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Comparison of two different methods for detecting periodontal pathogenic bacteria

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Abstract

Aim: To perform a comparative analysis between two methods for detecting *Porphyromonas gingivalis*, *Tannerella forsythia* and *Porphyromonas endodontalis* in periodontal plaque samples. **Methods:** The study sample consisted of twenty systemically healthy patients showing generalized chronic periodontitis. The subgingival samples for microbiological analysis were collected before (baseline) and 60 days after a basic periodontal therapy from 30 non-adjacent affected sites (Probing Depth (PD): 5-7 mm, Clinical Attachment Loss (CAL) ≥ 5 mm, positive for Bleeding on Probing (BOP)). Microbiological analysis was performed by PCR and qPCR. To allow a comparative analysis between both methods, qPCR was divided in three different scores (score 2: presence of more than 100 bacteria; score 1: presence of 10-100 bacteria, and score 0: absence of bacteria), in accordance to DNA quantity, while for PCR two scores were assigned: presence or absence of bacteria. **Results:** qPCR demonstrated higher sensitivity in the detection of these pathogens compared with PCR when scores 1 and 2 were considered positive. However, when only score 2 was considered positive, PCR and qPCR showed better agreement. **Conclusions:** qPCR demonstrated higher sensitivity than conventional PCR for detection of low numbers of microorganisms and can be useful for the quantification of periodontopathogens.

Keywords: Periodontal Diseases. Polymerase Chain Reaction. Bacteria.

Introduction

Periodontitis is an inflammatory oral disease caused by specific microorganisms that colonize the periodontal pocket and leads to destruction of the tooth-supporting tissues, including gingival connective tissue and alveolar bone[1]. Bacteria species that have been strongly associated with periodontitis include members of the red complex[2]: *Porphyromonas gingivalis (P. gingivalis), Tannerella forsythia (T. forsythia)* and *Treponema denticola (T. denticola)*. In addition, some studies have reported the presence of additional subgingival bacterial species in diseased periodontal sites[1,2]. Among these pathogens, *Porphyromonas endodontalis*, an asaccharolytic, black-pigmented, Gram-negative anaerobic bacteria, is noteworthy[3].

Mechanical therapy is considered as the gold standard treatment of periodontitis. This therapy consists in combining scaling and root planning performed by a professional and plaque removal achieved by the patient. In some cases, this treatment

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may not be sufficient to prevent the progression of periodontitis[4]. For these patients, an adjunctive antimicrobial therapy associated with mechanical therapy is often used[4].

Microbiological analysis is an important procedure to i) identify and quantify pathogens in periodontal pockets from patients with periodontitis, ii) clarify the diagnosis and etiology of severe forms of periodontitis, and iii) help to select the correct adjunctive antimicrobial therapy to treat patient[4,5]. Bacterial culture is considered the gold standard method for the identification of periodontopathogens, since it is the only technique capable of isolating viable pathogens and to test their susceptibility to antimicrobial agents. However, there are some limitations associated with this procedure. Indeed, cultivation cannot detect non-viable bacteria, as well as, hardly cultivated bacteria important in the etiology of periodontitis, such as *T. denticola*[6,7]. Nowadays, alternative nucleic acid-based methods, more sensitive than bacterial culture, have been used to detect and quantify pathogens present in oral cavity[5,6,8]. More specifically, polymerase chain reaction is a technique based on the detection of nucleic acids and has been used by many laboratories to quantify periodontopathogens[5-7].

Conventional PCR is a technique based on replication of DNA that is used to detect pathogens in periodontal pocket with a greater sensitivity and specificity than cultivation[9-11]. However, this technique has some limitations, such as the difficulties to detect pathogens in sample containing small quantities of DNA, since more than 100 bacteria in periodontal pockets is required for PCR[9,10]. In addition, the exact amount of bacteria cannot be determined since it is a qualitative method of diagnostic, and therefore cannot be used to differentiate health and disease samples[6,8,9,11,12]. In order to overcome some of these drawbacks, different strategies of PCR have been introduced in many laboratories. More specifically, quantitative PCR (qPCR) allows the quantification of low number of bacterial loads in subgingival plaque samples[12-14]. qPCR is able to detect approximately 10 bacteria and is able to quantify the exact amount of bacteria present in subgingival crevicular fluid[6,12,15].

