Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 69 (2011) 12–20
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Comparison of real-time polymerase chain reaction and DNA-strip technology in microbiological evaluation of periodontitis treatment

Sigrun Eick[a,b,*], Anna Straube[a], Arndt Guentsch[c], Wolfgang Pfister[a], Holger Jentsch[d]

[a]Institute of Medical Microbiology, University Hospital of Jena, D-07747 Jena, Germany
[b]Department of Periodontology, Laboratory of Oral Microbiology, University of Bern, CH-3010 Bern, Switzerland
[c]Department of Conservative Dentistry, University Hospital of Jena, D-07743 Jena, Germany
[d]Department of Conservative Dentistry and Periodontology, University Hospital of Leipzig, D-04103 Leipzig, Germany

Received 23 April 2010; accepted 23 August 2010

Abstract

The impact of a semiquantitative commercially available test based on DNA-strip technology (microIDent®, Hain Lifescience, Nehren, Germany) on diagnosis and treatment of severe chronic periodontitis of 25 periodontitis patients was evaluated in comparison with a quantitative in-house real-time PCR. Subgingival plaque samples were collected at baseline as well as at 3, 6, and 12 months later. After extracting DNA, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and several other periodontopathogens were determined by both methods. The results obtained by DNA-strip technology were analyzed semiquantitatively and additionally quantitatively by densitometry. The results for the 4 major periodontopathogenic bacterial species correlated significantly between the 2 methods. Samples detecting a high bacterial load by one method and negative by the other were always found in less than 2% of the total samples. Both technologies showed the impact of treatment on microflora. Especially the semiquantitative DNA-strip technology clearly analyzed the different loads of periodontopathogens after therapy and is useful in microbial diagnostics for patients in dental practices.

Keywords: Periodontopathogenic bacteria; Real-time PCR; DNA-strip technology

1. Introduction

Periodontitis is an infection that results from an imbalance between periodontopathogenic microorganisms and the local and systemic host defense and is characterized by a progressive destruction of the periodontal tissues. The progression of the disease is related to the colonization of the microorganisms, including *Aggregatibacter actinomycetemcomitans*, as well as the members of the so-called red complex *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (Holt and Ebersole, 2005). In addition, bacterial species such as *Prevotella intermedia*, *Eikenella corrodens*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Eubacterium nodatum*, and *Parvimonas micra* (formerly *Peptostreptococcus micros*) are considered to be closely related to periodontitis (consensus report, 1996).

Microbiological laboratory procedures have been involved in diagnosis and therapy control of severe forms of periodontitis for many years (Dahlen, 2006; Mombelli et al., 1994). Nucleic acid based methods being more sensitive for periodontopathogens than cultivation (Jervoe-Storm et al., 2005; Savitt et al., 1988) have become the standard methods in microbiological analysis of subgingival plaque samples.

Qualitative polymerase chain reactions (PCRs) including multiplex PCR were described by several authors (Ashimoto et al., 1996; Morikawa et al., 2008; Tran and Rudney, 1999). Nowadays, many laboratories introduced real-time PCR to quantify the bacterial loads in subgingival plaque (Lyons et al., 2000; Morillo et al., 2003; Rudney et al., 2003). Commercially available test systems have been developed, such as the IAI-PadoTest (IAI, IAI Institute Zuchwil, Switzerland), which uses specifically labeled probes directed against ribosomal RNA (Eguchi et al., 2008), and meridol® Perio Diagnostics (GABA International, Münchenstein, Switzerland), which is a real-time PCR-based test (Jervoe-

* Corresponding author. Tel.: +41-31-632-2542; fax: +41-31-632-8608.
E-mail address: sigrun.eick@zmk.unibe.ch (S. Eick).

0732-8893/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2010.08.017

Storm et al., 2005; Verner et al., 2006). Both test systems are available in a few specialized laboratories where samples must be sent. About 10 years ago, the company Hain Lifescience (Nehren, Germany) introduced the microIDent® test identifying 5 periodontopathogenic marker species (Eick and Pfister, 2002), and later added the microIDent®plus test for an additional 6 species (Haffajee et al., 2009). This test system can be made in each laboratory involved in microbiological diagnosis. A multiplex PCR is followed by a reverse hybridization where amplificates bind specifically to 16SrDNA, which has been commercially placed to strips.

The purpose of this study was to validate the microIDent® test in comparison to real-time PCR in the treatment schedule of chronic periodontitis. The comparison of the 2 methods should also demonstrate benefits and limitations of each method.

2. Material and methods

2.1. Study design

Samples were collected from 28 systemically healthy patients (12 male and 16 female) with generalized chronic periodontitis attending the university dental school clinics of Leipzig during their treatment schedule. Patients demonstrating attachment loss ≥5 mm at more than 30% of sites and an age of ≥35 years (mean age, 50.6 years) with generalized chronic periodontitis were included. Subjects were excluded if they had administration of medication such as antibiotics, steroids, or nonsteroidal anti-inflammatory drugs within the previous 6 months; had received any periodontal treatment in the previous 12 months; had systemic diseases (e.g., diabetes mellitus); were heavy smokers (more than 5 cigarettes per day); were pregnant or nursing.

