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Quantitative real-time polymerase chain reaction based on single copy gene sequence for detection of periodontal pathogens

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Abstract

Objective: To develop a method for quantification of *Actinobacillus actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Tannerella forsythensis* (*Tf*) from subgingival plaque samples based on TaqMan real-time polymerase chain reaction (PCR) technology.

Material and Methods: Bacterial cells from these species were obtained after culturing reference strains and were counted microscopically. Cellular suspensions in Tris-EDTA buffer were used for DNA extraction after boiling for 20 min. Primers for PCR were selected from sequences of the LktC (*Aa*), Arg-gingipain (*Pg*) and BspA antigen (*Tf*) genes in order to yield amplicons below 100 bp. TaqMan-based real-time PCR was adjusted to quantify each species separately. Cycle threshold (C_T) values were calculated for each species according to the initial number of copies. A reliability analysis was carried out using intra-class correlation coefficients (ICCs) with a two-way random effects model.

Results: A high sensitivity and specificity was obtained for the detection of the three bacterial species. The TaqMan real-time PCR technology yielded a good repeatability in the obtained cycle threshold (C_T) values for each initial number of copies, demonstrating coefficients of variation below 5% for each bacteria. The reproducibility of the technique was also demonstrated by the high ICCs (>0.98; $p<0.00001$) obtained for each bacteria with and without the addition of subgingival plaque.

Conclusion: A novel diagnostic method based on TaqMan real-time PCR was developed for the quantification of *Aa*, *Pg* and *Tf*. It has demonstrated good sensitivity and repeatability on pure cultures. Its diagnostic utility should be demonstrated in subgingival plaque samples.

Key words: *Actinobacillus actinomycetemcomitans*; bspA antigen; gingipain; leukotoxin; periodontitis; *Porphyromonas gingivalis*; real-time polymerase chain reaction; *Tannerella forsythensis*; TaqMan

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The role of specific bacterial species in the aetiology and pathogenesis of human periodontal diseases has been extensively reviewed, providing compelling evidence that a limited number of bacterial species (periodontal pathogens) contribute to the initiation and/or progression of destructive forms of periodontitis. The American Academy of Periodontology World Workshop (Consensus Report 1996) identified three species as the most strongly associated with periodontitis: *Actinobacillus actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Tannerella forsythensis* (*Tf*). These bacterial species, besides their positive association with destructive periodontitis, are able to secrete a wide array of potential virulence factors that support their potential pathogenicity. Specifi-

cally, *Aa* leukotoxin and *Pg* arginine and lysine proteases, also called gingipains, have been associated with periodontal tissues destruction (Fives-Taylor et al. 1999, He et al. 1999, Haraszthy et al. 2000, Baba et al. 2002, Henderson et al. 2002, Tada et al. 2002). Similarly, *Tf* has also shown the secretion of sialidase and trypsin-like proteases, together with the cell surface-associated leucine-rich repeat protein (BspA) involved in adhesion to fibronectin and fibrinogen (Sharma et al. 1998). The emphasis on the detection, quantification and therapeutic elimination of these species seems to be justified, even though it is recognised that other species contribute to the pathogenesis of different kinds of periodontal diseases.

Ecological studies on periodontitis patients have demonstrated that *Aa* and *Pg* are usually found in high numbers in sites with different forms of destructive periodontal diseases and, on the contrary, are not prevalent or detected in low numbers in healthy sites from diseased subjects or sites in periodontally healthy individuals (Slots & Ting 1999). Bacteriological culture has been the classic method of detection and enumeration of these microorganisms (Greenstein 1988, Lamster et al. 1993). However, these techniques have serious limitations, mostly associated with the difficulty in recovering species when they are found in low numbers. Moreover, significant bacterial species may be found in the subgingival biofilm, such as *Treponema* species and *Tf* that require stringent growing conditions and therefore are difficult to detect and quantify in culture (Tanner et al. 1986, Sakamoto et al. 2002). This fact justifies the scarcity of epidemiological studies carried out to demonstrate the relevance of these bacteria in periodontal diseases.