A large number of studies in the literature compared qPCR and anaerobic culture for the detection and quantification of pathogens in periodontal samples[6,16]. However, only few studies compared conventional PCR and qPCR for the detection of periodontopathogens. Therefore, the purpose of this study was to compare PCR and qPCR for the detection of *P. gingivalis*, *T. forsythia* and *P. endodontalis* in subgingival samples from patients with chronic periodontitis before and after periodontal treatment. This comparison of the two procedures should demonstrate benefits and limitations of each method.

Material and methods

Subject Population

This study was designed as a double-blind, controlled trial, which all patients were prospectively assigned for two months after a basic periodontal treatment of scaling and root planning, to evaluate the effect of periodontal treatment on the reduction of periodontopathogens on the periodontal pocket and to compare PCR and qPCR for the detection of *P. gingivalis*, *T. forsythia* and *P. endodontalis* in plaque samples from patients with 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[12,13,15]. Briefly, first we selected similar studies to find the approximate sample size that would be necessary (we found sample size between 20-30). Based on this number, we performed the sample calculation to find the smallest number that could be used in this study to get statistically significant results.

The sample size comprised twenty patients randomly selected (8 men and 12 women 35-55 years of age) with moderate to severe chronic periodontitis and without history of systemic diseases. Inclusion criteria were as follows: presence of at least 20 teeth; minimum of three non-adjacent teeth with bleeding on probing (BOP), clinical attachment level (CAL) > 5 mm and probing depth (PD) between 5 and 7 mm. No patients used antibiotic therapy in the 6 months prior to the study.

Forty-five days before beginning the periodontal treatment, all the patients selected for this study received oral hygiene instructions every week. After 30 days of oral hygiene instruction, periodontal examination was performed for patients with a visible plaque index of less than 30%.

Study design

After providing the oral hygiene instructions, 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. The selected teeth had no dysfunctions in relation to occlusion and had no prostheses.

In order to standardize the position of the manual probe (Williams®, São Paulo, Brazil) and the position of paper point to collect plaque samples in sites selected for microbiological analysis, alginate molds of the dental arches were made to prepare acetate stents. 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 plaque samples collections were performed at baseline and at 60 days after the periodontal treatment.

Clinical parameters

Clinical examination was performed by a trained and calibrated examiner, whose intra-exam repeatability was determined at baseline (Kappa score = 0.91). The clinical parameters measured included CAL, BOP, 0/1 (negative/positive) and PD, 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

Plaque samples for microbiological analyses were collected seven days after the initial clinical examination and

then 60 days after completion of the basic periodontal treatment and storage at -20ºC. Briefly, supragingival biofilm was removed and the selected sites were isolated with cotton rolls and gently air-dried. Two sterile paper points (No. 30; Dentsply, Maillefer, Petrópolis, RJ, Brazil) were inserted in the gingival pocket up to the apical portion for 30 seconds and the subgingival fluid was collected. 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 PCR and qPCR.

Basic periodontal treatment

The basic periodontal treatment was performed under local anesthesia and with the aid of manual instruments (McCall and Gracey Curettes and Hirschfeld File Scaler - Hu Friday®). Patients received a non-surgical periodontal treatment including scaling and root planning (SRP) followed by oral hygiene instructions seven days after completing the microbiological sample collection. Dental 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 weekly for 60 days, at which time the second sample plaque collection and periodontal examination was performed.

Bacterial strains and growth conditions

The bacterial species *P. gingivalis* (ATCC 33277), *T. forsythia* (ATCC 43037) and *P. endodontalis* (E203) were used to determine the specificity and detection levels of the PCR method. Briefly, bacteria were grown anaerobically on Tryptone soy blood agar plates supplemented with hemin (0.5 µg/ml) and menadione (1 µg/ml) at 37˚ C in 85% N_2, 5% CO_2 and 10% H_2 in an anaerobic chamber[17].

DNA extraction

The extraction of DNA from subgingival fluid samples and from bacterial reference cultures was performed using standard methods. To determine the concentration of DNA, the relationships between the absorbance at 260 and 280 nm were determined with a UV spectrophotometry. As a quality parameter, A260/A280 values between 1.8 and 2.0 were considered appropriate. Thereafter, DNA suspensions were divided and used for conventional PCR and qPCR.

