J Clin Periodontol 2004; 31: 1061–1069 doi: 10.1111/j.1600-051X.2004.00616.x

Journal of Clinical Periodontology

Quantitative real-time polymerase chain reaction versus culture: a comparison between two methods for the detection and quantification of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in subgingival plaque samples

Laura Lau[1], Mariano Sanz[1,2], David Herrera[1,2], Juan Manuel Morillo[1], Conchita Martín[3] and Augusto Silva[4]

[1]Laboratory of Microbiology; [2]Section of Graduate Periodontology; [3]Section of Orthodontics, Faculty of Odontology, University Complutense; [4]Center for Molecular Biology, National Center for Scientific Research, Madrid, Spain

Lau L, Sanz, M, Herrera D, Morillo JM, Martín C, Silva A: Quantitative real-time polymerase chain reaction versus culture: a comparison between two methods for the detection and quantification of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis in subgingival plaque samples. J Clin Periodontol 2004; 31: 1061–1069. doi: 10.1111/j.1600-051X.2004.00616.x. © Blackwell Munksgaard, 2004.

Abstract

Objective: The purpose of this investigation was to validate a real-time quantitative polymerase chain reaction (PCR) assay in identifying and quantifying *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* from subgingival plaque samples taken from subjects with different periodontal conditions, when compared with conventional cultural procedures.

Patients and Methods: Ninety-two adult subjects participated in this study, 32 with periodontitis, 30 with gingivitis and 30 healthy. A pooled subgingival sample was obtained from every patient. Culturing procedures were carried out using standard techniques. For real-time PCR analysis, primers were selected from sequences of the LktC (*A. actinomycetemcomitans*), Arg-gingipain (*P. gingivalis*) and BspA antigen (*T. forsythensis*) genes. Contingency tables were constructed to compare the qualitative results, while quantitative data were evaluated by paired *t*-test.

Results: *A. actinomycetemcomitans* was the least frequently recovered species with both techniques. Prevalence of *P. gingivalis* was low in healthy patients, increased in gingivitis and peaked in periodontitis patients. The frequency of detection of *T. forsythensis* showed marked differences between culture and PCR, although the same tendency of an increase in prevalence from health to gingivitis and to periodontitis was observed with both methods. Contingency tables demonstrated a good level of agreement between PCR and culture procedures for *A. actinomycetemcomitans* and *P. gingivalis*, especially in periodontitis patients. *P. gingivalis* culture counts were

significantly higher than those obtained by PCR. The opposite was true for *T. forsythensis*, and statistically significant higher counts were obtained by PCR for gingivitis and periodontitis patients.
Conclusion: This study demonstrated a good agreement between the quantitative PCR technology and the culture procedure. The high sensitivity and specificity of the quantitative PCR technology justify its use in epidemiological studies and as an adjunct in clinical diagnosis of periodontal patients.

Key words: *Actinobacillus actinomycetemcomitans*; culture; periodontitis; *Porphyromonas gingivalis*; real-time polymerase chain reaction; sensitivity; specificity; *Tannerella forsythensis*; TaqMan

Accepted for publication 8 March 2004

Culture techniques have been the classic diagnostic method to detect the bacterial species residing in the subgingival microflora. It is the only current method capable of identifying new species, determining the in vitro antimicrobial susceptibility of periodontal pathogens and able to identify and provide a quantitative measurement of viable microorganisms. As a result of this, it is still considered the gold standard in periodontal microbiology and remains an important means of characterizing the subgingival microbiota. However, these techniques have serious limitations, mainly the difficulty in recovering even cultivable species when they are found in low numbers. Moreover, significant bacterial species may be found in the subgingival biofilm, such as *Treponema* species and *Tannerella forsythensis*, which require stringent growing conditions and, therefore, are difficult to detect and quantify by means of culturing (Tanner et al. 1986, Sakamoto et al. 2002). Recently, novel subgingival bacteria have been identified, associated both with health and periodontal disease, which suggests that it is likely that many unidentified periodontal species still exist (Kumar et al. 2003). These intrinsic limitations of bacterial culturing together with its stringent requirements, such as the need for experienced personnel, time and relatively high cost, have led to the development of different non-cultural diagnostic methods, mainly based on immune-diagnosis or nucleic acid-based detection methods (Chen & Slots 1999). Overall, these methods have demonstrated a high degree of sensitivity and specificity in the detection of the putative periodontal pathogens; however, most of them are unable to adequately quantify the detected species and to test their antimicrobial susceptibility profiles. The recent advent of real-time polymerase chain reaction (PCR) with species-specific primers provides a very specific and sensitive method for an accurate detection of target microorganisms and, at the same time, allows for quantification of the individual bacterial species. Our research group has recently developed and tested a real-time PCR assay, based on single copy gene sequence and on the TaqMan chemistry, aimed at the quantification of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *T. forsythensis* in subgingival plaque samples (Morillo et al. 2003, 2004). This assay has shown a high degree of specificity and a very reproducible and consistent methodology to quantify these pathogenic species. However, this test has only been evaluated on reference strains and therefore, its diagnostic utility on bacterial samples from subjects with different periodontal conditions is unknown.

The purpose of this investigation was to validate this real-time quantitative PCR assay, by comparing it with conventional cultural procedures, in identifying and quantifying *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*, using subgingival plaque samples from subjects with different periodontal conditions.

