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Porphyromonas endodontalis in chronic periodontitis: a clinical and microbiological cross-sectional study

Telma Blanca Lombardo Bedran[1]*, Rosemary Adriana C. Marcantonio[1], Rubens Spin Neto[1], Marcia Pinto Alves Mayer[2], Daniel Grenier[3], Luis Carlos Spolidorio[4] and Denise Palomari Spolidorio[4]

[1]Department of Oral Diagnosis and Surgery, Araraquara Dental School, State University of São Paulo, Araraquara, Brazil; [2]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil; [3]Oral Ecology Research Group, Faculty of Dentistry, Université Laval, Quebec City, Quebec, Canada; [4]Department of Physiology and Pathology, Araraquara Dental School, State University of São Paulo, Araraquara, Brazil

Background: Although previous studies have shown the presence of *Porphyromonas endodontalis* in chronic periodontitis associated with periapical lesions, the occurrence of this pathogen in diseased periodontal sites without periapical lesions has been poorly investigated.

Objective: The aims of this study were to quantify *P. endodontalis* in patients with chronic periodontitis without periapical lesions, to evaluate the potential correlation of *P. endodontalis* with *Porphyromonas gingivalis* and *Tannerella forsythia,* and to evaluate the ability of periodontal treatment to reduce these pathogens.

Design: Patients with generalized chronic periodontitis were selected by recording clinical attachment level (CAL), probing depth (PD), and bleeding on probing (BOP). Subgingival samples from 30 diseased nonadjacent sites (CAL $\geq$5 mm, PD between 5 and 7 mm and positive BOP) and 30 healthy nonadjacent sites (PD $\leq$3 mm and negative BOP) were collected and subjected to microbial analysis by quantitative polymerase chain reaction (qPCR). The variables of age, PD, CAL and BOP of all individuals were analyzed using the paired t-test (GrapPad Prism5®). Data of bacteria quantification were subjected to a normality test (D'Agostino-Pearson Test). For bacterial correlation analysis, the Spearman correlation was used.

Results: Our results showed that diseased sites had significantly higher levels of *P. endodontalis* compared to healthy sites, similar to the results obtained for *P. gingivalis* and *T. forsythia*. The numbers of all bacterial species were reduced significantly after mechanical periodontal treatment. *P. endodontalis* was significantly correlated with the presence of *T. forsythia* and *P. gingivalis* in the diseased group.

Conclusion: Our results suggest that there is a high prevalence of *P. endodontalis, P. gingivalis* and *T. forsythia* in periodontitis sites and that mechanical periodontal treatment is effective at reducing the pathogens studied.

Keywords: *periodontitis*; *polymerase chain reaction*; *porphyromonas endodontalis*; *porphyromonas gingivalis*; *Tannerella forsythia*

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Periodontitis is an inflammatory disease initiated by specific bacterial species that colonize the subgingival area between the tooth surface and the marginal gingiva. This disease causes the destruction of tooth-supporting tissues, including connective tissue and alveolar bone, and can lead to tooth loss (1).

The main periodontal pathogens of periodontal disease include Gram-positive and Gram-negative bacteria, facultative and strictly anaerobic bacteria (2). *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* have been strongly associated with the chronic form of periodontitis and are thought to play important

roles in pathogenesis (1, 3, 4). Although individual bacterial species and groups of bacteria have been identified as etiological factors of periodontitis, interactions between microorganisms and the host also play a key role in the etiopathogenesis of the disease (5).

Several reports have shown that the presence of other bacterial species, including *Porphyromonas endodontalis*, is associated with chronic periodontitis (5, 6). Further studies are required to verify the relationship of these species with the disease and their exact contribution to the etiology and progression of periodontitis. *P. endodontalis* is primarily found in infections that originate in the pulp, but has also been isolated from the tonsillar area, the dorsum of the tongue and the periodontal pockets of patients with periodontal and pulp lesions (6, 7, 8). Tran et al. (6) were the first to report the detection of this species in periodontal pockets, although it was present at a low concentration. *P. endodontalis* is an asaccharolytic, black-pigmented, Gram-negative anaerobic bacterium. Originally known as *Bacteroides endodontalis*, *P. endodontalis* is highly sensitive to oxygen and is therefore difficult to cultivate from clinical samples (9). This species, along with other black-pigmented anaerobic rods, has been implicated in the etiology of infected root canals and periodontitis (4). However, additional studies are required to determine the role of *P. endodontalis* in patients with chronic periodontitis but without periapical lesions, as well as the contribution of this microorganism to the pathogenesis of periodontitis (10).