Conventional PCR

The presence of bacteria was confirmed using a non-specific oligonucleotide[18]. The positive samples for non-specific reaction were then processed in PCR Amplification with a specific primer: *P. gingivalis* (forward: 5'-AAT CGT AAC GGG CGA CAC AC-3', reverse: 5'-GGG TTG CTC CTT CAT CAC AC-3' – 593pb)[18], *T. forsythia* (forward: 5'-GCG TAT GTA ACC TGC CCG CA-3', reverse: 5'-TGC TTC AGT GTC AGT TAT ACC T-3' – 641pb)[18] and *P. endodontalis* (forward: 5'-GCT GCA GCT CAA CTG TAG TC-3', reverse: 5'-CCG CTT CAT GTC ACC ATG TC-3'– 672pb)[18] (Invitrogen Tech-LineSM). This Conventional PCR, which is an older method, was used as a gold standard.

PCR amplification was performed as described previously[18]. Briefly, the reaction was carried out in a volume of 25 µL with template DNA (50 ng/µl) and 1 mM oligonucleotide of specific bacteria (Invitrogen Tech-LineSM). The positive control consisted of genomic DNA (50 ng/µl) from tested bacteria. The reactions were carried out in a Thermocycler (MyCyclerTMthermal cycler - BioRad). PCR products were analyzed by 1.5% agarose gel electrophoresis at 100 V for 90 min. A 1 kb DNA ladder digest (Invitrogen Tech-LineSM) served as molecular weight marker.

Quantitative PCR reactions

For the quantitative analysis, plasmids containing the target genes were used as standard. 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*. The dilutions were 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[19,20].

The detection and quantification by qPCR was performed using universal (Applied Biosystems®)[21] and specific primers for *P. gingivalis* (forward: 5'-ACC TTA CCC GGG ATT GAA ATG-3', reverse: 5'-CAA CCA TGC AGC ACCT AC ATA GAA-3'- 83pb), *T. forsythia* (forward: 5'-AGC GAT GGT AGC AAT ACC TGT C-3', reverse: 5'-TTC GCC GGG TTA TCC CTC-3'- 88pb) and *P. endodontalis* (forward: 5'-GCT GCA GCT CAA CTG TAG TCT TG-3', reverse: 5'-TCA GTG TCA GAC GGA GCC TAG TAC-3'-110pb) (Applied Biosystems®)[22] .The species-specific primer sets were designed based on the variable regions of each target gene. The specificity of the primers was confirmed by multiple alignments of relevant sequences from closely related species and by a Basic Local Alignment Search Tool (BLAST) homology search[16].

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/µl template DNA. The thermocycling program included incubation at 95˚C for 10 minutes, 40 cycles of 95˚ C for 15 seconds and 60˚C for 1 minute. After the PCR reactions, 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 OneTM software (Applied Biosystems).

Analysis of data

The detection levels of conventional PCR and qPCR are 10^2 and 10 bacteria/subgingival sample, respectively. In the

present study, to allow the comparison between both techniques, the qPCR had the results adjusted (threshold level 10^2 bacteria/plaque sample). The results were presented as a score system in the following ranges for conventional PCR: presence (score 1) and absence (score 0), and for qPCR: $>10^2$ bacteria (score 2), 10-100 bacteria (score 1), and no detection (score 0).

Statistical analysis

The data were analyzed using the GraphPad Prism R 3.1 (GraphPad Software, Inc. La Jolla, CA, USA). Inter- and intra-group differences were analyzed for the various periods. The prevalence of bacteria determined by both techniques was analyzed using the Wilcoxon test. The sensitivities of the two techniques were determined by comparing the qPCR results with the conventional PCR results. Agreement between the conventional PCR and qPCR results was determined using the kappa test (k). To determine agreement, the percentages of total samples that were positive or negative for both techniques were used, as described previously[5,6].

Results

The average age of the study group was 46 ± 7.49, with 8 men (40%) and 12 women (60%). The average number of teeth was 24 ± 3. Since no statistically significant differences in age and number of teeth were observed in the sample set, it was considered as homogeneous.

The collection sites for the microbiological analysis had a prevalence of BOP of 100% at baseline that decreased to 13.33% after treatment ($P < 0.0001$). 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 (3.63 ± 0.76 mm and 3.83 ± 0.95 mm for PD and CAL, respectively).

Detection of Microorganisms

The standard curve for each bacterial species by qPCR was obtained with the use of specific primers and four serial dilutions (10^1-10^4 bacteria) of the genomic DNA of *P. gingivalis*, *T. forsythia* and *P. endodontalis*. The reaction efficiency (provided by software) for each organism was 96.3 (*P. gingivalis*), 97.8 (*T. forsythia*) and 97.1 (*P. endodontalis*). The correlation coefficient for the mean CT values was $R^2 > 0.99$. All 3 Sybr Green assays were highly specific and amplified only DNA extracted from the periodontopathogens.