Patients and Methods

Subjects

Ninety-two adult subjects participated in this study. They were recruited from among the patients referred for periodontal therapy to the Graduate Periodontal Clinic at the Faculty of Odontology, University Complutense, Madrid, Spain, as well as from among subjects working or studying in this academic institution who volunteered to participate in this study. This sample population received a standard periodontal examination (including full-mouth probing at six sites per tooth, evaluating probing pocket depth, clinical attachment level and bleeding on probing) and depending on their periodontal condition, they were assigned to one of the three groups according to the following criteria:

- Periodontitis group (32 subjects): adult patients with untreated periodontitis, demonstrating radiographic evidence of alveolar bone loss in each quadrant of the dentition, and the presence of $\geqslant 4$ sites with probing pocket depth $\geqslant 4$ mm and an attachment loss $\geqslant 3$ mm.
- Gingivitis group (30 subjects): adult patients with $\geqslant 3$ teeth in each quadrant of the dentition, and no periodontitis, as demonstrated by having less than three sites with probing depth > 3 mm and attachment loss > 2 mm. These subjects were included in this group if they showed bleeding on probing in more than 25% of the sites.
- Healthy group (30 subjects): they had to fulfil identical criteria to gingivitis patients, except that in bleeding on probing, they have to show bleeding in less than 25% of the sites.

All these subjects were otherwise healthy and had not taken systemic antibiotics in the last 2 months prior to the beginning of the study. Since this study was limited to a comparison between two diagnostic microbiological techniques, no additional criteria were applied other than willingness to participate by signing an informed consent.

Bacterial sampling

A pooled subgingival sample from the deepest bleeding site with bone loss from each quadrant was obtained from all patients in the periodontitis group. In the healthy and gingivitis groups, four interproximal buccal sites from the first molars were selected (one per quadrant).

After careful removal of supragingival plaque deposits, isolation of the sampling sites with cotton rolls and gentle air-drying, two sterile #30 paper points (Zipperer, United Dental MFRS Inc., West Palm Beach, FL, USA) were inserted simultaneously into the depth of the pockets and left in place for 10 s. One paper point from each site was

introduced into an empty 1.5 ml microfuge tube for PCR analysis, and the other point was introduced into a 1.5 ml reduced transport fluid (Syed & Loesche 1972). Samples for PCR analysis were stored at −70°C and culture samples were processed within 2 h after sampling.

Culture procedures

Culturing procedures were carried out in the Laboratory of Oral Microbiology, Faculty of Odontology, University Complutense, Madrid, Spain. Samples were vortexed for 30 s and 10-fold serially diluted in RTF; 100 μl of each dilution was plated on non-selective 5% horse blood agar plates (Oxoid no. 2, Oxoid Ltd, Basingstoke, UK) supplemented with haemin (5 mg/l) and menadione (1 mg/l) for determination of the total anaerobic bacterial counts and determination of specific periodontal pathogens. Samples were also plated on Dentaid-1 medium (Alsina et al. 2001) for isolation and counting of *A. actinomycetemcomitans*.

Blood agar plates were incubated at 37°C in 80% N_2, 10% CO_2 and 10% H_2. After 7–14 days of incubation, total anaerobic counts were assessed. The presence and numbers of the putative periodontal pathogens *P. gingivalis* and *T. forsythensis* were determined. Confirmation of identification was based on Gram staining and cell morphology, aero tolerance, production of catalase and other biochemical reactions (Rapid ID 32A, BioMerieux, SA, Le Balme les Grottes, France).

Dentaid-1 plates were incubated in air with 5% CO_2 at 37°C for 2 days. Total counts of *A. actinomycetemcomitans* were obtained while identification was based on its characteristic colony morphology (star-like inner structure), a positive catalase reaction with 3% hydrogen peroxide and a set of specific enzymes (APIZYM, BioMerieux).

Real-time quantitative PCR

DNA extraction

Every sample was diluted in 150 μl Tris-EDTA buffer, and dispersed in vortex for 1 min. A mineral oil drop was added to each sample to avoid evaporation during the boiling step. Samples were incubated at 100°C during 20 min in a thermal block. Then, the solution was transferred to a clean microfuge tube without mineral oil.

Primers and probes

Primer and probe design was described previously (Morillo et al. 2004). Definitive primers were:

Aa-forward 5′-ACGCAGACGATTG-ACTGAATTTAA-3′,
Aa-reverse 5′-GATCTTCACAGCTA-TATGGCAGCTA-3′,
Pg-forward 5′-CCTACGTGTACGG-ACAGAGCTATA-3′,
Pg-reverse 5′-AGGATCGCT-CAGCGTAGCATT-3′,
Tf-forward 5′-TCCCAAAGACGCG-GATATCA-3′ and
Tf-reverse 5′-ACGGTCGCGATGT-CATTGT-3′.

PCR products were 77, 71 and 65 bp length for *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*, 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′.

Real-time PCR assay

TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CD, USA) supplied at a 2 × concentration and containing AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1 and optimized buffer was used for PCR analysis. The final concentration used a total volume of 25 μl and contained 12.5 μl 2 × Master Mix, 3 μl of DNA template, 0.9 μM each primer and 0.25 μM probe and sterile distilled water to adjust volume. Real-time PCR was carried out in ABI Prism 7700 system (Applied Biosystems) with 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.

Data analysis

Counts of the selected pathogens identified by culture were calculated by direct measuring of the selected colonies with regard to the original sample. Results are expressed in colony-forming units/ml (CFU/ml) and log transformed.