Baseline of the study was set after hygiene phase, which included supragingival calculus removal and oral hygiene instructions and immediately before scaling and root planing (SRP). Furthermore, subjects were monitored at baseline as well as at 3, 6, and 12 months after SRP. At all these appointments, samples were obtained for microbiological analysis.

The study protocol was approved by the ethics committees of the Universities of Jena and Leipzig, Germany. All participants gave their informed consent.

2.2. Collection of subgingival plaque samples

Plaque samples from each subject were collected from 3 periodontal pockets with a depth of at least 5 mm. Three pockets (1 incisor, 1 premolar, 1 molar) were selected for determination of periodontopathogenic bacteria. At the selected sites, supragingival plaque was carefully removed, after which the sample sites were isolated with cotton rolls and gently air-dried. Each sterile paper point (ISO 35) was inserted into the periodontal pocket for 20 s. The paper points were stored at −20 °C until assayed. All specimens were blinded for laboratory analysis.

2.3. Microflora

The DNA was extracted using a DNA extraction system (High Pure PCR Template Preparation Kit; Roche, Mann-heim, Germany) according to manufacturer's recommendations. Afterward, the DNA was split into aliquots for real-time PCR and microIDent® test. Real-time PCR was carried out using a real-time rotary analyzer (RotorGene 2000; Corbett Research, Sydney, Australia). The primers for *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Campylobacter rectus*, and *Eikenella corrodens* were designed as previously described by Ashimoto et al. (1996); and those for *A. actinomycetemcomitans*, as described by Tran and Rudney (1999). Primers for *Parvimonas micra* (accession no. AF542231; forward: 5′-AACGACGATTAATACCGCATGAGACC-3′ reverse: 5′-CTTCCTCCTATGATACCGTCATTA-3′) and *Eubacterium nodatum* (accession no. U13041; forward: 5′-TTAAGTAAGCGTAGGGTTTAAGG-3′, reverse: 5′-AATTAAACCACATGCTCCGC-3′) have been chosen using a software (Rozen and Skaletsky, 2000). PCR amplifications were carried out in a reaction volume of 20 μL consisting of 2 μL template DNA and 18 μL of reaction mixture containing 2 μL 10× PCR buffer, 2.75 mmol/L $MgCl_2$, 0.2 mmol/L nucleotides, 0.5 μmol/L primer each, 10^{-4} Sybr Green, and 1 U taq polymerase (Fermentas Life Science, St. Leon-Rot, Germany). Negative and positive controls were included in each batch of specimens. The positive control consisted of 2 μL genomic DNA in concentrations in a range from 10^2 to 10^7 bacteria of the reference strains; the negative control was 2 μL of sterile water, both added to 18 μL reaction mixture. The cycling conditions comprised an initial denaturation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, at 60 °C (exception: *A. actinomycetemcomitans*, *Prevotella intermedia* 58 °C; *Eubacterium nodatum* 56 °C) for 20 s, and at 72 °C for 20 s. Furthermore, the specificity of the amplification was always assayed with the use of melting curves. For quantification, the results from unknown plaque specimens were projected on the counted pure culture standard curves of the target bacteria. The numbers of *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, and *Capnocytophaga sputigena* were summarized to *Capnocytophaga* sp.

The other aliquot of the plaque samples was used for performing the microIDent® including microIDent®plus test, which is able to identify 11 periodontopathogenic bacterial species (including 2 complexes) in 2 runs. Each PCR amplification was carried out in a reaction volume of 25 μL consisting of 2.5 μL of template DNA and 22.5 μL of reaction mixture containing 17.5 μL of primer–nucleotide mix (microIDent® and microIDent®plus, respectively), 2.5 μL of 10× PCR buffer, 2.5 μL of 25 mmol/L $MgCl_2$,

