Real-time Polymerase Chain Reaction Quantification of *Porphyromonas gingivalis* and *Tannerella forsythia* in Primary Endodontic Infections

Daniel Saito, DDS, PhD, Luiz Lehmann Coutinho, BSA, PhD,† Cristiane Pereira Borges Saito, DDS, PhD,* Siu Mui Tsai, BSA, PhD,‡ José Francisco Höfling, BS, PhD,* and Reginaldo Bruno Gonçalves, DDS, PhD**

Abstract

Introduction: *Porphyromonas gingivalis* and *Tannerella forsythia* are anaerobic bacteria commonly involved in root canal infections. Although previous investigations have assessed these species by strictly qualitative approaches, accurate determination of their cell levels by a sensitive quantitative technique may contribute with additional information regarding relevance in pain of endodontic origin. **Method:** The root canal levels of *P. gingivalis*, *T. forsythia*, and total bacteria were investigated by a quantitative polymerase chain reaction (PCR) assay based on unique copy molecular markers. A total of 32 symptomatic (n = 14) and asymptomatic (n = 18) cases of endodontic infections were analyzed. Root canal samples were collected; genomic DNA was extracted and submitted to SYBR Green I real-time PCR targeting the *rgpB* (*P. gingivalis*), *bspA* (*T. forsythia*), and *rpoB* (total bacteria) single copy genes. **Results:** Overall, *P. gingivalis*, *T. forsythia*, and the coexistence of both species were encountered in 28%, 66%, and 22% of the subjects, respectively. *P. gingivalis* and *T. forsythia* levels ranged from 5.65×10^{-6} to 1.20×10^{-2} and from 5.76×10^{-6} to 1.35×10^{-1}. *T. forsythia* was highly prevalent and numerous in the study groups, whereas *P. gingivalis* was moderately frequent and less abundant, displaying 19-fold lower average levels than the former. **Conclusions:** The endodontic levels of *P. gingivalis* and *T. forsythia*, individually or in conjunction, did not display significant associations with the manifestation of pain of endodontic origin. *(J Endod 2009;35:1518–1524)*

Key Words

Bacteria, endodontic infection, *Porphyromonas gingivalis*, real-time PCR, *Tennerella forsythia*

The microbiota of endodontic infections comprise only a fraction of that found in the oral cavity; notwithstanding, it still harbors sufficient diversity to request complementary characterization by molecular biology methodologies (1–3). Although no specific microorganism has been accredited as the sole etiologic agent of pulpal and periradicular pathologies, some species have been more consistently reported in the root canal space. These are primarily represented by members of the genera *Actinomyces*, *Campylobacter*, *Capnocytophaga*, *Eubacterium*, *Fusobacterium*, *Lactobacillus,* *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Selenomonas,* *Streptococcus*, and *Veillonella* (2–4). A substantial number of studies on root canal infections has relied on cultivation techniques and, more recently, on the polymerase chain reaction (PCR) for microbial identification. However, although cultivation lacks sufficient sensitivity for detecting the very fastidious and noncultivable species (2, 5), endpoint PCR does not offer quantitative aptitudes in pair with the current standards. The real-time PCR technology brings additional analytic power to conventional PCR by providing automatic fluorescence detection of DNA molecules at the log-linear phase of amplification. Also, because reaction products are analyzed directly in the test tube, automation is increased and the occurrence of procedural errors is minimized (6).

Porphyromonas gingivalis, a non–spore-forming gram-negative anaerobic rod, is the most widely investigated oral bacteria and possesses a plethora of pathogenic properties, including fimbriae, proteinases, exopolysaccharides, and hemin-binding proteins (7). *Tannerella forsythia*, a non–spore-forming gram-negative anaerobic fusiform rod, is frequently detected in subjects with various forms of periodontal disease, root canal infections, and perimplantitis (8). In humans, *P. gingivalis* and *T. forsythia* are more prevalent in subjects with periodontal destruction than in the healthy counterparts, and both have been implicated with risk factors in periodontitis, suggesting a relevant role in disease establishment and progression (9, 10). The Arg-gingipain proteinases (arginine-specific cysteine proteinases or Rgp) of *P. gingivalis* are its most well-characterized virulence factors (11). RgpB, in particular, has been shown to play a crucial role in *P. gingivalis*' virulence, conferring oral colonization capability, and immunogenic and bone resorptive properties (12). BspA is a multifunctional surface and secreted protein of *T. forsythia* that is responsible for many of its pathogenic properties, including bacterial coaggregation, fibronectin and fibrinogen binding, epithelial attachment and invasion, and induction of proinflammatory cytokines and chemokines (8, 13).

It is generally accepted that infection severity depends not only on the virulence of the infecting strain, but also on its abundance in the invaded tissues. In this sense, the

From the *Department of Oral Diagnosis, Microbiology and Immunology Division, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil; and †Department of Animal Science, and ‡Center of Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil.

Supported by grants from the Brazilian funding agency Fundação de Amparo à Pesquisa do Estado de São Paulo–FAPESP (04/01674-6, 04/13548-5).