Counts of the selected pathogens identified by PCR results are expressed in C_T. This is the cycle number at which the reaction begins to be exponential for a known number of DNA copies. Procedures were performed in triplicate, the mean value was calculated and the result was compared with a standard curve obtained with a known number of DNA copies (positive control) (Morillo et al. 2003, 2004). Means and standard deviations for the different bacterial counts using both techniques were obtained.

The qualitative results (presence or absence of the tested pathogens) from both diagnostic methods were plotted using contingency tables. From these tables, all the diagnostic parameters were calculated (sensitivity, specificity, positive and negative predictive values and positive and negative likelihood ratios) using culture as the diagnostic reference standard. This analysis was repeated using the PCR results as the reference standard. To evaluate the degree of agreement between both diagnostic methods, Pearson's coefficients were calculated. Conversely, the χ^2 with the Yates correction test was used to assess the independence between both tests.

To compare the quantitative results obtained with both methods, the data were transformed in order to account for the different thresholds of detection from both techniques. This was done by transforming all the negative results obtained in a given sample into positive results just below the technique's detection limit. Under this assumption, we assigned a value of nine for all negative PCR results; 99 for all negative *A. actinomycetemcomitans* results in culture; and 999 for all negative *P. gingivalis* and *T. forsythensis* results in culture. Once the results from both diagnostic techniques were transformed, they were compared using a paired *t*-test.

Results

Study population and microbiological results

Table 1 describes the study population and group distribution. Ninety-two subjects were included, 32 being allocated to the periodontitis group, 30 to gingivitis and 30 to health.

Table 2 describes the prevalence and mean counts of the examined species in the subgingival plaque samples from the

three periodontal conditions using both diagnostic methods. *A. actinomycetemcomitans* was the least frequently recovered species with both techniques (0–6.7% for culture and 3.3–18.8% for PCR). The mean counts in positive samples were low in samples from health and gingivitis patients and increased in the periodontitis group with both methods. In this group, the mean counts obtained with culture were higher than with PCR (1.9×10^5 versus 1.4×10^4).

The prevalence of *P. gingivalis* was low in healthy patients (20% for culture and 13.3% for PCR); increased in gingivitis (40% and 30%, respectively) and peaked in periodontitis patients (84.4% and 81.3%, respectively). The mean counts in positive samples were similar in both microbiological procedures, although higher in culture samples. A clear increase was observed from healthy patients to gingivitis patients, and from those to periodontitis patients.

The frequency of detection of *T. forsythensis* showed marked differences between culture and PCR, although the same tendency of an increase in prevalence from health to gingivitis and to periodontitis was observed with both methods. The prevalence of *T. forsythensis* was very high in all disease groups when PCR was used for diagnosis (73.3% in health, 93.3% in gingivitis and 100% in periodontitis). Conversely, the prevalence of this species was low for all groups when culture was used for the analysis (3.3% in health, 10% in gingivitis and 25% in periodontitis). The differences in recovery between both diagnostic methods showed the same tendency when the mean counts were calculated (between 2 and 3 log 10 differences), although the same tendency of increased number of bacteria from health to gingivitis and to periodontitis was seen in the results from both techniques. The mean counts in positive sites were also always higher for PCR in all groups.

Qualitative results

Table 3 shows the comparative results regarding the detection of *A. actinomycetemcomitans* with both diagnostic methods. Overall, 82 of the patients were negative for both tests, three positive in both tests, six were PCR positive and culture negative, while only one was culture positive and PCR negative. Considering culture results as the reference standard, the sensitivity of PCR in periodontitis patients was 1.0, while the specificity and negative predictive values were 0.86 and 1.0, respectively. In this group the likelihood ratio for PCR was 7.5. As a result of the low number of culture-positive samples in both health and gingivitis patients, the diagnostic parameters for PCR were difficult to generate. A statistically significant association between the results obtained with both techniques was obtained for all samples ($P < 0.001$), as well as for the gingivitis and periodontitis samples ($P < 0.001$), thus demonstrating a significant agreement between both diagnostic techniques.

Table 4 shows the comparative results regarding the detection of *P. gingivalis* with both diagnostic methods. Overall, 40 patients were negative in both tests, 32 were positive with both, seven were PCR positive and culture negative and the opposite occurred in 13 patients. The sensitivity of PCR was high when considering all patients or just the periodontitis group (0.711–0.926). However, it was low for healthy and gingivitis patients, because of the low frequency of detection of the pathogen. The same was true for the

Table 1. Demographic parameters of the population sample

Group	Age (years)		Gender	
	range	mean (SD)	female	male
periodontitis	36–71	49.4 (8.99)	16	16
gingivitis	30–62	46.6 (9.82)	17	13
healthy	30–63	37.8 (7.48)	16	14

Table 2. Microbiological results (frequency of detection, mean counts, mean counts in positive samples)