In order to overcome these difficulties, different technologies for microbiological diagnosis have been proposed, most of them based on molecular biology techniques. Among them, DNA hybridisation techniques have demonstrated the capacity of processing a large number of plaque samples, simultaneously assessing up to 40 bacterial species. This method, however, requires sophisticated laboratory equipment and expertise and although is highly specific, also requires a high number of bacteria for positive identification (10^3–10^4) (Socransky et al. 1994). Therefore, culture and DNA probes are labour intensive and require considerable expertise and expense, which limits their universal application.

The most sensitive method available for DNA sequence detection is the polymerase chain reaction (PCR). This technique may be able to amplify one copy of a DNA template by several million-fold (Saiki et al. 1988). PCR has already been used in the detection of periodontal pathogens in several epidemiological and longitudinal studies (Takamasu et al. 1999, Fujise et al. 2002). However, standard PCR only provides qualitative information on the specific bacteria assayed, and therefore, their use for diagnostic and prognostic purposes, although routinely used, is limited.

Real-time PCR with species-specific primers can provide a precise and sensitive method for an accurate quantification of individual bacterial species. Two main types of chemistries have been used in these assays: SYBR Green I dye and the fluorogenic 5′ nuclease probe or TaqMan assay. SYBR Green I dye is a highly specific double-stranded DNA binding dye, which also allows the detection of product accumulation during the PCR process. The difference between the two chemistries is that SYBR Green I assay will detect all double-stranded DNA, including non-specific reaction products, requiring a well-optimised reaction for accurate quantitative results (Morillo et al. 2003). The main advantage of this chemistry is that no probe is required, thus reducing the assay set-up and cost (Meuer et al. 2001). The fluorogenic 5′nuclease assay (TaqMan) assay uses a fluorescent probe to enable the detection of a specific PCR product as it accumulates during the amplification reaction. Its main advantage is the high specificity provided by each probe (Crockett & Wittwer 2001).

The primers mainly used in PCR-based diagnostic methods for the detection of periodontal bacteria are usually 16S rRNA sequences (Ashimoto et al. 1996). However, each bacteria may contain a variable amount of these sequences, and therefore these primers may be inadequate for quantitative analysis. For this purpose, primers based on single copy gene sequence are more appropriate. *Aa* leucotoxin operon genes, specifically the conserved region of LktC gene, *Pg* arginin-specific cystein–proteinase gene (Morillo et al. 2003) and *Tf* BspA antigen gene have been identified as specific primers for PCR-based quantitative assays.

The purpose of this investigation was to develop a standard real-time PCR TaqMan assay based on single copy gene sequence, in order to detect and specifically quantify the pathogens *Aa*, *Pg* and *Tf* using pure cultures with and without the addition of subgingival plaque bacteria

Material and Methods

Bacterial strains

Aa strain ATCC 33384, *Pg* strain ATCC 33277 and *Tf* (formerly *Bacteroides forsythus*) strain ATCC 43037 were obtained from the American Type Culture Collection (Manassas, VA, USA). Strains of *Prevotella intermedia*, *P. nigrescens*, *Peptostreptococcus micros* and *Fusobacterium nucleatum* used for the specificity analysis were obtained from subgingival plaque samples from periodontitis patients (Faculty of Odontology, University Complutense, Madrid, Spain).

DNA extraction

Bacterial species were cultured according to previously described techniques on agar plates. Colonies were removed with a disposable instrument and inserted into a microfuge tube with Tris-EDTA buffer. Bacterial cells were counted by microscopic visualisation in a Petroff–Hausser counting chamber (Hauser Scientific, Horsham, PA, USA). Aliquots of 150 μl of bacterial suspension for each species were obtained. Using a 1.5 ml microfuge tube, samples were incubated at 100°C during 20 min in a thermal block. A mineral oil drop was added to each aliquot to avoid sample evaporation during this boiling step. Then, the solution was transferred to a clean microfuge tube without mineral oil. Dilutions of known amounts of bacteria, as determined in the counting chamber, were used to determine the standard curve for real-time quantification.

Primer and probe design and synthesis

Complete sequences of LktC gene from *Aa*, Arg-gingipain from *Pg* and BspA antigen from *Tf* were obtained from the GeneBank (access # U51862, D64081 and AF054892, respectively). Oligonucleotides yielding PCR product of 50–150 bp length and an annealing temperature of 59°C were obtained by means of the Primer Express Software

(Applied Biosystems, Foster City, CA, USA), and checked through the web with the ''Basic Local Alignment Search Tool'' program (BLAST®, NCBI home page (http://ncbi.nlm.nih.gov/blast)).