Address requests for reprints to Dr Reginaldo Bruno Gonçalves, Department of Oral Diagnosis, Microbiology and Immunology Division, Piracicaba Dental School, State University of Campinas, 13414-903, Piracicaba, São Paulo, Brazil. E-mail address: reginald@fop.unicamp.br.

0099-2399/$0 - see front matter

doi:10.1016/j.joen.2009.08.005

application of sensitive molecular quantification techniques can help determine the levels of particular species of oral bacteria (14–16) and, thereafter, assess potential associations with disease status. Here, a SYBR Green I real-time quantitative PCR based on single-copy molecular markers was used to determine the cell levels of *P. gingivalis*, *T. forsythia*, and total bacteria in primary endodontic infections in an effort to investigate their relevance in pain of endodontic origin.

Material and Methods

Subjects

Working approval was granted by the Ethics Committee of the Piracicaba Dental School, State University of Campinas, São Paulo State, Brazil. Thirty-two subjects (14 males and 18 females) ranging from 15 to 61 years old (mean = 34.6, standard deviation = 13.4) were selected from those referred for endodontic treatment at the Piracicaba Dental School. Subjects who had undergone antibiotic therapy within 2 months before collection were not included. A comprehensive medical and dental inventory and a written consent were obtained from each of the participants. Teeth to be sampled consisted of incisors, canines, premolars, and molars harboring pulpal necrosis and no previous history of root canal treatment (primary endodontic infections). Cases were mostly composed of exposed endodontic infections consequent to advanced caries. All teeth were examined by probing, thermal stimulation, vertical and lateral percussion, palpation, and preoperative radiographic evaluation. A negative response to thermal stimulation; absence of pulpal bleeding during coronal access; and the presence of periapical radiolucency, fistula, purulent drainage or swelling were the foremost clinical parameters considered for diagnosis of pulpal necrosis. Patients were classified in two groups for correlation with the detected bacterial levels: symptomatic (n = 14), consisting of cases presenting spontaneous pain, and asymptomatic (n = 18), consisting of cases devoid of spontaneous symptoms.

Root Canal Sample Collection

Each patient was submitted to local anesthesia, and the tooth to be sampled was isolated from the oral cavity with a rubber dam. Antisepsis of the crown and operation field was conducted according to a previously described decontamination protocol (17). The coronal access cavity was gained with a high-speed bur irrigated with sterile saline solution. A sterile #15 K-file was carefully introduced inside the root canal space down to 3 mm from the root apex. The root canal was then briefly instrumented to emulsify the bacterial cells. The handle of the K-file was cut and the file's active portion inserted in a test tube containing 1 mL Tris-EDTA buffer (10 mmol/L Tris/HCl, 1 mmol/L EDTA, pH = 8.0). Four sterile #15 paper points were consecutively introduced inside the canal for 20 seconds each and inserted in the same test tube. Samples were placed on ice and immediately transported to the laboratory.

Bacterial Strains

The following reference strains were used as controls for conventional and real-time PCR assays: *Aggregatibacter actinomycetemcomitans* ATCC 29522, *Bacteroides fragilis* ATCC 25285, *Bacteroides merdae* M-36, *Bacteroides vulgatus* ATCC 8482, *Escherichia coli* ATCC 12795, *Porphyromonas assacharolytica* ATCC 25260, *Porphyromonas circumdentaria* ATCC 51356, *Porphyromonas endodontalis* ATCC 35406, *P. gingivalis* ATCC 33277, *Porphyromonas levii* ATCC 29147, *Porphyromonas salivosa* NCTC 11632, *Prevotella oulora* ATCC 43324, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* NCTC 9336, *Pseudomonas aeruginosa* ATCC 10145, *Streptococcus salivarius* ATCC 25975, *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 27607, *Streptococcus mutans* ATCC 25175, and *T. forsythia* ATCC 43037.

DNA Extraction

Samples were thawed in a water bath at 37°C for 10 minutes and vortexed for 30 seconds; the paper points and files were removed from the tubes. Samples were then pelleted by centrifugation at 20,000*g* for 10 minutes and the supernatant discarded. A DNA extraction protocol was adapted from a previous study (18) and used on both sample and reference strains. Briefly, a lysis buffer (1.4 mol/L NaCl, 100 mmo/L TrisHCl [pH = 8.0], 20 mmol/L EDTA [pH = 8.0], 1% polivinilpirrolidone, 2% hexadecyltrimethylammonium bromide, 100 μg proteinase K/mL, and 0.2% β-mercaptoetanol) was added to the pelleted cells followed by vortexing and incubation in a water bath at 65°C for 30 minutes with gentle shaking every 10 minutes. A chloroform:isoamyl alcohol solution (24:1) was added to the tubes followed by gentle shaking and centrifugation at 20,000*g* for 7 minutes. The supernatant was transferred to a new tube, and the chloroform:isoamyl alcohol procedure was repeated until no evidence of debris was observed. The DNA was precipitated with isopropanol at room temperature, centrifuged at 20,000*g* for 7 minutes, and washed with 70% ethanol. Samples were resuspended in 40 μL Tris-EDTA buffer with 10 μg/mL RNAse and incubated in a water bath at 37°C for 30 minutes. The DNA was quantified in a spectrophotometer at 260 nm and stored at –20°C until required.