	Actinobacillus actinomycetemcomitans		*Porphyromonas gingivalis*		*Tannerella forsythensis*	
	culture	PCR	culture	PCR	culture	PCR
Healthy						
n	30	30	30	30	30	30
frequency of detection (%)	0.0	6.7	20.0	13.3	3.3	73.3
mean counts	0.0E+00	1.1E+02	2.5E+03	4.4E+02	1.5E+02	2.6E+05
standard deviation	0.0E+00	6.0E+02	7.0E+03	1.7E+03	8.4E+02	1.4E+06
mean counts (positive)	NA	1.7E+03	1.3E+04	3.3E+03	4.6E+03	3.5E+05
Gingivitis						
n	30	30	30	30	30	30
frequency of detection (%)	6.7	3.3	40.0	30.0	10.0	93.3
mean counts	8.5E+01	1.4E+02	2.9E+04	1.4E+04	2.7E+03	2.5E+05
standard deviation	4.5E+02	7.6E+02	1.1E+05	3.7E+04	8.9E+03	4.6E+05
mean counts (positive)	1.3E+03	4.2E+03	7.2E+04	4.5E+04	2.7E+04	2.7E+05
Periodontitis						
n	32	32	32	32	32	32
frequency of detection (%)	6.3	18.8	84.4	81.3	25.0	100.0
mean counts	1.2E+04	2.6E+03	4.0E+06	9.0E+05	4.0E+05	1.2E+08
standard deviation	6.5E+04	1.0E+04	7.6E+06	1.5E+06	1.7E+06	2.1E+08
mean counts (positive)	1.9E+05	1.4E+04	4.8E+06	1.1E+06	1.6E+06	1.2E+08

PCR, polymerase chain reaction. ND, not available.

positive predictive value. Conversely, the specificity (0.800–0.875) and negative predictive values (0.667–0.808) were high in all groups. Also, with this pathogen, a statistically significant association between the results obtained with both techniques was obtained for all samples ($P<0.001$), as well as for the gingivitis ($P=0.02$) and periodontitis ($P<0.001$) samples, thus demonstrating a significant agreement between both diagnostic techniques for the detection of *P. gingivalis*.

Table 5 shows the comparative results regarding the detection of *T. forsythensis* with both diagnostic methods. Overall, 10 patients were positive by both methods, and 12 were negative. Seventy patients were PCR positive and culture negative, while no patient was positive by culture and negative by PCR. The sensitivity of PCR was maximum (1.000) because of the absence of patients being culture positive and PCR negative. Conversely, specificity and positive predictive values were low (0.12 and 0.14, respectively) considering both, all patients or just the periodontitis group (0.00 and 0.25). This pathogen was clearly identified more frequently with PCR than with culture, and therefore no significant association was found between the results obtained with both techniques for all samples, as well as for the gingivitis and periodontitis samples. This clearly shows that for *T. forsythensis*, PCR was more sensitive than culture.

Table 6 shows the sensitivity, specificity, positive and negative predictive values of culture when the PCR results were considered as the reference standard. For *A. actinomycetemcomitans* in periodontitis patients, the sensitivity was low (0.33) while the specificity and positive predictive values were maximum (1.0). For *P. gingivalis*, both the sensitivity and specificity were high (0.96 and 0.66, respectively), while the positive predictive value was also high (0.92). With regard to *T. forsythensis*, again the sensitivity was low (0.25) while the specificity and predictive values were maximum (1.0).

Table 3. Contingency tables for *Actinobacillus actinomycetemcomitans*

All	Cult+	Cult −	Healthy	Cult+	Cult −
PCR+	3	6	PCR+	0	2
PCR −	1	82	PCR −	0	28
Gingivitis	Cult+	Cult −	Periodontitis	Cult+	Cult −
PCR+	1	0	PCR+	2	4
PCR −	1	28	PCR −	0	26

	All	Healthy	Gingivitis	Periodontitis
sensitivity	0.750	NA	0.500	1.000
specificity	0.932	0.933	1.000	0.867
positive predictive value	0.333	0.000	1.000	0.333
negative predictive value	0.988	1.000	0.966	1.000
positive likelihood ratio	11.000	NA	NA	7.500
negative likelihood ratio	3.727	NA	2.000	NA
χ^2 (*p*-value)	0.0003*	NA	0.0700	0.0300
Pearson's (*p*-value)	0.0000†	NA	0.0002†	0.0008†

*Statistically significant rejection of independency.
†Statistically significant association.
Cult+, culture positive; Cult −, culture negative; PCR+, polymerase chain reaction positive; PCR −, polymerase chain reaction negative; NA, not available.

Table 4. Contingency tables for *Porphyromonas gingivalis*

All	Cult+	Cult −	Healthy	Cult+	Cult −
PCR+	32	7	PCR+	1	3
PCR −	13	40	PCR −	5	21
Gingivitis	Cult+	Cult −	Periodontitis	Cult+	Cult −
PCR+	6	3	PCR+	25	1
PCR −	6	15	PCR −	2	4

	All	Healthy	Gingivitis	Periodontitis
sensitivity	0.711	0.167	0.500	0.926
specificity	0.851	0.875	0.833	0.800
positive predictive value	0.821	0.250	0.667	0.962
negative predictive value	0.755	0.808	0.714	0.667
positive likelihood ratio	4.775	1.333	3.000	4.630
negative likelihood ratio	2.946	1.050	1.667	10.800
χ^2 (*p*-value)	0.0000*	1.0000	0.1200	0.001*
Pearson's (*p*-value)	0.0000†	0.3985	0.02†	0.0000†

*Statistically significant rejection of independency.
†Statistically significant association.
Cult+, culture positive; Cult −, culture negative; PCR+, polymerase chain reaction positive; PCR −, polymerase chain reaction negative; NA, not available.

Quantitative results

Table 7 shows the comparative analysis of the bacterial counts obtained by each method. Using the method to transform the data defined earlier, the results demonstrated that *P. gingivalis* culture counts were significantly higher ($P<0.001$) than those obtained by PCR, and this finding was consistent for all groups. The opposite was true for *T. forsythensis*, and statistically significant higher counts ($P<0.001$) were obtained by PCR, in comparison with culture, but only for gingivitis and periodontitis patients. Statistically significant differences were also observed for *A. actinomycetemcomitans*.

