; *T. forsythia*, *F. nucleatum*, and *P. gingivalis* from endodontic-periodontal lesions. Synergistic component observed separately in endodontic biofilm was found only between *T. forsythia* and *F. nucleatum* (r=0.380, P=0.03) while in periodontal biofilm *T. forsythia*, *F. nucleatum* and *P. gingivalis gave high* synergism result (P<0.0001). Correlation analysis showed that *T. forsythia* in primary endodontic infection and in periodontal lesion was significantly decreased with the increase of patients age (r=-0.308, P=0.017).

Conclusions: Correlation between targeted bacterial species levels from concurrent endodontic-periodontal diseases confirmed that coronal and cervical dentinal tubules may represent a viable pathway that allows spreading and maintaining of dual sites infection. Periodontal bacteria detected in root canal of concurrent endodontic-periodontal infections may originate from the local periodontal lesions.

Keywords: Microbiome correlation, dentinal tubules, endo-perio infection, pearson test, periodontal lesion

Introduction

The route of infection within endodontic-periodontal niche is still a subject of unresolved controversy as it is unclear if the bacterial migration occurs through dentinal tubules. The openings of dentinal tubules facing the periodontal ligament are sealed with *cementum*. Dentinal *cementum* can be mechanically removed, abraded, eroded, and destroyed by clinical, pathological and erosive processes. Until recently the gold standard in periodontal treatment would have been scaling and root planning, where the removal of infected *cementum* was considered a *condicio sine qua non*. Bacteria of the oral biofilm adhering on the root surface can obtain access to the adjacent dentinal

tubules [1]. Anatomical communications such as: apical fora-men, accessory canals, lateral canals and dentinal tubules link the two endodontic-periodontal niches [2-4]. While *cementum* may represent a barrier for bacterial migration through dentinal tubules, movement of bacteria from the outer surface of the root to the root canal system may reinforce the reservoir effect of the dentinal tubules. The different depths of penetration maybe related to the bacterial size, adhesive properties, and motility characteristics [5]. The degree of permeability varies between different areas of the tooth and the age of the patient [5,6]. In model systems it is shown that persistence in small space is mostly related to availability of nutrient in the context of spatial constriction that might be stricter than cell dimensions [7].

According to Harrington et al., [8] dental pulp is capable of surviving significant insults, unless periodontal disease extends all the way to the tooth apex, so the effect of periodontal disease and periodontal treatment on dental pulp is expected to be negligible. Conversely in other publications, it was suggested that periodontal disease is a direct cause of pulpal atrophy and necrosis, and periodontal diseases should be regarded as potential causes of pulpitis and pulpal necrosis [9-12].

The connection between the endodontic and periodontal niches in terms of pathogens involved has been investigated by traditional and molecular methods [13,14]. Considering the fact that the vital pulp tissue can immunologically respond to bacteria and bacterial bioproducts and preserve its vitality, the type of infection investigated in this study was primary endodontic infection with chronic periodontitis [15]. Our working hypothesis was that bacteria may migrate through dentinal tubules in both directions colonizing both endodontic-periodontal tissues, as supported by previous investigation [9,16-18].

The aim of this study was to explore quantitative distribution of periodontal pathogens between endodontic and periodontal samples of the same tooth. In that respect we have analyzed the occurrence of key periodontal pathogens such as: *Tannerella forsythia, Porphyromonas gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* using SYBR Green based real-time PCR assay. Furthermore, we assessed bacterial inter-relations in clinically validated cases of endodontic-periodontal infections using statistical correlation tests. The null hypothesis of the study was that no correlation existed between the pathogens sampled in periodontal and endodontic lesions.

Materials and methods
Subject recruitment
Study's participants were screened patients of the Department of Dental Pathology and Endodontics, School of Dentistry, University of Sarajevo. Thirty patients fulfilled enrollment criteria. Inclusion criteria included: age between 18-60 years, primary endodontic infection of single rooted tooth diagnosed as chronic apical periodontitis or acute apical periodontitis or localised absceses and concomitant periodontal lesions with chronic periodontitis. Periodontal pockets were not deeper than 6 mm. Endodontic-periodontal lesions were diagnosed clinically and

by long-cone periapical radiograph. Exclusion criteria were the use of antibiotic in the previous two months, the presence of fistula, radiographic evidence of endodontic-periodontal communication, and aggressive form of periodontitis. Clinical metadata and measurements were collected at the time of collection. All participants in this study were recruited on the basis of informed consent and procedures were done with respect to local ethical standards in accordance with Helsinki Declaration.