The detection of all periodontopathogens targeted was significantly higher using the qPCR technique. A marked difference was noted for all the bacteria (*P. gingivalis*, *T. forsythia* and *P. endodontalis*) between both methods.

The detection of microorganisms through conventional PCR technique at baseline was 46.6% for *P. gingivalis*, 53.3% for *T. forsythia*, and 56.6% for *P. endodontalis*. A significant statistical reduction at day 60 was observed: 10% for *P. gingivalis*, 3.3% for *T. forsythia*, and 20% for *P. endodontalis* (Figure 1).

On the other hand, the qPCR technique allowed the identification of more positive samples in regard to the presence of periodontopathogens. *P. gingivalis* was at 96.6% at baseline and at 93.3% at day 60 *T. forsythia* was detected at 90% at baseline with a statistically significant reduction to 66.6% at day 60. For *P. endodontalis*, the detection was 83.3% at baseline with a reduction to 43.3% at day 60 (Figure 1).

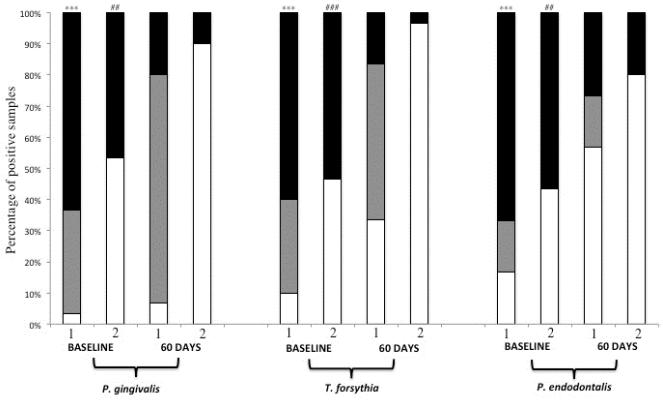

Fig.1. Bacteria detection (%) through qPCR and conventional PCR.

Comparison between Conventional PCR and qPCR without adjusting the threshold

Table 1, 2 and 3 summarizes the comparison between both techniques to detect pathogens. The detection of pathogens through qPCR without adjusting the results was significant higher when compared to conventional PCR detection.

At baseline, conventional PCR detected only 14 samples positive for P. gingivalis in opposite to 29 samples positive through qPCR, resulting a sensitivity of 100% and specificity only 6% for qPCR, and the kappa values showed poor agreement (k = 0.06). At 60 days post-treatment, only 3 of 28 samples positive for P. gingivalis by qPCR were also positive by conventional PCR, resulting in a sensitivity of 100% and specificity of only 7% and poor agreement (k = 0.02) (Table 1).

The comparison between techniques to detect *T. forsythia* at baseline showed a sensitivity of 94% and specificity of 14% for the qPCR. Same results were found at 60 days post-treatment with an increased sensitivity to 100% and specificity to 35%. The kappa values showed poor agreement between techniques, at baseline (k = 0.01) and at 60 days post-treatment (k = 0.03) (Table 2).

At baseline, only 15 of 25 samples positive for *P. endodontalis* by qPCR were also positive for this bacteria by conventional PCR, resulting in a sensitivity of 88%, a specificity of 23% and fair agreement between techniques (k = 0.25). Sixty days after periodontal treatment, the sensitivity was 83% and an increased specificity to 67% and agreement to k = 0.35 (Table 3).

Table 1 - Comparison between PCR and qPCR for the detection of *P. gingivalis*.

	PCR Result	N. (%) of samples with the qPCR result more than 10 bacteria/subgingival sample*			N. (%) of samples with the qPCR result more than 10^2 bacteria/subgingival sample#		
		Positive	Negative	Total	Positive	Negative	Total
BASELINNE	Positive	14 (46.6)	0	14	14 (46.6)	0	14
	Negative	15 (50)	1 (3.3)	16	5 (16.6)	11 (36.6)	16
	Total	29 (96.6)	1	30	19 (63.3)	11	30
		Positive	Negative		Positive	Negative	
60 DAYS	Positive	3 (10)	0	3	2 (6.7)	1 (3.3)	3
	Negative	25 (83.3)	2 (6,7)	27	4 (13.3)	23 (76.7)	27
	Total	28 (93.3)	2	30	6 (20)	24 (80)	30

Baseline: * Sensitivity 100%; Specificity 6%; k 0,06; # Sensitivity 100%; Specificity 69%; k 0,67
60 Days: *Sensitivity 100%; Specificity 7%; k 0,02; #Sensitivity 67%; Specificity 85%; k 0,36

Table 2 - Comparison between PCR and qPCR for the detection of *T. forsythia*.