Design of PCR Primers

Primers targeting the arginin-specific cystein-proteinase (Arg-gingipain or *rgpB*) gene of *P. gingivalis* spanning positions 1308-1379 (forward 5'-CCTACGTGTACGGACAGAGCTATA-3', reverse 5'-AGGATCGCTCAGCGTAGCATT-3') were previously published (16). Primers for the surface antigen *bspA* gene of *T. forsythia* spanning positions 1911-2043 (forward 5'- TCACTATTGTGTCTCGCTG-3', reverse 5'-TCTCTCCGATTGTGGTTA-3') were designed based on the complete gene sequence acquired from the GenBank database (GI 3005672). Universal primers for the *rpoB* gene spanning positions 1528-1710 of *E. coli* (forward 5'-CAGTTGTCTCAGTTCATGGACC-3', reverse 5'- ACCGATGTTTGGACCTTCAG-3') were designed based on the gene sequences of 34 bacterial species of medical importance from Genbank (http://www.ncbi.nlm.nih.gov/Genbank) (GIs 50196905, 42779081, 52783855, 60679597, 58036264, 15791399, 10636177, 18308982, 38232642, 29374661, 49175990, 19703352, 16271976, 32265499, 15611071, 42518084, 54295983, 45655914, 57116681, 15793034, 34539880, 50841496, 15595198, 26986745, 16758993, 16763390, 30061571, 49482253, 57865352, 24378532, 15902044, 15674250, 42516522, and 15638995). Sequences were aligned with ClustalW (19) and searched for conserved domains with Bioedit (http://www.mbio.ncsu.edu). The Primer3 software was used for the design of candidate oligonucleotide sequences for all primer pairs (http://frodo.wi.mit.edu). A final set of primers was chosen according to the lowest potential to form secondary structures, as determined by Netprimer (http://www.premierbiosoft.com/netprimer). Validation of primer specificity was performed by submitting the primer sequences to the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a 100% nucleotide identity and coverage threshold with the corresponding taxa. In the case of the species-specific markers (*bspA* and *rgpB*), substantially lower identities and/or coverages with unrelated taxa was also required.

Validation of Markers by Endpoint PCR

PCR pilots were performed in 25-μL reactions with 1 × PCR Buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.2 μmol/L primers,

and 1.5 U Platinum *Taq* DNA polymerase (Invitrogen). The temperature profiles were: (1) for *rgpB,* initial denaturation at 96°C for 3 minutes: 36 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes; (2) for *bspA*, initial denaturation at 96°C for 3 minutes, 36 cycles of denaturation at 94°C for 1 minute, annealing at 46°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes; and (3) for *rpoB*, initial denaturation at 96°C for 3 minutes, 36 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes.

Preparation of Standard DNA

Ten-fold standard DNA dilutions from 10^7 to 10^2 genome copies per reaction were prepared for *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans*, considering their respective genome sizes as 2.34 Mb, 3.40 Mb, and 2.9 Mb (http://www.brop.org). Dilutions were achieved based on the formula: DNA weight (pg) = genome size (bp) × 1.023 E-9 (20). Genome copy levels were considered as numerically equivalent to bacterial cell levels, assuming that each cell contained a single genome copy. The standard dilutions were subsequently used for the generation of *rgpB, bspA*, and *rpoB* amplification profiles.

Optimization of the Real-time PCR Assay

Reactions were conducted in a Roche LightCycler 1.0 System running with LightCycler 3 Run software, version 4.24 (Roche Applied Science). Standard DNA corresponding to 10^3 cells of *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans* were used as templates for *rgpB*, *bspA*, and *rpoB* (total bacteria) markers, respectively. Reaction optimization was performed with various $MgCl_2$ and primer concentrations as stated by the manufacturer (Roche). Optimal reactions were performed in total volumes of 10 μL containing 2.0 μL DNA, 1.0 μL LightCycler FastStart DNA Master SYBR Green I, 4 mmol/L $MgCl_2$, and 0.5 μmol/L of each primer. The temperature profiles were (1) for *P. gingivalis* (*rgpB* gene): hot-start denaturation at 95°C for 10 minutes and 42 cycles of denaturation at 94°C for 10 seconds, annealing at 60°C for 7 seconds, extension at 72°C for 7 seconds, and fluorescence acquisition at 78°C for 3 seconds; (2) for *T. forsythia* (*bspA* gene): hot-start denaturation at 95°C for 10 minutes and 50 cycles of denaturation at 95°C for 10 seconds, annealing at 46°C for 7 seconds, extension at 72°C for 7 seconds, and fluorescence acquisition at 83°C for 3 seconds; and (3) total bacteria (*rpoB* gene): hot-start denaturation at 95°C for 10 minutes and 38 cycles of denaturation at 95°C for 10 seconds, annealing at 56°C for 7 seconds, extension at 72°C for 7 seconds, and fluorescence acquisition at 83°C for 3 seconds.