Specimen sampling
Periodontal samples were taken following supragingival biofilm removal and cotton roll isolation by inserting two paper points (ISO 20) into the deepest periodontal pockets and left *in situ* for 1 minute each. Samples from the root canal of the diagnosed tooth were collected under rubber-dam isolation and under strict aseptic conditions. Samples were collected from single root during endodontic treatment. The tooth and the surrounding field were cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite solution. Complete access preparations were made using sterile burs without irrigation, and the operative field including the pulp chamber, was then swabbed with 2.5% NaOCl, which was then inactivated by sterile 5% sodium thiosulphate. Samples were collected using a size 20 K-type file, held with sterile locking pliers, and introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs. Two sequential sterile paper points (ISO 20) were placed to the working length level and used to soak up the fluid in the root canal. Each paper point was left in position for 1 minute. The file and the two paper points were then transferred to cryotubes containing 1 ml of TE buffer (10 mmol/l tris-HCl, 0.1 mmol/l EDTA, pH 7.6). Samples were immediately frozen and kept at -80°C prior to further analysis.

DNA extraction
Before the DNA extraction, frozen endodontic and periodontal specimens were resuspended and thawed at room temperature. Total DNA from endodontic and periodontal samples was extracted using Qiamp DNA (Qiagen, Hilden, Germany) mini kit according to manufacturer's instructions. DNA concentration and purity were evaluated using UV mini spectrophotometer 1240 (Shimadzu, Japan).

Primers description and amplification
Detection of all targeted periodontal pathogens was performed using PCR with previously published strain specific primers pairs listed below: for *T. forsythia*, *Tf* forward 5'-GCG TAT GTA ACC TGC CCG CA-3' *Tf* reverse 5'-TGC TTC AGT GTC AGT TAT ACC T-3' with amplicon length 641 base pairs [19]; for *F. nucleatum Fn* forward 5'-AGA GTT TGA TCC TGG CTC AG-3' *Fn* reverse 5'-GTC CTC GTG CAC ACA GAA TTG CTG -3' with amplicon length 360 bp [20]; *P. gingivalis Pg* forward 5'-AGG CAG CTT GCC ATA CTG CG-3' *Pg* reverse 5'-ACT GTT AGC AAC TAC CGA TGT-3' with amplicon length 404 bp [21]; and for *A. actinomycetemcomitans* [1], *Aa* forward 5'-AAA CCC

ATC TCT GAG TTC TTC TTC AG-3' *Aa* reverse 5'-ATG CCA ACT TGA CGT TAA AT-3' with amplicon length 557 bp [22].

Amplification reaction mixture for temperature gradient optimization contained 200 ng of DNA template, 2.5μl PCR buffer, 2 mmol $MgCl_2$, 0.5μmol/l dNTP, 1 pmol of each primer, 0.25 units of Taq DNA polymerase, 16.6μl ddH_2O and 0.25μl of glycerol in a total volume of 25μl. The PCR program with temperature profile for all primers included an initial denaturation step at 90°C for 3 min, followed by 35 cycles of a denaturation step at 95°C for 30 s, then 35 cycles annealing step at 55-65°C for 1 min, and 35 cycles of elongation step at 72°C for 1 min 30 s, and final elongation at 72°C for 5 min.

The purpose of temperature gradient optimization was to achieve proper individual temperature for primers amplification, before SYBR Green Real-Time PCR program was carried out. Adequate temperature profile for primers annealing was necessary preconditioning for the SYBR Green Real-Time PCR program to achieve appropriate quantitation results.

SYBR Green real-time PCR
Real Time PCR reactions were carried out in total volume of 12 μl containing PCR SYBR Green premix (SYBR Green I dye, Ampli Taq DNA polymerase, dNTP with dUTP, passive reference dye and optimized buffer component) in a volume 6.25 μl, each primer 20 pmol, 200 ng of DNA template and sterile distilled water to adjust the total volume of 12 μl.

Amplification and detection of bacterial DNA by SYBR Green was performed in a 7300 Real-Time PCR System (Applied Biosystems Foster City USA) with the programs for cycling conditions which started with initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation step at 95°C for 45 s, then 35 cycles of annealing at 60°C for 1 min and 35 cycles of elongation step at 72°C for 1min 30 s and finally 3 cycles of dissociation at 95°C for 15 s, at 60°C for 30 s, at 95°C for 15 s. All samples were run in duplicate. Amplification results of *T. forsythia, P. gingivalis, F. nucleatum,* and *A. actinomicetemcomitans* were collected in form of Ct values or threshold cycle which is the intersection between an amplification curve and a threshold line to derive the concentration of the target in the reaction. Efficacy of SYBR Green Real Time PCR quantitation experiment was validated by dissociation curve analysis method.