Discussion

The results from this study have shown that the tested real-time quantitative PCR assay is able to identify and quantify *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*, with a high degree of sensitivity and specificity when compared with standard culturing techniques.

When tested for the detection of *A. actinomycetemcomitans*, real-time quantitative PCR yielded a prevalence of 18.8%. The evaluation of *A. actinomycetemcomitans* prevalence results in

Table 5. Contingency tables for *Tannerella forsythensis*

All	Cult+	Cult −	Healthy	Cult+	Cult −
PCR+	12	70	PCR+	1	21
PCR −	0	10	PCR −	0	8
Gingivitis	Cult+	Cult −	Periodontitis	Cult+	Cult −
PCR+	3	25	PCR+	8	24
PCR −	0	2	PCR −	0	0

	All	Healthy	Gingivitis	Periodontitis
sensitivity	1.000	1.000	1.000	1.000
specificity	0.125	0.276	0.074	0.000
positive predictive value	0.146	0.045	0.107	0.250
negative predictive value	1.000	1.000	1.000	NA
positive likelihood ratio	1.143	1.381	1.080	1.000
negative likelihood ratio	NA	NA	NA	NA
χ^2 (*p*-value)	0.4237	1.0000	1.0000	NA
Pearson's (*p*-value)	0.0993	0.2779	0.3198	NA

Cult+, culture positive; Cult − , culture negative; PCR+, polymerase chain reaction positive; PCR − , polymerase chain reaction negative; NA, not available.

Table 6. Diagnostic validity of bacterial culture using PCR as the reference standard

	All	Healthy	Gingivitis	Periodontitis
Actinobacillus actinomycetemcomitans				
sensitivity	0.333	NA	1.000	0.333
specificity	0.987	1.000	0.965	1.000
positive predictive value	0.750	NA	0.500	1.000
negative predicitive value	0.932	0.933	1.000	0.867
Porphyromonas gingivalis				
sensitivity	0.8205	0.25	0.666	0.9615
specificity	0.7547	0.8076	0.7142	0.666
positive predictive value	0.711	0.167	0.500	0.926
negative predictive value	0.851	0.875	0.833	0.800
Tannerella forsythensis				
sensitivity	0.130	0.045	0.107	0.250
specificity	1.000	1.000	1.000	NA
positive predictive value	1.000	1.000	1.000	1.000
negative predicitive value	0.125	0.276	0.074	0.000

NA, not available; PCR, polymerase chain reaction.

periodontitis patients using PCR technology gives very heterogeneous results when assessing the scientific literature, from very high prevalences (over 60%) reported in China (Tan et al. 2001b), Singapore (Tan et al. 2002), Korea (Choi et al. 2000), Brazil (Avila-Campos & Velásquez-Meléndez 2002) and an oriental population in the USA (Umeda et al. 1998), to relatively low prevalences reported in Taiwan (Yuan et al. 2001), Japan (Takamatsu et al. 1999, Okada et al. 2000), Ireland (Mullally et al. 2000), Greece (Kamma et al. 2001). In the UK, there are three studies, one showing high frequencies of detection (Doungudomdacha et al. 2001), another intermediate (Riggio et al. 1996) and another low (Darby et al. 2000). The explanations for this heterogeneity may vary in terms of patient selection, methodological issues, different bacterial serotypes and true geographical differences, although large ecological studies in specific populations around the world using standardized highly specific quantitative PCR should be performed in order to understand the true prevalence and geographical distribution of this bacterial species.

When the presence of *A. actinomycetemcomitans* was evaluated with culturing techniques, it demonstrated a prevalence of 6.3% in periodontitis patients. A similar figure was also reported using bacterial culture (3.2%), in a previous study by our group with a comparable population in Spain (Sanz et al. 2000), although a different culture medium was used in both studies. Low prevalences of this pathogen (20–40%) have been reported using bacterial culture throughout the world, although a geographical influence must not be discarded (for a review, see Sanz et al. 2000). In this study, we have reported a prevalence of this bacterial species of 18.8% using PCR.

Comparing the culture and PCR results, a significant diagnostic agreement was found, especially when considering all groups of patients together. In six cases, PCR yielded a positive result, associated with a negative by culture. This could be explained by the lower limit of detection and the stringent growing conditions required by culture. Conversely, one gingivitis patient was positive by culture and negative for PCR. This case could mean a false positive identified by culture. This has been reported with the species *Haemophilus aphrophilus*, which can show cross-reactivity with some of the biochemical tests used to identify *A. actinomycetemcomitans* (Olsen et al. 2000). Another possibility is the presence of false negatives with PCR. This could be justified by the presence of genetic variations in the sequence of the leukotoxin-C gene used as a primer for this PCR assay.