Real-time PCR Amplification of Standard DNA

Amplification profiles for *rgpB, bspA* and *rpoB* markers were generated in duplicates with standard DNA dilutions (Table 1). The computer-assisted second derivative maximum algorithm was used for crossing point inference for each dilution. A standard curve was generated for each marker by linear regression analysis and used as a basis for further quantification of target DNA from the clinical samples (Table 1). Amplification efficiencies were inferred according to the following formula: $E = 10^{(-1/s)}$, where s is the slope of the standard curve (21).

Real-time PCR Amplification of DNA From Clinical Samples

Real-time PCR amplification assays were conducted in duplicates, as described for standard DNA. Bacterial cell levels were inferred for each sample based on the previously obtained standard curves.

Melting Curve Analysis

Reaction specificities were verified by melting curve analysis with a progressive temperature increase from 70°C to 95°C at a 0.1°C/s transition rate and continuous fluorescence acquisition. To minimize potential primer-dimer artifacts during the analysis of clinical samples, fluorescence acquisition temperatures were set to approximately 4°C below the denaturing temperature of each amplification product, as previously established by melting curve analysis; 3.0 μL of each real-time PCR product was submitted to 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized on ultraviolet light. PCR products with melting temperatures or fragment sizes divergent from those established for standard DNA were considered as false-positives; for such cases, a null fluorescence value was attributed.

Sequencing of Selected PCR Products From Clinical Samples

Five representative electrophoresis gel bands per species-specific marker were selected to ascertain amplicon identities. DNA was purified with the GFX DNA Purification kit (Amersham Biosciences) as stated by the manufacturer. DNA sequencing reactions were performed in 10-μL mixtures containing approximately 100 ng template DNA, 1 μL Big Dye Terminator Ready version 3.0 (Applied Biosystems), 0.5 mmol/L primer, and 3 μL sequencing buffer (200 mmol/L Tris/ HCl [pH = 9.0] and 5 mmol/L $MgCl_2$). Nucleotide sequences were visualized with Bioedit and submitted to the BLASTN program for comparison with sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). A 99% minimum nucleotide identity was required for positive identification at the species level.

Statistical Analysis

R (www.r-project.org) and BioEstat (http://www.mamiraua.org.br) softwares were used for statistical testing of data. Preliminary exploration with the Lilliefors test indicated that data were nonhomogeneous even after log transformation. The Mann-Whitney nonparametric test was then chosen to evaluate the null hypothesis that the target species were not associated with endodontic symptoms, with a significance level $\alpha = 0.05$. In order to assess the correlation between the concomitant presence of target bacteria and endodontic pain, the integrated levels of *P. gingivalis* and *T. forsythia* were used in the cases in which both appeared concurrently; in all other cases (ie, exclusive presence of either species or absence of both), the integrated levels were assigned as null.

Results

Real-time PCR Amplification of Standard DNA

The number of cycles required to amplify the full range of serially diluted DNA ranged from 23 to 42 for *rgpB* (10^7 to 10^2), from 24 to 50 for *bspA* (10^7 to 10^2), and from 21 to 38 for *rpoB* (10^7 to 10^2). Linear regression equations used for the inference of cell levels of the target species are shown in Table 1, along with the corresponding r^2 and amplification efficiency values. No evidence of nonspecific or cross-reaction products was observed for both species-specific markers, as determined by conventional endpoint PCR using standard DNA from various reference strains (data not shown). Dissociation profiles of standard DNA amplicons revealed well-depicted peaks for all markers, with melting temperatures of 82.8°C (*rgpB*), 87.5°C (*bspA*), and 88.7°C (*rpoB*).

TABLE 1. Mean Crossing Points (CPs) and Standard Curve Formulas Obtained From 10-fold Serially Diluted DNA of Reference Bacteria

	P. gingivalis (*rgpB*)		*T. forsythia* (*bspA*)		Total bacteria (*rpoB*)	
Cell levels	Mean CP	SD	Mean CP	SD	Mean CP	SD
10^7	23.56	0.03	24.92	0.06	21.02	0.00
10^6	26.89	0.05	30.42	0.32	24.19	0.04
10^5	30.00	0.09	35.53	0.11	27.88	0.01
10^4	34.11	0.16	40.02	0.03	31.09	0.09
10^3	38.01	0.58	45.07	0.36	33.78	0.09
10^2	41.19	2.49	49.67	0.00	37.25	0.00
Standard curve formula	y = −3.42x + 47.52 (r^2 = 0.989) (E = 0.96)		y = −4.94x + 59.86 (r^2 = 0.999) (E = 0.59)		y = −2.98x + 43.77 (r^2 = 0.998) (E = 1.16)	

SD, standard deviation, E, amplification efficiency.

Real-time PCR Amplification of DNA From Clinical Samples

Overall, *P. gingivalis*, *T. forsythia*, and the coexistence of both species were detected in 9 of 32 (28%), 21 of 32 (66%), and 7 of 32 (22%) of the subjects, respectively. Absolute cell levels in clinical samples ranged from 2.27×10^2 to 6.32×10^4 for *P. gingivalis* and from 1.35×10^2 to 3.94×10^6 for *T. forsythia* (means 5.26×10^3 and 2.28×10^5 cells per sample, respectively). Relative cell levels ranged from 5.65×10^{-6} to 1.20×10^{-2} for *P. gingivalis* and from 5.76×10^{-6} to 1.35×10^{-1} for *T. forsythia* (means 6.47×10^{-4} and 1.22×10^{-2} per sample, respectively) (Table 2).