Recent advances in diagnostic methods using molecular biology techniques that are more sensitive than bacterial culture assays have led to a better understanding of the roles that previously unidentified microbial species play in the pathogenesis of periodontitis. Most conventional microbiological tests, such as standard bacterial culture, do not distinguish between *P. endodontalis* and other closely related black-pigmented pathogens. However, molecular techniques, such as polymerase chain reaction (PCR), allow for the specific identification of *P. endodontalis* and other Gram-negative anaerobic bacteria found in the oral cavity (6, 11). Therefore, the purpose of this study was to use quantitative PCR (qPCR) to detect and quantify *P. endodontalis* in patients with chronic periodontitis but without infected root canals, and to investigate how these data correlate with data on pathogens that have been previously shown to be related to chronic periodontitis, i.e. *P. gingivalis* and *T. forsythia*. In addition, the levels of *P. endodontalis*, *P. gingivalis* and *T. forsythia* following a mechanical periodontal treatment were determined.

Material and methods

Subject population

This study was designed as a double-blind, controlled trial with a two-month duration to quantify pathogens in chronic periodontitis before and after basic periodontal treatment. The study was approved by the Ethics Committee in Human Research (Protocol # 26/08), and all study participants signed a free and informed consent form. The sample size calculation was based on previous studies using qPCR for the detection of periodontal pathogens (12, 13). For this calculation, we used the following criteria: 90% test power, 5% significance level and 1.0 mm detection difference in probing depth (PD). The standardized difference of 0.95 in study power ($1-\beta=0.95$) and a confidence level of $a=0.05$ necessitated a sample size of at least 17 patients that needed to receive periodontal treatment.

This study comprised 20 randomly selected patients (8 men and 12 women of 35–55 years of age) with moderate chronic periodontitis according to the general criteria established by the American Academy of Periodontology (14). Inclusion criteria were as follows: the presence of at least 20 teeth; a minimum of three non-adjacent teeth with bleeding on probing (BOP), a clinical attachment level (CAL) ≥ 5 mm and a probing depth (PD) between 5 and 7 mm; and three healthy teeth with no BOP and a PD ≤ 3 mm. Radiographic analyses were performed to complement the periodontal diagnosis and to exclude teeth with periapical lesions. No patients had a history of systemic disease or antibiotic therapy in the 6 months prior to the study.

Study design

We selected 30 non-adjacent posterior sites from 20 patients with a PD of 5–7 mm, positivity for BOP and a CAL ≥ 5 mm as well as 30 healthy non-adjacent anterior sites with no clinical attachment loss or BOP and a PD ≤ 3 mm. The selected teeth had no dysfunctions in relation to occlusion and had no prostheses.

Alginate molds of the dental arches were made to prepare acetate stents for standardizing the position of the manual probe (Williams®, São Paulo, Brazil) of sites selected for microbiological analyzes. All of the periodontal clinical measurements were performed by a single trained examiner, while another oral health professional performed the basic periodontal treatment followed by oral hygiene instruction and microbiological assessment. The clinical measurements and the sample plaque collections were performed at baseline and at 60 days after the periodontal treatment.

Oral hygiene program

Forty-five days before beginning the periodontal treatment, patients received oral hygiene instructions every week according to the individual needs of each patient. After 30 days of oral hygiene instruction, periodontal examination was performed for patients with a visible plaque index of less than 30%.

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Clinical parameters

Clinical examination was performed by a trained, calibrated examiner whose intra-examiner repeatability was determined at baseline (Kappa score = 0.91). The clinical parameters assessed included the Visible Plaque Index (VPI, 0–1) and Marginal Bleeding Index (MBI, 0–1) (15) determined at four points per tooth (mesial, distal, buccal and lingual), and the Probing Depth (PD), Clinical Attachment Level (CAL) and Bleeding on Probing (BOP, 0–1) (16) determined at six different sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) using a periodontal probe (Williams®, São Paulo, Brazil).