P. gingivalis was detected in 81.3% of the patients with periodontits using PCR and in 84.4% using culture. These results are higher than what has been reported in previous prevalence studies using culture (range between 27% and 51%) (for a review, see Sanz et al. 2000). It is likely that there are important geographical differences, and Spain demonstrates a high prevalence of these bacteria. This fact was clearly demonstrated in a previous study comparing oral bacterial prevalence between the Netherlands and Spain (Sanz et al. 2000). Using PCR, however, most of the studies have shown a high prevalence of *P. gingivalis* (over 50%), although some heterogeneity also exists among different geographical locations: in Japan between 78% and 95% (Amano et al. 1999, 2000, Nozaki et al. 2001, Okada et al. 2001, Takeuchi et al. 2001, Fujise et al. 2002); in Korea 100% (Choi et al. 2000); in USA between 50% and 87% (McClellan et al. 1996, Tuite-McDonnell et al. 1997, Umeda et al. 1998, Griffen et al. 1999); in Brazil 78% (Avila-Campos & Velásquez-Meléndez

Table 7. Quantitative results: comparison of counts (log transformed) obtained by each method, in each group

	Mean	Mean Diff.*	95% CI		*t*	Sig.
			Lower	Upper		
Periodontitis						
Aa-PCR	1.403	− 0.7725	− 0.1216	− 0.4236	− 4.51	0.0000
Aa-culture	2.175					
Pg-PCR	4.493	− 1.0732	− 1.5964	− 0.5500	− 4.18	0.0002
Pg-culture	5.566					
Tf-PCR	7.138	3.4912	3.0392	3.9432	15.75	0.0000
Tf-culture	3.647					
Gingivitis						
Aa-PCR	1.043	− 0.9998	− 1.0868	− 0.9129	− 25.5	0.0000
Aa-culture	2.043					
Pg-PCR	1.888	− 1.5799	− 2.1491	− 1.0107	− 5.68	0.0000
Pg-culture	3.468					
Tf-PCR	4.324	1.1872	0.6428	1.7307	4.46	0.0001
Tf-culture	3.138					
Healthy						
Aa-PCR	1.064	− 0.9319	− 1.1118	− 0.7523	− 10.6	0.0000
Aa-culture	1.996					
Pg-PCR	1.230	− 1.9369	− 2.2270	− 1.6468	− 13.6	0.0000
Pg-culture	3.167					
Tf-PCR	2.641	− 0.3802	− 0.9472	0.1868	− 1.37	0.181
Tf-culture	3.022					

*Negative values means higher counts for culture.
Aa: *A. actinomycetemcomitans*; *Pg*; *P. gingivalis*; *Tf*: *T. forsythensis*; PCR: polymerase chain reaction.

2002); and in the UK between 50% and 65% (Darby et al. 2000, Doungudomdacha et al. 2001). In this study, we have reported a prevalence of 13% in healthy subjects and 81.3% in periodontitis, which is in agreement with most of the studies using standard PCR.

When the results in the detection of *P. gingivalis* were compared between both techniques, they showed a significant agreement, especially in periodontitis patients. Overall, in 13 cases a positive culture result was associated with a negative PCR, and the opposite was true in seven cases. These seven cases can be easily explained by the culture lower detection limits. However, it is more difficult to justify the 13 culture positive PCR negative. In order to discard a possible technical failure, after evaluating the results, these negative samples were again processed using a standard PCR technique with 16S rRNA-specific primers (Wahlfors et al. 1995), and the results were again negative for *P. gingivalis*. Another possible explanation could be the existence of a non-pathogenic *P. gingivalis*-like phylotype with identical phenotypic features, since 11 out of the 13 cases were found in healthy or gingivitis patients. These species have only been described associated with animals (*P. macacae* and *P. gulae*); however, the presence of non-pathogenic subtypes, genetically related to *P. gingivalis* and very similar phenotypically, cannot be discarded (Allaker et al. 1997, Fournier et al. 2001, Norris & Love 2001). This finding deserves further investigation.

T. forsythensis was detected in 100% of the patients with periodontitis using real-time PCR. This high prevalence has also been reported in Singapore (91%) (Tan et al. 2001a), in the USA (between 84.5% and 100%) (Leys et al. 2002, Umeda et al. 1998), in Brazil (82%) (Avila-Campos & Velásquez-Meléndez 2002) and in Ireland (78%) (Mullally et al. 2000). However, in healthy subjects we obtained a prevalence of 73.3%, which is higher than what has been reported in other studies (between 20% and 50%). This discrepancy could be because of the primers used in this study, which can detect a sequence of very low size, since it is a single copy gene. When higher size sequences are used, there is less available DNA for the PCR amplification in the samples that have less number of target bacteria. Another possibility is that the selected primers might have also detected a recently reported phylotype (BU063) of this bacterial species, which is associated with gingival health, and with a possible antagonism mechanism with the pathogenic *T. forsythensis* (Leys et al. 2002). These possibilities deserve further study.

Conversely, only 25% of the periodontitis patients were detected positively with culture for *T. forsythensis*. This percentage is much lower than the 64.5% reported previously by our group, and also lower than other previous results based on culture technology (Sanz et al. 2000).

Comparing both procedures, PCR yielded a significantly higher prevalence than culture in all patient categories. These results could be partially explained by the lower detection threshold of PCR, but most likely the low detection demonstrated in culture is because of fastidious growth demonstrated by this bacterial species. *T. forsythensis* is a strict anaerobe, which grows very slowly in common media. The blood agar plates used in this study are not probably the most suitable medium for its growth, since it requires the presence of neighbouring colonies, such as *P. gingivalis* or *Fusobacterium nucleatum* that provides the essential *N*-acetyl-muramic acid for its growth. This significant difference between PCR and culture was also demonstrated when comparing counts in positive samples, the PCR counts being clearly higher, which is exactly the opposite to what was seen with *P. gingivalis*. In this latter case, because of the presence of haemin and menadione in the culture medium, if the pathogen was present, their growth could have been clearly favoured by the culture media used. Based on these results, the use of selective media for *T. forsythensis* enriched with *N*-acetyl-muramic acid should be recommended when culture is used for its detection.