Absolute quantitation method
In order to determine exact number of bacterial DNA copies for all investigated strains we used a calibration curve analysis based on serial dilutions of pure DNA extracts from $5x10^1$-$5x10^6$ genome copies. Strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA): *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 25586, *T. forsythia* ATCC 43037. Absolute number of genome copies in tested samples was extrapolated from respective calibration curve.

Biostatistical analysis
Student-T test (P<0.05) was used to check the endodontic-peri-

odontal microbial interrelationships for each respective sample. Analyses of variance (ANOVA) was implemented in order to detect possible bacterial influence on clinical symptoms and endodontic-periodontal diagnosis. Pearson correlation test was used to assess correlation in copy number of targeted bacterial strains in the concurrent endodontic-periodontal lesion. The same test was used to estimate correlation between absolute quantitation of targeted bacteria separately in endodontic and periodontal biofilm. Also, correlation test was implemented to perceive possible association between copy number of targeted bacterial strains and patient's age. The SPSS 10 software (SPSS Inc.; Chicago, IL) was used for statistical analysis.

Results
Thirty subjects (age 37±13.74; 57% females and 43% males) fulfilled recruitment criteria. In total sixty specimens were sampled from endodontium and periodontium. The most frequently detected species was *F. nucleatum* (100% of both endodontic and periodontal samples), followed by *T. forsythia* (93% of periodontal pocket samples; 90% in primary endodontic infection), *P. gingivalis* (53% of periodontal pocket samples; 70% in primary endodontic infection), and *A. actinomycetemcomitans* (13% of periodontal pocket samples; 3% in primary endodontic infection). There was no statistically significant difference in the quantity of targeted pathogens between the endodontium and periodontium of the same tooth as found by T-test (**Table 1**). As *A. actinomycetemcomitans* was detected in both endodontic and periodontal samples with a low representation among the participants as well as at low levels, this bacteria was excluded from further analyses.

Pearson correlation test detected association between targeted bacteria from endodontic-periodontal lesions and co-localization among detected bacteria from endodontium and periodontium separately. Analysis between bacteria in endodontic-periodontal infection confirmed statistically significant correlation between absolute quantitation of *T. forsythia* and *F. nucleatum* (r=0.547, P<0.0001), *F. nucleatum* and *P. gingivalis* (r=0.427, P<0.01), while *T. forsythia* and *P. gingivalis* showed tendency toward statistical correlation in endodontic-periodontal infections (r=0.244, P=0.06). Pearson test showed statisticaly significant correlation between *T. forsythia* and *F. nucleatum* (r=0.682, P<0.0001), *F. nucleatum* and *P. gingivalis* (r=0.977, P<0.0001),

Table 1. Comparsion of bacterial quantity by T-test from endodontium and periodontium of the same tooth using absolute quantitation real time PCR method.

	P	Average difference in copy number	Std.error
T. forsythia	0.571	-5584.1	9801.4487
F. nucleatum	0.319	-49241287319	48991947514
P. gingivalis	0.724	216373.8	610683.46
A. actinomicetemcomitans	0.324	-174.59	175.57

as well as *T. forsythia* and *P. gingivalis* (r=0.691, P<0.0001) indicating a potential sinergistic component in periodontal biofilm. Significant correlation between targeted bacteria from endodontic biofilm was only observed between *T. forsythia* and *F. nucleatum* (r=0.380, P=0.03).

Correlation analysis showed that only *T. forsythia* in primary endodontic infection and in periodontal lesion was significantly decreased with the increase of patients age (r=-0.308, P=0.017). Only *F. nucleatum* in endodontic-periodontal lesions showed statistically significant difference in cases of acute apical periodontitis and clinical features such as localised abscesses (F=3.586, P>0.05).

Discussion
SYBR Green real-time PCR was used in this study for its high level of detection sensitivity and specificity. Potential PCR inhibitors in our reaction mixture were either absent or present in amounts not sufficient to significantly interfere with the reaction. The high frequency of the targeted bacteria in this study is a result of the SYBR Green real-time PCR detection method high sensitivity, and confirms previous results [23,24]. Positive statistical correlation of absolute quantitation of the targeted species and the increase of pocket depth was not observed, possibly due to the measuring depth not deeper than 6 mm. The purpose of this limitation was to facilitate the demonstration that the coronal and middle part of the root is frequently used bacterial pathway in both, endodontic and periodontal direction. Differences in size, number and diameter of dentinal tubules depend on the area of the root. Progressive and pathological sclerosis and obliteration of dentinal tubules affect the size of dentinal tubules diameter [6]. Cervical and mid-root dentin has a higher tubular density while the apical region is consisted of lower number of dentinal tubules with sclerosis and obliteration tendency [25].