Subgingival sample collection

Samples for microbiological analysis were collected seven days after the initial clinical examination and 60 days after completion of the basic periodontal treatment. After removal of the supragingival biofilm, the selected sites were isolated with cotton rolls and gently air-dried. Subgingival fluid was collected using two sterile paper points (no. 30; Dentsply, Maillefer, Petrópolis, RJ, Brazil) that were inserted in the gingival pocket up to the apical portion for 30 seconds. The paper points were immediately placed in sterile Eppendorf vials containing 500 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and stored at −20°C until DNA was extracted for microbial analysis by qPCR.

Basic periodontal treatment

Seven days after the microbiological sample collection, patients underwent a non-surgical periodontal treatment including scaling and root planing (SRP) followed by oral hygiene instruction; the treatment was performed under local anesthesia and with manual instruments (McCall and Gracey Curettes and Hirschfeld File Scaler – Hu Friday®). Polishing was performed immediately after each session of SRP with rubber cups and paste. After SRP, supragingival biofilm control (maintenance phase) was performed via prophylaxis and oral hygiene instruction was conducted weekly for 60 days, at which time the second collection of crevicular fluid and periodontal examination occurred.

Bacterial strains and growth conditions

The bacterial species *P. endodontalis* (E203), *P. gingivalis* (ATCC 33277) and *T. forsythia* (ATCC 43037) were grown anaerobically on Tryptone soy blood agar plates supplemented with hemin (5 mg/l) and menadione (1 mg/l) for 7–15 days at 37°C in 85% N_2, 5% CO_2 and 10% H_2 in an anaerobic chamber (Plas Labs, Lansing, MI, USA). *Escherichia coli* strain DH5-alpha was used as the host for plasmids and was grown aerobically at 37°C in LB medium (17).

DNA extraction

The extraction of DNA from subgingival fluid samples and from bacterial reference cultures was performed using standard methods. Briefly, cells were lysed by boiling, and DNA was extracted with phenol:chloroform:iso-amyl alcohol (25:24:1). After the addition of 3 M NaCl, the DNA was precipitated with ethanol (99%) and re-suspended in TE buffer. The DNA concentration was determined by UV spectrophotometry (Biomate 3 Spectrophotometer, Thermo Electron Corporation), and the relationships between the absorbances at 260 and 280 nm were analyzed. As a quality parameter, A_{260}/A_{280} values between 1.8 and 2.0 were considered appropriate.

Quantitative PCR reactions

For the quantitative analysis, plasmids containing the target genes were used as standards. PCR amplification was initially performed for the 16S rRNA of *P. endodontalis, P. gingivalis* and *T. forsythia*. The amplicons were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and plasmids were transformed into *E. coli*. After growth of the transformants, the plasmids were extracted using a PureLink Quick sHRNA Miniprep kit (Invitrogen) and then sequenced. The plasmid solutions were diluted in sterile water to a concentration of 10^8 copies/μL and then serially diluted (final concentrations from 10^4 to 10^1 copies/μL). The dilutions were then used as template DNA in the qPCR reactions. In each reaction, data obtained from the standard curve were used to convert the CT scores (cycle threshold) obtained with patient samples into the exact numbers of DNA copies (18, 19).

The detection and quantification by qPCR was performed using universal (20) and specific primers for *P. endodontalis* (5′-GCT GCA GCT CAA CTG TAG TCT TG-3′, 5′-TCA GTG TCA GAC GGA GCC TAG TAC-3′-110 pb) (21), *P. gingivalis* (5′-ACC TTA CCC GGG ATT GAA ATG-3′, 5′-CAA CCA TGC AGC ACCT AC ATA GAA-3′- 83 pb) (22), and *T. forsythia* (5′-AGC GAT GGT AGC AAT ACC TGT C-3′, 5′-TTC GCC GGG TTA TCC CTC-3′- 88 pb) (22) (Applied Biosystems®). The species-specific primer sets were designed based on the variable regions of each target gene. The specificities of the primers were confirmed by multiple alignments of relevant sequences from closely related species and by a Basic Local Alignment Search Tool (BLAST) homology search (1).