Definitive primers were: *Aa*-forward 5′-ACGCAGACGATTGACTGAATT-TAA-3′ (from 736 to 759 position), *Aa*-reverse 5′-GATCTTCACAGCTATA-TGGCAGCTA-3′ (from 788 to 812 position), *Pg*-forward 5′-CCTACGTG-TACGGACAGAGCTATA-3′ (from 1308 to 1332 position), *Pg*-reverse 5′-AGGATCGCTCAGCGTAGCATT-3′ (from 1358 to 1379 position), *Tf*-forward 5′-TCCCAAAGACGCGGA-TATCA-3′ (from 2586 to 2605 position) and *Tf*-reverse 5′-ACGGTCGCG-ATGTCATTGT-3′ (from 2651 to 2633 position). PCR products were 77, 71 and 65 bp length for *Aa*, *Pg* and *Tf*, respectively.

Definitive TaqMan probes were:

*Aa*S 5′-FAM-TCACCCTTC-TACCGTTGCCATGGG-3′,
*Pg*S 5′-TET-TCGCCCGGGAA-GAACTTGTCTTCA-3′ and
*Tf*S 5′-VIC-CCGCGACGT-GAAATGGTATTCCTC-3′.

TaqMan chemistry requires optimisation of the primer and probe concentrations. Following the manufacturer's instructions, several master mixes were prepared with different amounts of the primers (from 0.05 to 0.9 μM) and probes (from 0.05 to 0.25 μM). TaqMan® Universal PCR Master Mix (Applied Biosystems), supplied at a 2 × concentration and containing AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1 and optimised buffer, was used for the PCR analysis. The final concentration used a total volume of 25 μl and contained 12.5 μl of 2 × Master Mix, 3 μl of DNA template, primers, probe solutions and sterile distilled water to adjust volume. Real-time PCR was carried out in an ABI Prism 7700 system (Applied Biosystems) using the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Real-time PCR assay

A primer and probe concentration of 0.9 and 0.25 μM, respectively, were finally used for the three species. The PCR reaction was carried out according to standard conditions. Known amounts of bacterial-specific DNA were used to construct a standard curve. To examine the influence of the plaque environment on the PCR yield, *Aa*-, *Pg*- and *Tf*-negative subgingival plaque samples from clinical isolates were added to the PCR reaction and the PCR assay was run in parallel with and without plaque.

Data analysis

The outcome variable used to evaluate the reproducibility of this assay was cycle threshold (C_T). This is the cycle number at which the reaction begins to be exponential for a known number of DNA copies. The reaction at each DNA concentration was repeated fivefold. Means and standard deviations were obtained to calculate the coefficient of variation (CV) for each DNA concentration. A reliability analysis was carried out using intra-class correlation coefficients (ICCs) with a two-way random effects model (absolute agreement) (SPSS software v. 11.0).

Results

Specificity of primers

All primers tested yielded specific products. To assess the primer specificity, PCR assays were run in parallel against other subgingival bacteria (*P. intermedia*, *P. nigrescens*, *P. micros*, *F. nucleatum*). No cross-reaction was demonstrated with any of these tested bacterial species.

Quantification of *Aa*

A DNA solution of 10^6 cells/3 μl was prepared from pure *Aa* cultures. For the PCR assay, 10-fold dilutions were prepared in order to obtain different cell concentrations (10^1–10^6). The assay was run five times for each dilution. Each PCR contained 3 μl of DNA template. Table 1 shows the mean C_T values for each initial number of copies, and its variability. The obtained CV was around 1%, between 10^2 and 10^6 copies. For 10^1 copies the coefficient was 2.2%. The fluorescent curve at the six different cell concentrations demonstrated both high sensitivity and reproducibility in the C_T values (Fig. 1). Addition of subgingival plaque to the PCR reaction slightly increased the C_T value (23.37 versus 22.78), although differences were not statistically significant and the variability was minimal. The reliability analysis (Table 2) provided values of ICCs higher than 0.99 ($p < 0.00001$), therefore demonstrating a highly statistically significant reproducibility.