In periodontal pockets, *T. forsythia*, *F. nucleatum* and *P. gingivalis* high statistical correlations of their quantitation, as found by Pearson test, suggest optimal environmental conditions for bacterial growth in periodontal pocket. In contrast, the same strains from the root canal of same tooth resulted in statistically less significant correlation. Those findings underline the environmental influence on bacterial growth, as the root canal condition may be less favorable than the periodontal niche for a synergistic interaction among those organisms. Environmental influence and conditions can lead to nonspecific microbial presence in the root canal, and is one of the reasons why endodontic specialists do not have root canal filling for all endodontic infections [26]. The environmental effect as a direct consequence for the subjects as the observed periapical and periradicular radiolucencies associated event promote development of marginal bone loss and consequently should be regarded as a risk factor in periodontitis progression [8]. For clinicians this means that in their treatment protocol they always need to first complete endodontic treatment and then terminate periodontal treatment [26].

T. forsythia in primary endodontic infection and in periodontal lesion showed significant decrease in correlation with the increase of patients age. The detection of *T. forsythia* both in primary endodontic infection and periodontal lesion is in accordance with previous investigation using non targeted molecular methods [27]. *F. nucleatum* levels in endodontic-periodontal lesions correlated with symptoms of severe pain in cases of acute apical periodontitis and localized abscesses.

The low prevalence of *A. actinomycetemcomitans* in the primary endodontic infection is in agreement with previous research [28], and may be associated in our study with the exclusion of aggressive form of periodontitis and an average periodontal pocket depth of 4 mm, and no deeper than 6 mm. *A. actinomycetemcomitans,* found in this study, is not a relevant endodontic pathogen and the environmental differences between the root canal and periodontal pocket probably determined the low prevalence of this bacteria in the root canal system.

T. forsythia, P. gingivalis, and *F. nucleatum* detected in this study were positively associated with endodontic-periodontal combined infection. *F. nucleatum* in endodontic-periodontal lesions correlation with the symptoms of severe pain in cases of acute apical periodontitis as well as clinical features such as localised abscesses echoe the findings of Villanueva [29].

The number of detected bacterial copies between endodontium and periodontium space had no significant difference, confirming the working hypothesis. Endodontic infections are sustaining periodontal lesions and periodontal lesions are most likely a bacterial source for endodontic infections [13]. Correlation between targeted bacterial species from concurrent endodontic-periodontal diseases confirmed that coronal and cervical dentinal tubules may represent a viable pathway that allows spreading and maintaining of dual sites infection. Periodontal bacteria detected in root canal of concurrent endodontic-periodontal infections may originate from the local periodontal lesions.

The limitations of the study are associated with the targeted nature of the approach and the number of participants. The targeted nature of this approach limits to known species that have been associated with dysbiosis in and around the tooth structure. Future studies should include untargeted approaches such as targeted gene sequencing using next-generation sequencing [30,31], which has yet to be applied to this topic. The number of participant while limited to thirty individuals allowed an in depth analysis of two adjacent sites from the same tooth, limiting the scale of recruitment. Future studies could include the microbiota of adjacent teeth to validate the findings.

Conclusions
In this study, we investigated the controversial issue related to clinical questions whether bacteria from periodontium could migrate through coronal and middle part of the root dentinal tubules and infect the pulp tissue and vice-versa Absolute quantitation of the frequent periodontal and endodontic pathogens using SYBR Green real-time PCR confirmed working hypothesis and reveled no statistically significant difference in the presence

of the targeted bacteria in endodontic-periodontal infections. Molecular detection method facilitated the demonstration of the coronal and middle part of the root as frequently used bacterial pathway in both, endodontic and periodontal direction.

This research emphasized that endodontic bacteria most likely originate from periodontal pockets and that endodontic infections maintain periodontal pockets and periodontal infection.

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Citation:
Lačević A, Foschi F, Pojskić L, Pojskić N, Bajrović K and Izard J. **Correlation of periodontal pathogens in concurrent endodontic-periodontal diseases**. *Oral Biol Dent*. 2015; **3**:5.
http://dx.doi.org/10.7243/2053-5775-3-5