The qPCR reactions were performed with the use of a Step One™ qPCR System (Applied Biosystems®). All reactions were performed in duplicate, and average values were used to calculate the bacterial load. The total volume of each reaction was 10 μL containing 5 μL of SYBR Green ER qPCR SuperMix Universal (Invitrogen Tech-Line℠), 0.1 μM of each primer pair (Applied

Biosystems®) and 50 ng/uL template DNA. The thermocycling program included an incubation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and an incubation at 60°C for 1 minute. After the qPCR reaction, the dissociation curve (melting curve) was obtained using temperatures between 60°C and 95°C to determine primer specificity. Melting curve analysis revealed only one peak of amplification. All reactions were performed in 48-well MicroAmp optical plates covered with optical adhesive (Applied Biosystems). Data were analyzed by Step One™ software (Applied Biosystems®).

Statistical analysis

The clinical and microbiological data were statistically analyzed using the software GraphPad Prism 5®. Inter- and intra-group analyses were conducted for the various periods. The variables age, PD, CAL and BOP of all individuals were analyzed using the paired t-test. Quantitative variables (PD and CAL) were subjected to the normality test (D'Agostino-Pearson Test). PD and CAL data were analyzed by the Wilcoxon test for comparisons between groups and between periods. To analyze the BOP categorical variable, the Cochran test was used. To identify statistically significant results, we used the exact McNemar test. Bacterial quantification data were subjected to a normality test (D'Agostino-Pearson Test) and showed a non-normal distribution. Thus, the Mann-Whitney test was applied. For bacterial correlation analysis, the Spearman rank correlation was used. $p < 0.05$ was considered statistically significant.

Results

Clinical parameters

The general characteristics of the sampled population are summarized in Table 1. Since no statistically significant differences in age or number of teeth were observed in the sample set, it was considered homogeneous. The average CAL was divided into three CAL scores (1–2 mm, 3–4 mm and ≥5 mm), and the average PD was divided into three PD scores (1–3 mm; 4–5 mm; ≥6 mm). After treatment, there was a statistically significant improvement of both parameters ($p < 0.001$).

The collection sites for the microbiological analysis had a prevalence of bleeding on probing of 100% at baseline that decreased to 13.33% after treatment in the disease group ($p < 0.0001$). In addition, PD (5.33 ± 0.54 mm, baseline) and CAL (5.4 ± 0.62 mm, baseline) showed statistically significant reductions ($p < 0.0001$) after periodontal therapy in the diseased group (3.63 ± 0.76 mm and 3.83 ± 0.95 mm for PD and CAL, respectively). In the healthy sites, PD (1.6 ± 0.56 mm) and CAL (1.63 ± 0.56) showed no significant reduction ($p = 0.035$) over the 60-day period.

Table 1. Mean (± SD) clinical parameters of full mouth of subjects in the study

$N = 20$ subjects	BASELINE	60 DAYS
Age (years)	46 ± 7.49	
Gender (M/F)	8/12	
Average number of teeth	23.55 ± 2.95	
	% of sites with	
Probing depth (1–3 mm)	86%	94%
Probing depth (4–5 mm)	10%	5.3%
Probing depth (≥6 mm)	4%	0.7%
Clinical attachment level (1–2 mm)	60%	78%
Clinical attachment level (3–4 mm)	28.5%	20%
Clinical attachment level (≥5 mm)	11.5%	2%
Bleeding on probing	47%	10%

Detection of microorganisms by qPCR

The standard curve for each microorganism was obtained with the use of specific primers and four serial dilutions (10^1–10^4) of the genomic DNA of *P. endodontalis, P. gingivalis* and *T. forsythia*. The reaction efficiencies for each organism were 97.1 (*P. endodontalis*), 96.3 (*P. gingivalis*) and 97.8 (*T. forsythia*). The correlation coefficient for the mean C_T values was $R^2 \geq 0.99$. All 3 Sybr Green assays were highly specific and amplified only DNA extracted from periodontopathogens.

The average numbers of *P. endodontalis, P. gingivalis* and *T. forsythia* detected in the patient group were $1.7.10^4$, $1.72.10^4$ and $1.5.10^4$ cells, respectively. There were no statistically significant differences among the three pathogens tested. However, after periodontal treatment, we observed significant reductions of these pathogens to $1.3.10^2$, $6.33.10^2$ and $1.6.10^2$, respectively, for *P. endodontalis P. gingivalis* and *T. forsythia*. *P. gingivalis* remained in higher concentrations after periodontal treatment (Fig. 1).