Quantification of *Pg*

This experiment was conducted in a similar manner as it was described for *Aa*. From a DNA solution of 10^6 cells/3 μl, 10-fold dilutions were prepared for PCR, and five replicates for each dilution were analysed in the experiment. Each PCR contained 3 μl of DNA template. Table 3 shows the mean C_T values for each quantity and its variability. This reaction offered good reproducibility, demonstrated by a CV below 2% between 10^2 and 10^6 copies. The sensitivity obtained was lower than with *Aa*, since the smallest number of copies detected was 10^2 cells/reaction. The fluorescent curve of PCR (Fig. 2) also showed good reproducibility in the C_T values for each cell concentration. PCR reactions when subgingival plaque was added did not significantly change the C_T values when compared with negative-plaque reactions (23.64 versus 22.5). The reliability analysis (Table 2) provided values higher than 0.98 ICC ($p < 0.00001$), demonstrating excellent reproducibility.

Quantification of *Tf*

A DNA solution of 10^6 cells/3 μl, 10-fold dilutions were prepared for PCR,

Table 1. Variability of the *Actinobacillus actinomycetemcomitans* assay

Predicted copy number	Mean C_T	Standard deviation	CV (%)
10^6/reaction	22.78	0.127	0.559
10^5/reaction	26.07	0.269	1.031
10^4/reaction	28.8	0.028	0.098
10^3/reaction	31.845	0.219	0.688
10^2/reaction	34.685	0.290	0.836
10^1/reaction	36.835	0.827	2.246

C_T, cycle number at which the reaction begins to be exponential for a known number of DNA copies; CV, coefficient of variation.

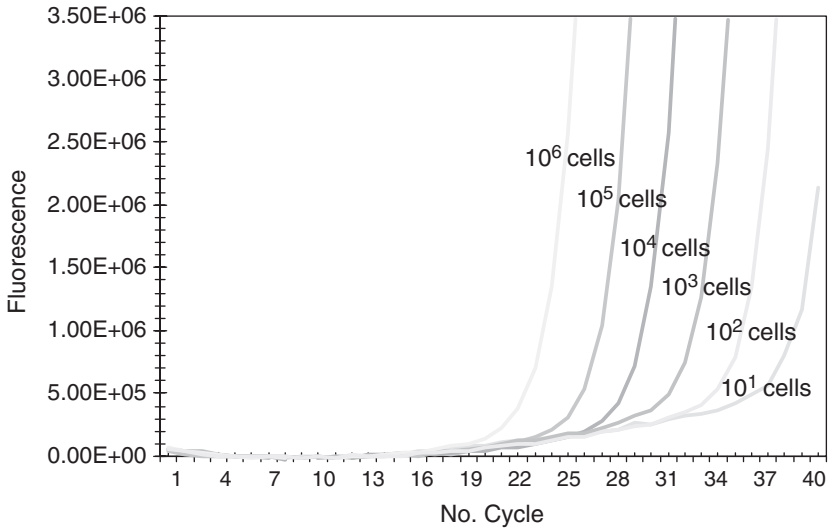

Fig. 1. Quantification curve of *Actinobacillus actinomycetemcomitans* (*Aa*) real-time polymerase chain reaction. Replicate curves generated by 10-fold dilutions of DNA template from 10^6 to 10^1 cells/reaction.

Table 2. Reliability analysis for the PCR assay

Bacteria	ICC value	95% confidence interval	*F*-value	Significance
Actinobacillus actinomycetemcomitans	0.9953	0.9846–0.9990	1763.84	0.0000
Porphyromonas gingivalis	0.9881	0.9599–0.9977	685.68	0.0000
Tannerella forsythensis	0.9903	0.9657–0.9979	998.48	0.0000

ICC, intra-class correlation coefficient; PCR, polymerase chain reaction.