Statistical Analysis

No statistical difference in the relative levels of *P. gingivalis*, *T. forsythia*, or their integrated levels was observed between the symptomatic and asymptomatic groups as determined with the Mann-Whitney test (all $p > 0.05$).

Discussion

Real-time PCR is a DNA amplification technique that allows precise determination of nucleic acid levels by monitoring fluorescent signals at a cycle-to-cycle rate (22), bringing important contribution to the detection of pathogenic bacteria in mixed oral infections (14, 15, 23). It has been shown that real-time PCR can provide up to 41-fold greater sensitivity when compared with colony counting for selected anaerobic species, offering 36% to 51% detection increases in the particular cases of *T. forsythia* and *P. gingivalis* (5, 24).

SYBR Green I is an intercalating dye that binds to the minor groove of double-stranded DNA. It has been widely applied in concert with the real-time PCR technology because of its versatility, simplicity of use, and

TABLE 2. Average Cell Levels of *P. gingivalis* (*Pg*), *T. forsythia* (*Tf*), and Total Bacteria Detected in 32 Subjects

Subject	*Pg*	*Tf*	*Pg*/total bacteria	*Tf*/total bacteria	Total bacteria
Symptomatic					
S1	—	6.38 E + 3	—	8.45 E-5	7.54 E + 7
S2	—	—	—	—	4.67 E + 7
S3	—	3.94 E + 6	—	1.24 E-1	3.19 E + 7
S4	—	2.12 E + 3	—	2.94 E-5	7.22 E + 7
S5	6.32 E + 4	1.06 E + 4	4.35 E-4	7.32 E-5	1.45 E + 8
S6	—	2.10 E + 3	—	6.70 E-5	3.13 E + 7
S7	—	9.95 E + 2	—	1.43 E-5	6.97 E + 7
S8	—	—	—	—	2.06 E + 8
S9	1.64 E + 3	—	4.87 E-4	—	3.38 E + 6
S10	—	—	—	—	1.67 E + 7
S11	—	—	—	—	2.61 E + 7
S12	2.27 E + 2	1.35 E + 2	2.11 E-4	1.26 E-4	1.07 E + 6
S13	—	1.06 E + 5	—	1.41 E-4	7.50 E + 8
S14	—	—	—	—	2.86 E + 5
Asymptomatic					
S15	—	3.50 E + 2	—	1.39 E-5	2.51 E + 7
S16	3.86 E + 4	—	4.61 E-3	—	8.36 E + 6
S17	—	4.01 E + 4	—	1.92 E-5	2.09 E + 9
S18	—	—	—	—	4.14 E + 5
S19	1.00 E + 3	4.09 E + 4	5.65 E-6	2.31 E-4	1.77 E + 8
S20	—	4.79 E + 3	—	5.76 E-6	8.32 E + 8
S21	4.53 E + 4	9.61 E + 5	2.07 E-3	4.39 E-2	2.19 E + 7
S22	—	6.02 E + 4	—	1.18 E-3	5.12 E + 7
S23	—	—	—	—	3.10 E + 8
S24	—	3.74 E + 2	—	2.06 E-4	1.82 E + 6
S25	1.23 E + 4	3.71 E + 2	5.96 E-4	1.80 E-5	2.06 E + 7
S26	—	6.92 E + 5	—	1.35 E-1	5.14 E + 6
S27	—	—	—	—	4.01 E + 6
S28	2.62 E + 3	3.49 E + 5	2.25 E-4	2.99 E-2	1.16 E + 7
S29	0	1.08 E + 6	—	5.45 E-2	1.98 E + 7
S30	3.38 E + 3	2.00 E + 2	1.20 E-2	7.14 E-4	2.80 E + 5
S31	—	—	—	—	5.19 E + 5
S32	—	1.15 E + 3	—	6.34 E-4	1.81 E + 6

reduced cost (6). This dye has proved to be a sensitive and accurate reporter molecule for nucleic acid quantification, with specificity and robustness comparable to those of other available chemistries (25). However, because SYBR Green I may bind to any double-stranded DNA, a careful reaction optimization protocol should be preferably conducted to avoid simultaneous quantification of nonspecific products and/or primer-dimer artifacts (6, 25). In this regard, we have used preventive procedures to minimize such technical limitation, including preliminary evaluation of primer sequences and melting curve analysis of amplification products. The evidence of well-defined unique peaks in the dissociation profiles ensured satisfactory reaction specificities for all markers, with no evidences of primer-dimer formation. As an additional precaution, monitoring of reaction products was conducted by fluorescence acquisition at temperatures in which double-stranded DNA was presumably composed only by the target gene products (22).