	PCR Result	N. (%) of samples with the qPCR result more than 10 bacteria/subgingival sample*			N. (%) of samples with the qPCR result more than 10^2 bacteria/subgingival sample#		
		Positive	Negative	Total	Positive	Negative	Total
BASELINNE	Positive	15 (50)	1 (3.3)	16	13 (43.3)	3 (10)	16
	Negative	12 (40)	2 (6.7)	14	5 (16.7)	9 (30)	14
	Total	27 (90)	3 (10)	30	18 (60)	12 (40)	30
		Positive	Negative		Positive	Negative	
60 DAYS	Positive	1 (3.3)	0	1	1 (3.3)	0	1
	Negative	19 (63.3)	10 (33.3)	29	4 (13.3)	25 (83.4)	29
	Total	20 (66.6)	10	30	5 (16.6)	25	30

Baseline: * Sensitivity 94%; Specificity 14%; k 0,01; # Sensitivity 81%; Specificity 64%; k 0,46
60 Days: *Sensitivity 100%; Specificity 35%; k 0,03; #Sensitivity 100%; Specificity 86%; k 0,53

Table 3 - Comparison between PCR and qPCR for the detection of *P. endodontalis*.

	PCR Result	N. (%) of samples with the qPCR result more than 10 bacteria/subgingival sample*			N. (%) of samples with the qPCR result more than 10^2 bacteria/subgingival sample#		
		Positive	Negative	Total	Positive	Negative	Total
BASELINNE	Positive	15 (50)	2 (6,7)	17	15 (50)	2 (6.7)	17
	Negative	10 (33.3)	3 (10)	13	5 (16.7)	8 (26.7)	13
	Total	25 (83.3)	5 (16.7)	30	20 (66.7)	10 (33.4)	30
		Positive	Negative		Positive	Negative	
60 DAYS	Positive	5 (16.6)	1 (3.3)	6	5 (16.7)	1 (3.3)	6
	Negative	8 (26.7)	16 (53.3)	24	3 (10)	21 (70)	24
	Total	13 (43.3)	17 (56.6)	30	8 (26.7)	22 (73.3)	30

Baseline: * Sensitivity 88%; Specificity 23%; k 0,25; # Sensitivity 88%; Specificity 61%; k 0,51
60 Days: *Sensitivity 83%; Specificity 67%; k 0,35; #Sensitivity 83%; Specificity 87%; k 0,63

Comparison between Conventional PCR and qPCR adjusting the threshold

When the detection threshold of qPCR to 102 bacteria (score 2) was adjusted, the agreement between both methods increased. At baseline, the comparison between techniques for the detection of *P. gingivalis* showed a good agreement (k = 0.67) that corresponded to sensitivity of 100% and specificity of 69%. Similar results were obtained at 60 days post-treatment with a sensitivity of 67% and a specificity of 85%, although the kappa value showed a fair agreement between techniques (k = 0.36) (Table 1).

After adjusting the threshold, 13 samples were positive for *T. forsythia* for both techniques at baseline. Three samples were positive only for Conventional PCR and 5 samples were positive only through qPCR, resulting in a sensitivity of 81%, specificity of 64%, and kappa value showed a moderate agreement (k = 0.46). After periodontal treatment, the agreement between both methods increased (k = 0.53), with corresponding a sensitivity of 100% and specificity of 86% (Table 2).

The comparison between both techniques for the detection of P. endodontalis at baseline showed a moderate agreement between techniques (k = 0.51), each one corresponding to a sensitivity of 88% and a specificity of 61%. At 60 days post-treatment, the kappa value increased and showed a good agreement (k = 0.63), corresponding to a high sensitivity (83%) and specificity (87%) (Table 3).

Discussion

The detection of periodontopathogens in samples from patients with periodontitis has become important in order to monitor the effect of periodontal treatments, especially patients that need complementary therapy[4,5]. As described by Socransky et al.[2] (1998), all cases of periodontitis are associated with the presence of periodontopathogens, although, the disease will not necessarily develop as long as the microorganisms does not exceed the threshold for the host.

Periodontal therapy is not exclusively based on microbiological diagnostic. Indeed, for some cases, the clinical analysis can be associated with microbiological diagnostic, for example, metronidazole is recommended for patients that have the presence of the red complex pathogens (*P. gingivalis*, *T. forsythia* and *T. denticola*) but not for patients showing the presence of *A. actinomycetemcomitans*[5,23]. This statement supports the importance of quantifying pathogens in periodontal pockets[6].