Table 3. Variability of the *Porphyromonas gingivalis* assay

Predicted copy number	Mean C_T	Standard deviation	CV (%)
10^6/reaction	22.5	0.240	1.068
10^5/reaction	26.87	0.481	1.789
10^4/reaction	30.015	0.021	0.071
10^3/reaction	32.465	0.049	0.152
10^2/reaction	37.535	0.686	1.827

C_T, cycle number at which the reaction begins to be exponential for a known number of DNA copies; CV, coefficient of variation.

and five replicates for each dilution were analysed in the experiment. Each PCR contained 3 μl of DNA template. Table 4 shows the mean C_T values for each initial number of copies, and its variability. The CV was below 5% for all the concentrations. The fluorescent curve at different cell concentrations is shown in Fig. 3, demonstrating a good reproducibility in the C_T values for the different number of copies. For these bacterial species, the PCR assay demonstrated the highest sensitivity. The PCR reactions, when subgingival plaque was added, slightly decreased the C_T value with respect to negative-plaque reactions (16.315 versus 16.825). However, the repeatability was improved (CV, 0.13% versus 1.387%). The reliability analysis (Table 2) provided values higher than 0.99 ICC ($p < 0.00001$), demonstrating excellent reproducibility.

Table 5 shows the reliability analysis for the three bacteria combined with and without the addition of subgingival plaque. The results demonstrated almost identical ICCs, therefore showing similar results.

Discussion

Among the various detection methods used in diagnostic microbiology, PCR has demonstrated the highest sensitivity, being able to identify few cells with a high specificity, under optimised amplification conditions. When compared with bacterial culturing in the detection of putative periodontal pathogens, different PCR assays have demonstrated higher specificity and sensitivity values (Riggio et al. 1996).

Most of the PCR assays developed and tested to identify *Aa*, *Pg* or *Tf* have used, as primers, 16S rRNA sequences (Umeda et al. 1998, Mullaly et al. 2000, Kamma et al. 2001, Okada et al. 2001). However, since there might be different number of these molecules per cell, the use of these primers in PCR assays aiming for reproducible quantification is not indicated. In spite of this, only a small number of PCR assays have used as primers oligonucleotides derived from a single copy gene. For *Pg* the collagenase prtC gene has been used (Fujise et al. 2002) and the fimbrillin fimA gene (Riggio et al. 1996) have been used; for *Aa* the leukotoxin lktA gene (Riggio et al. 1996); and for *Tf* the protease prtH gene (Tan et al. 2001). Since the PCR tested in the present study was aimed for quantification, we have selected the complete sequences of lktC gene from *Aa*, Arg-gingipain from *Pg* and BspA antigen from *Tf*, yielding excellent sensitivity (10^1 copies for *Aa* and *Tf* and 10^2 for *Pg*) and specificity (no cross-reaction was demonstrated with any of the subgingival species tested).

Standard PCR technology, although useful to determine the presence or absence of a specific target microorganism, has significant limitations when used for quantification in clinical samples. This bacterial mixture may contain inhibitors that could reduce the amplification process, thus limiting the reproducibility for any quantitative analysis and, therefore, reducing its utility in microbiological diagnosis, since the mere detection of target bacteria, if it is at low levels, may have no clinical relevance. In this study, the PCR assay was tested on pure cultures and after the addition of subgingival plaque samples with known absence of the target bacterial. In both instances, the quantitative method gave similar results, thus demonstrating a well-optimised reaction.

The DNA extraction method used in different PCR assays may also be

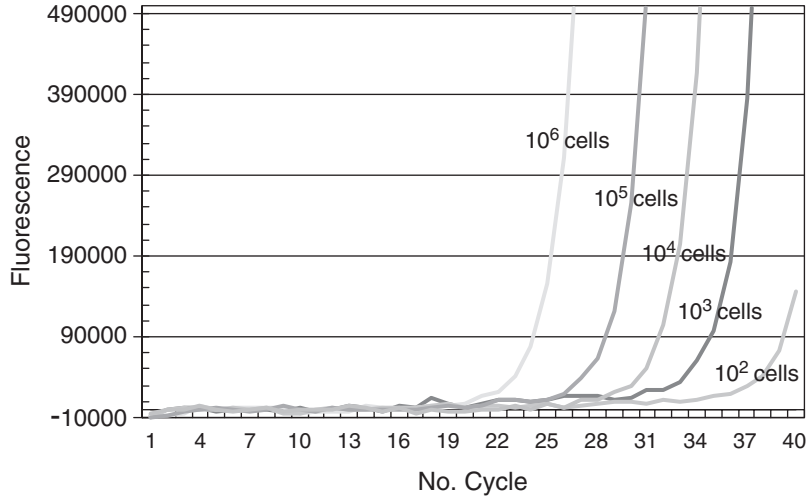

Fig. 2. Quantification curve of *Porphyromonas gingivalis* (*Pg*) real-time polymerase chain reaction. Replicate curves generated by 10-fold dilutions of DNA template from 10^6 to 10^2 cells/reaction.