When culture was used as the diagnostic reference standard, PCR demonstrated a high sensitivity, specificity and positive predictive value in the detection of *P. gingivalis* in periodontitis patients (Table 4). For *A. actinomycetemcomitans*, the sensitivity and specificity were also high, although the positive predictive value was low (Table 3). For *T. forsythensis*, only the sensitivity was high, the specificity and predictive values being low (Table 5). The high sensitivity and specificity of the PCR detection of *A. actinomycetemcomitans* shown in this study in periodontitis patients (100% sensitivity and 86% specificity) is clearly better than the results obtained by Papapanou et al. (1997) using checkerboard detection

(17% sensitivity and 89% specificity) and by Loesche et al. (1992) using DNA probes (14% sensitivity and 85% specificity). In the case of *P. gingivalis*, the obtained results in periodontitis patients (92% sensitivity and 80% specificity) are comparable with the 83% sensitivity and 67% specificity reported with checkerboard detection (Papapanou et al. 1997) but clearly superior to the 90% and 23%, respectively, reported with DNA probes (Loesche et al. 1992). In the case of *T. forsythensis*, our results demonstrate a high sensitivity but very low specificity, which is again comparable with the results obtained with checkerboard detection (Papapanou et al. 1997) and standard PCR (Lotufo et al. 1994), which probably reflects the difficulties in recovering this bacterial species by culture.

These difficulties in culturing and the development of improved new technologies always raise the issue as to what kind of appropriate reference standard should be used. In this study, we have also calculated the diagnostic validity of culture using quantitative PCR as the reference standard. Bacterial culture demonstrated a high sensitivity, specificity and predictive value for the detection of *P. gingivalis.* However, for detecting *A. actinomycetemcomitans*, and *T. forsythensis*, their respective sensitivity and positive predictive values were low (Table 6). These different results in diagnostic validity for both techniques can be explained by the different detection limits, being 10 bacteria for PCR and 10^2–10^4 for culture, what results in the higher sensitivity demonstrated by PCR. The differences between the pathogens might be because of the specific growing conditions that limit their recovery by culture. This was clearly demonstrated for *T. forsythensis*.

When both techniques were compared for their ability to quantify the tested bacteria, without taking into account the different detection limits, culture showed significantly higher counts than PCR for *P. gingivalis*, while PCR showed significantly higher counts that culture for *T. forsythensis*. These results could be explained by the difficulties for growing in culture media demonstrated by *T. forsythensis* previously discussed, and the converse enhancement of *P. gingivalis* in culture because of the enrichment with haemin and menadione in the medium used. *A. actinomycetemcomitans* results are difficult to interpret due to the low frequency of detection.

In conclusion, our study demonstrated a good agreement between the quantitative PCR technology and the culture procedure. The discrepancy was attributed mostly to the lower detection limits of bacterial culture and the difficulties in growth demonstrated by *T. forsythensis*. The high sensitivity and specificity of the quantitative PCR technology justify its use in epidemiological studies of periodontal diseases. Moreover, its capability of accurate quantification would also justify its use as an adjunct in the clinical diagnosis of periodontal patients. However, it is important to keep in mind a major advantage of culture techniques, its capability to detect multiple bacterial species coincidentally, to detect unexpected bacteria, as well as allowing the determination of antibiotic resistance.

References

Allaker, R. P., Young, K. A., Langlois, T., de Rosayro, R. & Hardie, J. M. (1997) Dental plaque flora of the dog with reference to fastidious and anaerobic bacteria associated with bites. *Journal of Veterinary Dentistry* **14**, 127–130.

Alsina, M., Olle, E. & Frias, J. (2001) Improved, low-cost selective culture medium for *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology* **39**, 509–513.

Amano, A., Kuboniwa, M., Nakagawa, I., Akiyama, S., Morisaki, I. & Hamada, S. (2000) Prevalence of specific genotypes of *Porphyromonas gingivalis* fimA and periodontal health status. *Journal of Dental Research* **79**, 1664–1668.

Amano, A., Nakagawa, I., Kataoka, K., Morisaki, I. & Hamada, S. (1999) Distribution of *Porphyromonas gingivalis* strains with fimA genotypes in periodontitis patients. *Journal of Clinical Microbiology* **37**, 1426–1430.

Avila-Campos, M. J. & Velásquez-Meléndez, G. (2002) Prevalence of putative periodontopathogens from periodontal patients and healthy subjects in Sao Paulo, Brazil. *Revista do Instituto de Medicina Tropical de Sao Paulo* **44**, 1–5.

Chen, C. & Slots, J. (1999) Microbiological tests for *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Periodontology 2000* **20**, 53–64.

Choi, B. K., Park, S. H., Yoo, Y. J., Choi, S. H., Chai, S. K., Cho, K. S. & Kim, C. H. K. (2000) Detection of mayor putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach. *Journal of Periodontology* **71**, 1387–1394.

Darby, I. B., Hodge, P. J., Riggio, M. P. & Kinane, D. F. (2000) Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. *Journal of Clinical Periodontology* **27**, 417–424.

Doungudomdacha, S., Rawlinson, A., Walsh, T. F. & Douglas, C. W. I. (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.

Fournier, D., Mouton, C., Lapierre, P., Kato, T., Okuda, K. & Ménard, C. (2001) *Porphyromonas gulae* sp. nov., an anaerobic, Gram-negative coccobacillus from the gingival sulcus of various animal hosts. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1179–1189.

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.

Griffen, A. L., Lyons, S. R., Becker, M. R., Moeschberger, M. L. & Leys, E. J. (1999) *Porphyromonas gingivalis* strain variability and periodontitis. *Journal of Clinical Microbiology* **37**, 4028–4033.