Despite the inexistence of primer dimmer artifacts and spurious reaction products in control PCR, a notably high number of PCR cycles was required to amplify the full range of DNA dilutions. This was more clearly noticeable in the case of the *bspA* primer set. Accordingly, the use of an amplification efficiency formula displayed values of 0.96 for *rgpB*, 0.59 for *bspA*, and 1.16 for *rpoB*, with 1.0 considered as the gold standard (21). Bearing this in mind, additional care was taken when analyzing the clinical samples in order to minimize the possibility of cross-reactions with external bacteria. This was performed by adding negative controls to every LightCycler run and also by submitting representative reaction products to DNA sequencing for taxonomic confirmation. According to this procedure, no amplification was observed in the negative controls, and all sequenced products unveiled nucleotide identities ≥99% with the corresponding taxa, ensuring reliability in the detection protocol.

Molecular detection and quantification of oral bacteria have been mostly achieved by analysis of the ribosomal genes (5, 14). However, it has been shown that variations in ribosomal operon copy numbers among species and strains (26) may impair proper determination of cell levels in complex assemblages (27). In this sense, the *rpoB* protein-encoding gene has been suggested as a replacement marker for microbial ecology research, offering phylogenetic resolutions comparable to those of the 16S rRNA gene at various taxonomic levels (28). In a similar way, the *rgpB* and *bspA* single-copy protein encoding genes have been used as alternative markers for quantification of *P. gingivalis* and *T. forsythia*, both providing satisfactory detection sensitivities in dental biofilm samples (15, 16).

Many high-throughput 16S rDNA clone library studies failed to find either *P. gingivalis* or *T. forsythia* in endodontic infections (1-3). This could be a consequence of DNA amplification biases commonly generated by the use of broad-detection PCR primers or the lack of primer specificity for these bacterial species (29, 30). Conversely, the use of PCR with species-specific primers has shown greater detection rates for *P. gingivalis* and *T. forsythia* in root canal infections (30). Truly, Siqueira et al (31) and Fouad et al (32) were able to detect *P. gingivalis* in 4% of the samples by species-specific 16S rDNA PCR, whereas Foschi et al (33) revealed the species in 13% of patients using a similar approach. Higher prevalences were obtained by Gomes et al (34) (36%), Gomes et al (35) (44%), Tomazinho et al (36) (43%), and Siqueira et al (37) (41%) using PCR methodologies. In this study, *P. gingivalis* was detected in 28% of the subjects, corroborating the results of Siqueira et al (37) using DNA-DNA hybridization (28%), Tomazinho et al (36) using cultivation (27%) and Jung et al (38) using DNA probing (27%). Siqueira et al (31) and Foschi et al (33) were able to detect *T. forsythia* in 4% and 7% of the patients by conventional and by nested 16S rDNA PCR, respectively. Gomes et al (35), Rôças et al (39), Fouad et al (32), and Jung et al (38) detected the species in 18% to 24% of primary endodontic infections by conventional PCR. In this study, *T. forsythia* was observed in 66% of the subjects, an estimate closer to those obtained by Siqueira and Rôças (40) with nested PCR (52%) and by Gonçalves and Mouton (41) with immunocapture PCR (54%).

Selected clinical features have been attributed to the presence of *P. gingivalis* in root canal infections. Gomes et al (34, 35) were able to find significant associations between *P. gingivalis* and tenderness to percussion, purulent drainage, swelling, and abscess. Jacinto et al (42) revealed a correlation of this species with pain on palpation and purulent drainage. Siqueira et al (43) found a higher prevalence of *P. gingivalis* in cases of acute apical abscesses than in chronic or acute apical periodontitis. Hashioka et al (44) found representatives of the genus *Porphyromonas* to be associated, as a group, with subacute symptoms in a cultivation-based study; however, the specific contribution of *P. gingivalis* was not assessed in a direct manner. To the breadth of our knowledge and despite its correlation with various clinical parameters, no clear evidence of *P. gingivalis*' relevance in the manifestation of spontaneous pain has been yet successfully achieved (33, 38, 39). Our results support this concept because no statistically significant differences in *P. gingivalis* root canal levels were detected between the symptomatic and asymptomatic study groups.

In a previous report, Gomes et al (45) were able to detect significant association between the presence of *T. forsythia* and tenderness to percussion in root-filled teeth by a nested PCR approach, whereas Sassone et al (46) found a direct relationship between *T. forsythia* counts and pain of endodontic origin by DNA-DNA hybridization analysis. In this study, no statistical association between *T. forsythia*'s relative levels and spontaneous pain could be observed in contempt of its relatively high prevalence in the sample set. The result accords with those of previous nonquantitative molecular studies (33, 38-40).

Positive associations among endodontic bacteria have been previously described in root canal infections (47, 48). In addition, it has been shown that specific combinations of bacteria are more frequent in cases of persistent periapical lesions than single species (49). In a clinical scope, this adds to the idea that bacterial complexes in endodontic infections might be more relevant than individual species alone (4, 50). *P. gingivalis* and *T. forsythia* are part of a bacterial complex responsible for disease severity in periodontitis (51). The synergistic pathogenic effect of *P. gingivalis* and *T. forsythia* was clearly shown in an animal model with abscess formation and sepsis after subcutaneous coinoculation (52). Intercellular binding and growth induction of *P. gingivalis* by *T. forsythia* isolates have been observed *in vitro* (53). In addition, it has been shown that *P. gingivalis* accelerates growth of *T. forsythia* and that *rgp* and *kgp* gene products are directly involved in the synergistic pathogenic activities observed in mixed infections (54). In this study, in order to analyze *P. gingivalis* and *T. forsythia* as a unique consortium, their respective cell levels were integrated, and their potential association with pain of endodontic origin was assessed. Accordingly, no linkage with the presence of symptoms could be verified. It is possible that the exceedingly selective environmental conditions established inside the canal might downmodulate the cellular interactions between these particular oral species, but more specific research would be required to validate such hypothesis.