Table 4. Variability of the *Tannerella forsythensis* assay

Predicted copy number	Mean C_T	Standard deviation	CV (%)
10^6/reaction	16.825	0.233	1.387
10^5/reaction	20.85	0.269	1.289
10^4/reaction	22.43	0.933	4.161
10^3/reaction	27.83	0.452	1.626
10^2/reaction	31.685	0.615	1.941
10^1/reaction	34.435	1.336	3.881

C_T, cycle number at which the reaction begins to be exponential for a known number of DNA copies; CV, coefficient of variation.

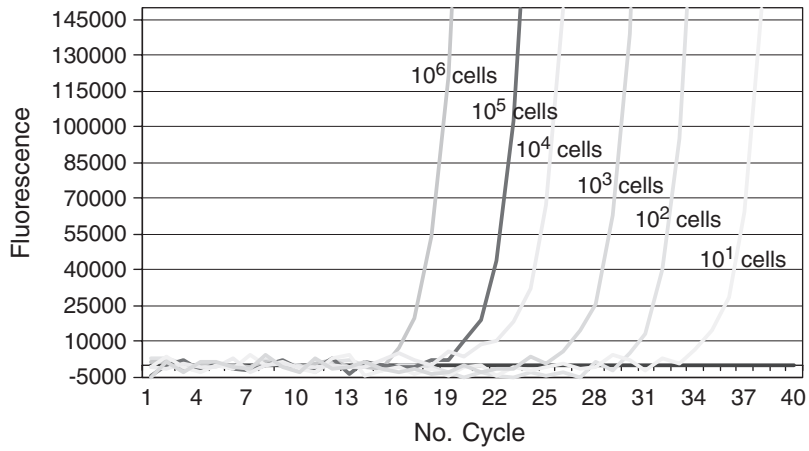

Fig. 3. Quantification curve of *Tannerella forsythensis* (*Tf*) real-time polymerase chain reaction. Replicate curves generated by 10-fold dilutions of DNA template from 10^6 to 10^1 cells/reaction.

critical when used for quantification of bacteria. Most of the assays reported use complex chemical extraction methods, such as detergents, enzymes or silica columns (Lyons et al. 2000, Sakamoto et al. 2001). While most of these methods yield a high DNA quality, a different quantity of DNA may be lost during processing, which discard it for any quantitative evaluation. The method we used in our real-time PCR assay was based on the dilution of bacterial sample cells in Tris-EDTA buffer and boiling. To overcome the possible heterogeneous distribution of the boiling temperature in the sample, a mineral oil drop was used to cover each sample. The amplification process with the three bacterial species showed excellent repeatability (Tables 1–3).

Recently, several quantitative PCR assays have been developed and tested. Fujise et al. (1995) developed a quantitative PCR method for *Aa* using the leukotoxin gene as a primer. Similarly, Doungudomdacha et al. (2001) developed a quantitative PCR based on ethidium bromide detection using as primers fimA gene for *Pg* and lktA gene for *Aa*. They showed a good correlation between the number of *Pg* and the clinical outcome variables studied in their longitudinal evaluation (probing depth and attachment loss). However, both studies used the end-point PCR methodology. PCR product synthesis usually starts slowly until it suffers an exponential growth and then it reaches the plateau phase. Since the end-point technology only measures the PCR yield after the exponential growth phase, there is usually a weak correlation between the end-point PCR product and the initial DNA quantity. This methodology, therefore, may not be accurate for a reproducible quantification. In order to overcome some of these difficulties, the real-time PCR technology was developed, since it measures the product synthesis during the early exponential phase. Few studies have used real-time PCR in periodontal microbiological diagnosis. Lyons et al. (2000) aimed to quantify *Pg* using nested PCR with primers based on 16S rRNA sequences. Shelbourne et al. (2000) also used TaqMan chemistry to quantify *Tf* with primers based on 16S rRNA sequences. Although in these reports a good correlation was encountered with the number of cells tested, their approach may not be appropriate since there might be a variable number of these molecules per cell, which limits their use for quantitative analysis. Sakamoto et al. (2001) have used real-time PCR for the detection and quantification of five periodontal pathogens using the LightCycler system, which also allows a good real-time quantification. Recently, our research group (Morillo et al. 2003) have also developed and tested a real-time PCR assay