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

Kumar, P. S., Griffen, A. L., Barton, J. A., Paster, B. J., Moeschberger, M. L. & Leys, E. J. (2003) New bacterial species associated with chronic periodontitis. *Journal of Dental Research* **82**, 338–344.

Leys, E. J., Lyons, S. R., Moeschnerger, M. L., Rumpf, R. W. & Griffen, A. L. (2002) Association of *Bacteroides forsythus* and a novel *Bacteroides* phylotype with periodontitis. *Journal of Clinical Microbiology* **40**, 821–825.

Loesche, W. J., Lopatin, D. E., Giordano, J., Alcoforado, G. & Hujoel, P. (1992) Comparison of the benzoyl-DL-arginine-naphthylamide (BANA) test, DNA probes, and immunological reagents for ability to detect anaerobic periodontal infections due to *Porphyromonas gingivalis*, *Treponema denticola*, and *Bacteroides forsythus*. *Journal of Clinical Microbiology* **30**, 427–433.

Lotufo, R. F., Flynn, J., Chen, C. & Slots, J. (1994) Molecular detection of *Bacteroides forsythus* in human periodontitis. *Oral Microbiology and Immunology* **9**, 154–160.

McClellan, D. L., Griffen, A. L. & Leys, E. J. (1996) Age and prevalence of *Porphyromonas gingivalis* in children. *Journal of Clinical Microbiology* **34**, 2017–2019.

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.

Morillo, J. M., Lau, L., Sanz, M., Herrera, D., Martin, C. & Silva, A. (in press) Quantitative real-time PCR based on single copy gene sequence for detection of periodontal pathogens. *Journal of Clinical Periodontology* **31**.

Mullally, 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.

Norris, J. M. & Love, D. N. (2001) Serum antibody responses of cats to soluble whole cell antigens and isolated fimbriae of feline *Porphyromonas salivosa* (macacae) and associations with periodontal disease. *Veterinary Microbiology* **79**, 225–237.

Nozaki, T., Kusumoto, Y., Kitamura, M., Hirano, H., Kohyama, A., Hayakawa, M., Takiguchi, H., Abiko, Y., Murakami, S. & Okada, H. (2001) A sensitive method for detecting *Porphyromonas gingivalis* by polymerase chain reaction and its possible clinical application. *Journal of Periodontology* **72**, 1228–1235.

Okada, M., Hayashi, F. & Nagasaka, N. (2000) Detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in dental plaque samples from children 2 to 12 years of age. *Journal of Clinical Periodontology* **27**, 763–768.

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.

Olsen, I., Shah, H. N. & Gharbia, S. E. (2000) Taxonomy and biochemical characteristics of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Periodontology 2000* **20**, 14–52.

Papapanou, P. N., Madianos, P. N., Dahlen, G. & Sandros, J. (1997) ''Checkerboard'' versus culture: a comparison between two methods for identification of subgingival microbiota. *European Journal of Oral Sciences* **105**, 389–396.

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.

Sakamoto, M., Suzuki, M., Umeda, M., Ishikawa, I. & Benno, Y. (2002) Reclassification of *Bacteroides forsythus* (Tanner et al. 1896) as *Tannerella forsythensis corrig., gen. nov., comb. nov*. *International Journal of Systematic and Evolutionary Microbiology* **52**, 841–849.

Sanz, M., van Winkelhoff, A. J., Herrera, D., Dellemijn-Kippuw, N., Simon, R. & Winkel, E. (2000) Differences in the composition of the subgingival microbiota of two periodontitis populations of different geographical origin. A comparison between Spain and The Netherlands. *European Journal of Oral Sciences* **108**, 383–392.

Syed, S. A. & Loesche, W. J. (1972) Survival of human dental plaque flora in various transport media. *Applied Microbiology* **24**, 638–644.

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.

Takeuchi, Y., Umeda, M., Sakamoto, M., Benno, Y., Huang, Y. & Ishikawa, I. (2001) *Treponema socranskii*, *Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction. *Journal of Periodontology* **72**, 1354–1363.

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

Tan, K. S., Song, K. P. & Ong, G. (2002) Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. *Journal of Periodontal Research* **37**, 268–272.

Tan, K. S., Woo, C. H., Ong, G. & Song, K. P. (2001b) Prevalence of *Actinobacillus actinomycetemcomitans* in an ethnic adult Chinese population. *Journal of Clinical Periodontology* **28**, 886–890.

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.

Tuite-McDonnell, M., Griffen, A. L., Moeschberger, M. L., Dalton, R. E., Fuerst, P. A. & Leys, E. J. (1997) Concordance of *Porphyromonas gingivalis* colonization in families. *Journal of Clinical Microbiology* **35**, 455–461.

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.

Wahlfors, J., Meurman, J. H., Vaisanen, P., Alakuijala, P., Korhonen, A., Torkko, H. & Janne, J. (1995) Simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by a rapid PCR method. *Journal of Dental Research* **74**, 1796–1801.

Yuan, K., Chang, C. J., Hsu, P. C., Sun, H. S., Tseng, C. C. & Wang, J. R. (2001) Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. *Journal of Periodontal Research* **36**, 18–24.

Address:
Mariano Sanz
Faculty of Odontology
Universidad Complutense
Plaza. Ramón y Cajal, s/n
28040 Madrid,
Spain
Fax: +34-91-394-19-10
E-mail: marianosanz@odon.ucm.es