Bacterial cultures, considered a gold standard microbiological test, is routinely used in many microbiological laboratories, but hassome limitations, such as the need of at least 10^3 bacteria samples to allow the detection by culture. Nowadays, other detection methods that might be more sensitive than microbiological culture, including methods based on nucleic acid detection, are routinely used, but still need evaluation and validation[24].

Conventional PCR is 10-100 times more sensitive than anaerobic culture to detect periodontopathogens, although the intrinsic limitations of PCR cause a loss of qualitative information, because at least 10^2 pathogens are required for positive detection[5,13,23]. In some cases, conventional PCR shows false negative results and, therefore, can hide the real presence of pathogens and lead to a misdiagnosis.

The qPCR is a more sensitive technique than conventional PCR, because only 10 bacteria are required for positive detection

and allows a real quantification of periodontopathogens in periodontitis sites, turning it an important criteria to distinguish among healthy and diseased sites. qPCR is able to detect and quantify small changes in the number of pathogens following periodontal treatments[5,16]. On the other hand, the most important disadvantage of qPCR is related to its cost and the fact that it requires special equipment[6]. In this study, we compared qPCR with conventional PCR for the detection of *P. gingivalis*, *T. forsythia* and *P. endodontalis* in patients with chronic periodontitis before and after periodontal treatment. Conventional PCR, the oldest method, was used as a gold standard[4].

As reported by Mullis[10] (1990) and Higuchi et al.[25] (1992) the detection limit by qPCR is 10 bacteria while 10^2 bacteria is required by conventional PCR. For this reason, in this study, the agreement between both techniques was analyzed at two different threshold levels. Therefore, the threshold level of qPCR was adjusted to 10^2 bacteria to allow an unbiased comparison between PCR and qPCR[6].

Our results are in accordance with other microbiological studies that reported the presence of most periodontopathogens even in low quantity and proportions following a periodontal treatment[5]. Through conventional qualitative PCR, small differences before and after periodontal treatment can be masked[5,13]. These results showed the importance of qPCR to quantify low proportion of periodontophatogens, that cannot be detected though conventional PCR[21].

Since microbiological analysis are often used for a therapeutic strategy for patients who do not initially respond to conventional mechanical treatment, qPCR may be an interesting tool for the selection of antibiotics or for monitoring the efficacy of the antibiotic treatment[16].

The comparative analysis of both techniques showed a higher sensitivity of qPCR, resulting in a low chance of false negative results, when compared with conventional PCR. Even though both PCR and qPCR techniques are more sensitive in the detection of pathogens when compared with anaerobic culture, a detection without quantification may be irrelevant in terms of disease activity, since the presence of microorganisms will not necessarily lead to the development of disease[12].

Our results showed a poor agreement between both techniques with a high sensitivity and low specificity associated with qPCR when was compared with conventional PCR without adjusting the threshold. However, when the threshold of qPCR was adjusted, the agreement between techniques increased. This results, confirms the requirement of a good microbiological test diagnostic with high sensitivity that allow the detection of low number of periodontopathogens, because sometimes the number of bacteria present in periodontal pockets can be very low due to the difficulty to collect samples from subgingival pockets. This may be related to the insertion of paper points in the subgingival pocket and the capacity of paper points to absorb enough amount of gingival fluid.

Nowadays, the qPCR technique becomes an important tool to quantify the exact number of microorganisms and differentiate the stages of health and periodontal disease in combination with the clinical data. This technique allows identifying the presence of periodontopathogens in subgingival pockets and helps to choose the best treatment for some patients that need additional periodontal therapy[12]. The qPCR in comparison with conventional PCR has the advantages that it does not require the post-PCR processing of amplicons, eliminating some contaminations and the assay can be completed within 2,5 h.

Considering the limitation of this study and the small number of samples, qPCR showed to be a fast, efficient and a sensitive technique to detect and quantify *P. gingivalis*, *T. forsythis*, and *P. endodontalis* in subgingival samples from patients with chronic periodontitis. The qPCR approache can contribute to recognize new putative pathogens present in subgingival pocket from patients with periodontitis, and allow the development of new periodontal therapy. Additional studies with larger sample size are required to validate this difference between conventional PCR and qPCR.

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Conflicts of interest

This manuscript does not have any kind of conflict of interest.

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