Table 5. Reliability analysis for the PCR assay with and without the addition of subgingival plaque (for the three bacteria combined)

Measure	ICC value	95% confidence interval	*F*-value	Significance
without plaque	0.9918	0.9781–0.9970	1070.86	0.0000
with plaque	0.9921	0.9721–0.9988	968.92	0.0000

ICC, intra-class correlation coefficient; PCR, polymerase chain reaction.

for periodontal pathogens using the SYBR Green I dye chemistry. Although similar results were obtained and these chemistries are cheaper than TaqMan, they require a more complex process for optimisation, until the conditions for optimal specificity are met. Moreover, the results are less specific, because of the mechanism of union of the colorant to the DNA chain. All these factors make the TaqMan technology more specific and versatile for its use in microbiological testing of subgingival samples.

In summary, we have developed and tested a real-time PCR assay, based on single copy gene sequence and on the TaqMan chemistry, aimed at the quantification of *Aa*, *Pg* and *Tf* in subgingival plaque samples. This assay has shown a high degree of specificity and has proved to be a very reproducible and consistent method to quantify these pathogenic species. More research is needed to validate the diagnostic utility of this methodology with the use of clinical samples.

References

Ashimoto, A., Chen, C., Bakker, I. & Slots, J. (1996) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiology and Immunology* **11**, 266–273.

Baba, A., Kadowaki, T., Asao, T. & Yamamoto, K. (2002) Roles for Arg- and Lys-gingipains in the disruption of cytokine responses and loss of viability of human endothelial cells by *Porphyromonas gingivalis* infection. *Biological Chemistry* **383**, 1223–1230.

Consensus report (1996) Periodontal diseases: pathogenesis and microbial factors. *Annals of Periodontology* **1**, 926–932.

Crockett, A. O. & Wittwer, C. T. (2001) Fluorescein-labeled oligonucleotides for real-Time PCR: using the inherent quenching of deoxyguanosine nucleotides. *Analytical Biochemistry* **290**, 89–97.

Doungudomdacha, S., Rawlinson, A., Walsh, T. F. & Douglas, C. W. (2001) Effect of non-surgical periodontal treatment on clinical parameters and the numbers of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* at adult periodontitis sites. *Journal of Clinical Periodontology* **28**, 437–445.

Fives-Taylor, P. M., Meyer, D. H., Mintz, K. P. & Brissette, C. (1999) Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontology 2000* **20**, 136–167.

Fujise, O., Hamachi, T., Hirofugi, T. & Maeda, K. (1995) Colorimetric microtiter plate based assay for detection and quantitation of amplified *Actinobacillus actinomycetemcomitans* DNA. *Oral Microbiology and Immunology* **10**, 372–377.

Fujise, O., Hamachi, T., Inoue, K., Miura, M. & Maeda, K. (2002) Microbiological markers for prediction and assessment of treatment outcome following non-surgical periodontal therapy. *Journal of Periodontology* **73**, 1253–1259.

Greenstein, G. (1988) Microbiologic assessments to enhance periodontal diagnosis. *Journal of Periodontology* **59**, 508–515.

Haraszthy, V. I., Hariharan, G., Tinoco, E. M. B., Cortelli, J. R., Lally, E. T., Davis, E. & Zambon, J. J. (2000) Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. *Journal of Periodontology* **71**, 912–922.

He, T., Nishihara, T., Demuth, D. R. & Ishikawa, I. (1999) A novel insertion sequence increases the expression of leukotoxicity in *Actinobacillus actinomycetemcomitans* clinical isolates. *Journal of Periodontology* **70**, 1261–1268.

Henderson, B., Wilson, M., Sharp, L. & Ward, J. M. (2002) *Actinobacillus actinomycetemcomitans*. *Journal of Medical Microbiology* **51**, 1013–1020.