In the healthy sites, we detected average numbers of 70, 160 and 58 cells for *P. endodontalis, P. gingivalis* and *T. forsythia,* respectively. After periodontal treatment, there were significant reductions of all bacteria tested (Fig. 1). These data suggest that even in the healthy sites, *P. gingivalis* is present at higher levels than *T. forsythia* and *P. endodontalis.*

Correlations in the detection of analyzed bacteria

When the correlations between the three bacterial species were assessed, we observed statistically significant correlations between *P. gingivalis* and *T. forsythia* ($r = 0.624$, $p \leq 0.001$) and between *T. forsythia* and *P. endodontalis* ($r = 0.756$, $p \leq 0.01$) in the diseased group (baseline). However, there was only a moderate correlation between *P. gingivalis* and *P. endodontalis* ($r = 0.385$, $p \leq 0.05$). We found similar patterns of correlation in the diseased

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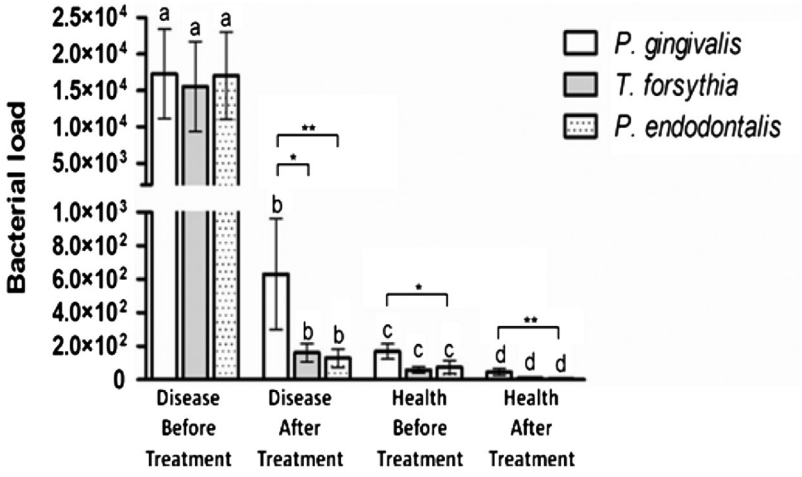

Fig. 1. Quantification of *P. endodontalis, P. gingivalis* and *T. forsythia* in subgingival plaque of patients with chronic periodontitis by qPCR. Letters represented the evaluating of each bacterium separately before and after periodontal treatment. So those bacteria that had statistically significant reduction after periodontal treatment were represented with different letters.

group after 60 days of periodontal treatment, although the correlation values were less significant. The correlations between *T. forsythia* and *P. gingivalis* ($r = 0.390$, $p \leq 0.05$) and between *T. forsythia* and *P. endodontalis* ($r = 0.483$, $p \leq 0.01$) were statistically significant, but the correlation was not significant between *P. gingivalis* and *P. endodontalis*. The healthy group showed no correlations between the analyzed bacteria between both periods (baseline and 60 days) possibly due to low levels of detection (data not shown).

Discussion

The primary goal of this study was to detect and quantify the pathogen *P. endodontalis* in patients with chronic periodontitis without periapical lesions and to determine a possible correlation with the pathogens that are already related to chronic periodontitis, i.e. *P. gingivalis* and *T. forsythia*. Microorganisms and their products are the initiators of periodontal lesions. The main pathogens associated with periodontal disease include *P. gingivalis*, *T. forsythia* and *T. denticola*. However, Kumar et al. (5), Tran et al. (6) and Dahlén and Leonhardt (7) suggested that other poorly characterized microorganisms (e.g. *P. endodontalis*) might also be associated with chronic periodontitis.

P. endodontalis was initially found in endodontic infections by Sundqvist et al. (23) and Van Winkelhoff et al. (8). This pathogen has already been identified as a member of the subgingival microbiota in humans; however, its association with periodontal disease is not well established. Few studies have reported the prevalence of this microorganism in diseased periodontal sites in patients without periapical lesions or the effect of a periodontal treatment in reducing the levels of *P. endodontalis* (6, 24).