Many pain-producing substances have been described, including bacterial metabolic products (ie, ammonia, urea, and indole) and membrane components (ie, lipopolysaccharide, lipoteichoic acid, and peptidoglycan) (55). However, because of the overwhelming diversity of bacteria residing in the oral cavity (56), a multitude of virulence factors might still remain undetermined. Here, we have assessed the cell levels of two oral bacteria by a quantification approach targeting two restricted virulence factors. Because *rgpB* and *bspA* are both single-copy genes, the methodology has also permitted the determination of

rgpB and *bspA* gene levels in the clinical samples. Although no implications with symptoms could be verified, complementary research at the messenger RNA or protein standpoints could bring additional insight into the clinical relevance of these factors.

In conclusion, the application of a real-time PCR methodology based on unique copy genes in primary endodontic infections has shown that *T. forsythia* can be highly prevalent and abundant in endodontic infections, whereas *P. gingivalis* is moderately frequent and less abundant, displaying 19-fold lower average levels than those observed for the former. Within the technical boundaries of this study, the results indicated that the root canal levels of *P. gingivalis* and *T. forsythia*, individually or in conjunction, do not play a significant role in the development of pain of endodontic origin.

References

1. Munson MA, Pitt-Ford T, Chong B, et al. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002;81:761–6.
2. Rolph HJ, Lennon A, Riggio MP, et al. Molecular identification of microorganisms from endodontic infections. J Clin Microbiol 2001;39:3282–9.
3. Saito D, Leonardo RT, Rodrigues JLM, et al. Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries. J Med Microbiol 2006; 55:101–7.
4. Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surg Oral Med Oral Pathol 1994;78:522–30.
5. Martin FE, Nadkarni MA, Jacques NA, et al. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J Clin Microbiol 2002;40: 1698–704.
6. Zhang T, Fang HHP. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Appl Microbiol Biotechnol 2006;70:281–9.
7. Holt SC, Kesavalu L, Walker S, et al. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000;1999(20):168–238.
8. Tanner AC, Izard J. *Tannerela forsythia*, a periodontal pathogen entering the genomic era. Periodontol 2000;2006(42):88–113.
9. Grossi SG, Genco RJ, Machtei EE, et al. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol 1995;66:23–9.
10. van Winkelhoff AJ, Loos BG, van der Reijden WA, et al. *Porphyromonas gingivalis, Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. J Clin Periodontol 2002;29:1023–8.
11. Shi Y, Ratnayake DB, Okamoto K, et al. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA, rgpB, kgp*, and *hagA*. J Biol Chem 1999; 274:17955–60.
12. Pathirana RD, O'Brien-Simpson NM, Brammar GC, et al. Kgp and RgpB, but not RgpA, are important for *Porphyromonas gingivalis* virulence in the murine periodontitis model. Infect Immun 2007;75:1436–42.
13. Inagaki S, Onishi S, Kuramitsu HK, et al. *Porphyromonas gingivalis* vesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by "*Tannerella forsythia*". Infect Immun 2006;74:5023–8.
14. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J Clin Microbiol 2000;38:2362–5.
15. Morillo JM, Lau L, Sanz M, et al. Quantitative real-time PCR based on single copy gene sequence for detection of periodontal pathogens. J Clin Peridontol 2004;31: 1054–60.
16. Morillo JM, Lau L, Sanz M, et al. Quantitative real-time PCR based on single copy gene sequence for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. J Periodontal Res 2003;38:518–24.
17. Ng YL, Spratt D, Sriskantharajah S, et al. Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. J Endod 2003;29:317–20.
18. Smith GL, Sansone C, Socransky SS. Comparison of two methods for the small-scale extraction of DNA from subgingival microorganisms. Oral Microbiol Immunol 1989; 4:135–40.
19. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22: 4673–80.
20. Dolezel J, Bartos J, Voglmayr H, et al. Nuclear DNA content and genome size of trout and human. Cytometry A 2003;51:127–8.
21. Peters IR, Helps CR, Hall EJ, et al. Real-time RT-PCR: considerations for efficient and sensitive assay design. J Immunol Methods 2004;286:203–17.
22. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 1998;24: 954–8, 960, 962.
23. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, et al. The additional value of real-time PCR in the quantitative detection of periodontal pathogens. J Clin Periodontol 2006;33:427–33.
24. Verner C, Lemaitre P, Daniel A, et al. Carpegen real-time polymerase chain reaction vs. anaerobic culture for periodontal pathogen identification. Oral Microbiol Immunol 2006;21:341–6.
25. Maeda H, Fujimoto C, Haruki Y, et al. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ* gene and total bacteria. FEMS Immunol Med Microbiol 2003;39:81–6.