Kamma, J. J., Contreras, A. & Slots, J. (2001) Herpes viruses and periodontopathic bacteria in early-onset periodontitis. *Journal of Clinical Periodontology* **28**, 879–885.

Lamster, I. B., Celenti, R. S., Jans, H. H., Fine, J. B. & Grbic, J. T. (1993) Current status of tests for periodontal disease. *Advances in Dental Research* **7**, 182–190.

Lyons, S. R., Griffen, A. L. & Ley, E. S. (2000) Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *Journal of Clinical Microbiology* **38**, 2362–2365.

Meuer, S., Wittwer, C. & Nakagawara, K. (2001) *Rapid Cycle Real-Time PCR. Methods and Applications*. Berlin: Springer-Verlag.

Morillo, J. M., Lau, L., Sanz, M., Herrera, D. & Silva, A. (2003) Quantitative real-time PCR based on single copy gene sequence for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Journal of Periodontal Research* **38**, 518–524.

Mullaly, B. H., Dace, B., Shelbourne, C. E., Wolff, L. F. & Coulter, W. A. (2000) Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. *Journal of Periodontal Research* **35**, 232–241.

Okada, M., Hayashi, F. & Nagasaka, N. (2001) PCR detection of 5 putative periodontal pathogens in dental plaque samples from children 2 to 12 years of age. *Journal of Clinical Periodontology* **28**, 576–582.

Riggio, M. P., Macfarlane, T. W., Mackenzie, D., Lennon, A., Smith, A. J. & Kinane, D. (1996) Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. *Journal of Periodontal Research* **31**, 496–501.

Saiki, R., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* **239**, 487–491.

Sakamoto, M., Suzuki, M., Umeda, M., Ishikawa, I. & Benno, Y. (2002) Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythensis corrig, gennov, comb nov International*. *Journal of Systematic and Evolutionary Microbiology* **52**, 841–849.

Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. & Benno, Y. (2001) Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. *Microbiology and Immunology* **45**, 39–44.

Sharma, A., Sojar, H. T., Glurich, I., Honma, K., Kuramitsu, H. K. & Genco, R. J. (1998) Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *Bacteroides forsythus* ATCC 43037. *Infection and Immunity* **66**, 5703–5710.

Shelbourne, C. E., Prabhu, A., Gleason, R. M., Mullally, B. H. & Coulter, W. A. (2000) Quantitation of *Bacteroides forsythus* in subgingival plaque. Comparation of immunoassay and quantitative polymerase chain reaction. *Journal of Microbiology* **39**, 97–107.

Slots, J. & Ting, M. (1999) *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontology* **20**, 82–121.

Socransky, S. S., Smith, C., Martin, L., Paster, B. J., Dewhirst, T. E. & Levin, A. E. (1994) ''Checkerboard'' DNA–DNA hybridization. *Biotechniques* **17**, 788–792.

Tada, H., Sugawara, S., Nemoto, E., Takahashi, N., Imamura, T., Potempa, J., Travis, J., Shimauchi, H. & Takada, H. (2002) Proteolysis of CD14 on human gingival fibroblasts by arginine-specific cysteine proteinases from *Porphyromonas gingivalis* leading to down-regulation of lipopolysaccharide-induced interleukin-8 production. *Infection and Immunity* **70**, 3304–3307.

Takamatsu, N., Yano, K., He, T., Umeda, M. & Ishikawa, I. (1999) Effect of initial periodontal therapy on the frequency of detecting *Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*. *Journal of Periodontology* **70**, 574–580.

Tan, K. S., Song, K. P. & Ong, G. (2001) *Bacteroides forsythus* prtH genotype in periodontitis patients: occurrence and association with periodontal disease. *Journal of Periodontal Research* **36**, 398–403.

Tanner, A. C. R., Listgarten, M. A., Ebersole, J. L. & Strzempko, M. N. (1986) *Bacteroides forsythus sp. nov.*, a slow-growing, fusiform *Bacteroides sp* from human oral cavity. *International Journal of Systematic Bacteriology* **36**, 213–221.

Umeda, M., Chen, C., Bakkeer, I., Contreras, A., Morrison, J. L. & Slots, J. (1998) Risk indicators for harboring periodontal pathogens. *Journal of Periodontology* **69**, 1111–1118.

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