Our study showed a high prevalence of *P. endodontalis* in addition to *P. gingivalis* and *T. forsythia,* in diseased periodontal sites when compared to healthy sites, with a statistically significant reduction after periodontal therapy. A previous study also has shown that *P. endodontalis* can be found in diseased sites in higher proportions than in healthy sites and in proportions similar to those of other pathogens already associated with periodontitis, such as *P. gingivalis* (13). On the other hand, Tran et al. (6) reported the presence of *P. endodontalis* in periodontal pockets but only at a low concentration.

Similar reports have shown that putative pathogens may be present in healthy periodontal sites but at lower levels than in diseased sites. This would explain why the pathogenicity of the microbiota differs from area to area and also from one individual to another (25). Haffajee et al. (25) demonstrated that the presence of pathogenic species is essential during the development of periodontal disease, but often they are not sufficient in numbers to establish disease and the onset of periodontitis depends on the presence of other predisposing factors (25).

The similarity between *P. endodontalis* and *P. gingivalis*, one of the main bacterial species found in diseased periodontal sites, is well known. However, the microorganisms possess distinct phenotypes, and no cross-reactions between them have been detected using molecular techniques. Thus, the results obtained in this study could not be related to the detection of *P. gingivalis*, as specific primers for *P. endodontalis* were used (7).

As noted in our results, *P. gingivalis* showed the smallest reduction after periodontal treatment among the microorganisms studied. At the end of the 60-day period between collections, it remained present at higher concentrations than the other bacteria. Reports in the literature indicated that *P. gingivalis* have fimbriae, which are potent virulence factors involved in bacterial adhesion to oral tissues, allowing the microorganism to invade the periodontal tissue more easily and increasing its survival in the gingival tissue (26).

The adhesion of *P. endodontalis* to the epithelial cells of the gingival sulcus differs from the adhesion of *P. gingivalis*. *P. endodontalis* is less able to adhere to gingival cells compared to *P. gingivalis* because it does not have fimbriae (27). *P. endodontalis* also does not produce a trypsin-like enzyme that other pathogens use for the destruction of periodontal tissue and does not exhibit hemagglutination activity (6). However, *P. endodontalis* has collagenases and proteases, enzymes that can also aid in the destruction of supporting tissues (27). *T. forsythia* also does not have fimbriae, reducing its capacity to colonize and invade periodontal tissue. However, it expresses a protein, BspA, that has a domain similar to a domain present in the fimbriae protein of *P. gingivalis* and induces inflammatory cytokine production in mammalian cells. These characteristics may contribute to the greater correlation between *P. endodontalis* and *T. forsythia* when compared to the correlation between *P. endodontalis* and *P. gingivalis* (3).

The present study demonstrated significant correlations between the numbers of bacterial cells of the three pathogens tested in the diseased group. The diseased sites had increased numbers of pathogens compared to the healthy sites. Therefore, the presence and amounts of *P. endodontalis*, *P. gingivalis* and *T. forsythia* in the subgingival environment may influence the local development of periodontal disease. Our data support the study of Dahlén and Leonhardt (7) that proposed the addition of *P. endodontalis* to the list of microorganisms that are used in the microbiological diagnosis of periodontal disease.

Thus, through a sensitive and specific qPCR method that is routinely used as a diagnostic tool for periodontitis, the association of *P. gingivalis* and *T. forsythia* was confirmed; these pathogens were found in higher proportions in affected sites than in healthy sites. We also noted a higher incidence of *P. endodontalis* in sites with periodontal disease than in healthy sites.

Despite the short term evaluation and small number of patients in this study, our results suggest that *P. endodontalis,* in addition to *P. gingivalis* and *T. forsythia,* can be found at high levels in diseased periodontal sites of periodontitis patients without periapical lesions when compared to healthy sites. Furthermore, a significant reduction in the numbers of *P. endodontalis, P. gingivalis,* and *T. forsythia* after mechanical periodontal therapy was observed.

Longitudinal studies with longer evaluation periods of 6 months to 1 year after periodontal treatment and controlled studies involving higher numbers of patients should be conducted to confirm our data and to clearly assess the pathogenic potential of *P. endodontalis* during the course of periodontal disease.

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Conflicts of interest and funding

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***Telma Blanca Lombardo Bedran**
Departament of Phisiology and Patology
Rua Humaitá, 1680
14801-903 Araraquara
SP – Brazil 55-16-33016402.
Email: telmabedran@hotmail.com