26. Lee ZM, Bussema C 3rd, Schmidt TM. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res 2009;37:489–93.
27. Farrelly V, Rainey FA, Stackebrandt E. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 1995;61:2798–801.
28. Case RJ, Boucher Y, Dahllöf I, et al. Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. Appl Environ Microbiol 2007;73:278–88.
29. Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 1996;62:625–30.
30. Vickerman MM, Brossard KA, Funk DB, et al. Phylogenetic analysis of bacterial and archaeal species in symptomatic and asymptomatic endodontic infections. J Med Microbiol 2007;56:110–8.
31. Siqueira JF Jr., Rôças IN, Alves FR, et al. Selected endodontic pathogens in the apical third of infected root canals: a molecular investigation. J Endod 2004;30:638–43.
32. Fouad AF, Barry J, Caimano M, et al. PCR-based identification of bacteria associated with endodontic infections. J Clin Microbiol 2002;40:3223–31.
33. Foschi F, Cavrini F, Montebugnoli L, et al. Detection of bacteria in endodontic samples by polymerase chain reaction assays and association with defined clinical signs in Italian patients. Oral Microbiol Immunol 2005;20:289–95.
34. Gomes BPFA, Pinheiro ET, Jacinto RC, et al. Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction. J Endod 2008;34:537–40.
35. Gomes BPFA, Montagner F, Jacinto RC, et al. Polymerase chain reaction of *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia* in primary endodontic infections. J Endod 2007;33:1049–52.
36. Tomazinho LF, Avila-Campos MJ. Detection of *Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia*, and *Prevotella nigrescens* in chronic endodontic infection. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:285–8.
37. Siqueira JF Jr., Rôças IN, De Uzeda M, et al. Comparison of 16S rDNA-based PCR and checkerboard DNA-DNA hybridisation for detection of selected endodontic pathogens. J Med Microbiol 2002;51:1090–6.
38. Jung IY, Choi BK, Kum KY, et al. Molecular epidemiology and association of putative pathogens in root canal infection. J Endod 2000;26:599–604.
39. Rôças IN, Siqueira JF Jr., Santos KRN, et al. "Red complex" (*Bacteroides forsythus, Porphyromonas gingivalis*, and *Treponema denticola*) in endodontic infections: a molecular approach. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91: 468–71.
40. Siqueira JF Jr., Rôças IN. *Bacteroides forsythus* in primary endodontic infections as detected by nested PCR. J Endod 2003;29:390–3.
41. Gonçalves RB, Mouton C. Molecular detection of *Bacteroides forsythus* in infected root canals. J Endod 1999;25:336–40.
42. Jacinto RC, Gomes BP, Shah HN, et al. Incidence and antimicrobial susceptibility of *Porphyromonas gingivalis* isolated from mixed endodontic infections. Int Endod J 2006;39:62–70.
43. Siqueira JF Jr., Rôças IN, Silva MG. Prevalence and clonal analysis of *Porphyromonas gingivalis* in primary endodontic infections. J Endod 2008;34:1332–6.
44. Hashioka K, Yamasaki M, Nakane A, et al. The relationship between clinical symptoms and anaerobic bacteria from infected root canals. J Endod 1992; 18:558–61.
45. Gomes BP, Jacinto RC, Pinheiro ET, et al. Molecular analysis of *Filifactor alocis, Tannerella forsythia*, and *Treponema denticola* associated with primary endodontic infections and failed endodontic treatment. J Endod 2006;32:937–40.
46. Sassone LM, Fidel RA, Faveri M, et al. A microbiological profile of symptomatic teeth with primary endodontic infections. J Endod 2008;34:541–5.
47. Peters LB, Wesselink PR, vanWinkelhoff AJ. Combinations of bacterial species in endodontic infections. Int Endod J 2002;35:698–702.
48. Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol Immunol 1992;7:257–62.

49. Fabricius L, Dahlén G, Sundqvist G, et al. Influence of residual bacteria on periapical tissue healing after chemomechanical treatment and root filling of experimentally infected monkey teeth. Eur J Oral Sci 2006;114:278–85.
50. Gomes BP, Pinheiro ET, Gadê-Neto CR, et al. Microbiological examination of infected dental root canals. Oral Microbiol Immunol 2004;19:71–6.
51. Socransky SS, Haffajee AD, Cugini MA, et al. Microbial complexes in subgingival plaque. J Clin Periodontol 1998;25:134–44.
52. Takemoto T, Kurihara H, Dahlen G. Characterization of *Bacteroides forsythus* isolates. J Clin Microbiol 1997;35:1378–81.
53. Yoneda M, Yoshikane T, Motooka N, et al. Stimulation of growth of *Porphyromonas gingivalis* by cell extracts from *Tannerella forsythia*. J Periodontal Res 2005;40:105–9.
54. Yoneda M, Hirofuji T, Anan H, et al. Mixed infection of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a murine abscess model: involvement of gingipains in a synergistic effect. J Periodontal Res 2001;36:237–43.
55. Hahn C, Liewehr FR. Relationship between caries bacteria, host responses, and clinical signs and symptoms of pulpitis. J Endod 2007;33:213–9.
56. Aas JA, Paster BJ, Stokes LN, et al. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005;43:5721–32.