and 1 U taq polymerase (Fermentas Life Science). PCR cycling was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The cycling conditions comprised an initial denaturation step at 95 °C for 5 min; 10 cycles at 95 °C for 30 s and at 60 °C for 2 min; 20 cycles at 95 °C for 10 s, at 55 °C for 30 s, and at 72 °C for 30 s; and a final extension step at 72 °C for 10 min. The subsequent reverse hybridization was performed according to the microIDent® including microIDent®plus kit. In short, the biotinylated amplicons were denatured and incubated at 45 °C with hybridization buffer and strips coated with 2 control lines and 5 or 6 species-specific probes. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any nonspecific bound DNA. Streptavidin-conjugated alkaline phosphatase was added, the samples were washed, and hybridization products were visualized by adding a substrate for alkaline phosphatase. Finally, the ends of the strips were fixed with tapes on analysis forms belonging to the microIDent® kits. Each 4 of the strips were then scanned using Adobe Photoshop® Elements (Adobe Systems, San Jose, CA). After adjusting the contrast (autocontrast), the luminescence of the bands was measured. The range of the white background of the strips and the conjugate control was set to 100%, and the value of each measured band was set with relation to the resulting percentage of staining to the control. Because of the normally semiquantitative analysis of the strips, the quantitative results were additionally set to a semiquantitative score: 1 (0.01–9.99%), 2 (10.00–39.99%), 3 (40–69.99%), 4 (≥70.00%). Strips of microIDent® test contain 2 mixed probes: one with *F. nucleatum* and *Fusobacterium periodonticum*; the second with *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, and *Capnocytophaga sputigena*. Two controls, one for amplification (detection of PCR inhibition) and a second for hybridization, are included.

For adjusting the methods (sensitivity), the following species have been used in a concentration of 10–100 000 000: *Porphyromonas gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, *A. actinomycetemcomitans* ATCC 33384, *Treponema denticola* ATCC 35405, *F. nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611, *Campylobacter rectus* ATCC 33238, *Eikenella corrodens* ATCC 23834, *Eubacterium nodatum* ATCC 33099, *Parvimonas micra* ATCC 33270, *Capnocytophaga gingivalis* ATCC 33624, *Capnocytophaga ochracea* ATCC 27872, *Capnocytophaga sputigena* ATCC 33612. Sensitivities for real-time PCR and microIDent® were 100–1000 and 1000–10 000, respectively. No cross-reactivity between the used species has been found. Furthermore, specificity of the microIDent® and the primers used in real-time PCR have previously been checked by database analysis (Ashimoto et al., 1996; Eick and Pfister, 2002; Tran and Rudney, 1999).

2.4. Data analysis

Differences in the numbers of positive samples were determined by using χ^2 test. Correlation was determined by using Spearman test. These 2 analyses were site-based, whereas other analysis was made subject-based. The changes in microbiological parameters between baseline and 3, 6 and 12 months were analyzed using the Friedman test. The Wilcoxon test was used to compare differences between baseline and each follow-up. Analyses were performed with statistical software (SPSS 13.0 for Windows, SPSS, Chicago, IL).

3. Results

Of the 28 patients who began the study, 3 had to be removed. One patient received therapy with antibiotics because of a general health problem; the other 2 patients did not complete all follow-up visits. In total, 300 subgingival plaque samples were analyzed.

3.1. Coincidence of real-time PCR with DNA-strip technology

Real-time PCR was more accident-sensitive than microIDent®kit resulting in re-runs. Furthermore, all negative results were confirmed in a second run.

The number of positive results was significantly ($P < 0.001$) higher for *Parvimonas micra*, *Campylobacter rectus*, and *Eubacterium nodatum* using real-time PCR and for *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia*, and *F. nucleatum* using strip technology. Nevertheless, samples detecting a high bacterial load by one method (>100,000 real-time PCR, score 4 microIDent) and negative by the other were always found in less than 2% of total samples.

The sensitivity of microIDent® using real-time PCR as reference ranged between 83.5% (*A. actinomycetemcomitans*) and 96.4% (*Porphyromonas gingivalis*) for the major pathogens and between 40.9% (*Eubacterium nodatum*) and 97.9% (*F. nucleatum*) for the others; the specificity for all was between 18.9% (*F. nucleatum*) and 97.3% (*Eubacterium nodatum*). The respective sensitivity values for the real-time PCR using microIDent® as reference are 42.5% (*Porphyromonas gingivalis*), 76.6% (*A. actinomycetemcomitans*), 79.2% (*F. nucleatum*), and 88.7% (*Eubacterium nodatum*); specificity ranged between 33.7% (*Capnocytophaga* sp.) and 93.8% (*Treponema denticola*) (Table 1).

All correlations between the 2 methods were highly significant; the correlation coefficients (R) of the 2 methods were between 0.62 (*Treponema denticola*) and 0.74 (*Porphyromonas gingivalis*) for the major pathogens and 0.33 (*Capnocytophaga* sp.) and 0.64 (*Prevotella intermedia*) for the others (Fig. 1, Table 1).

3.2. Real-time PCR in treatment of periodontitis

Analyzing real-time PCR-results, *A. actinomycetemcomitans* did not differ significantly between the different

Table 1
Correlation between real-time PCR and microIDent® using each one method as reference[a]

	Reference real-time PCR		Reference microIDent®		Spearman	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	*R*	*P*
A. actinomycetemcomitans	83.5	76.2	57.3	92.4	0.68	<0.001
Porphyromonas gingivalis	96.4	42.5	81.0	82.2	0.74	<0.001
Tannerella forsythia	88.4	66.0	72.5	84.9	0.69	<0.001
Treponema denticola	89.0	70.0	55.5	93.8	0.62	<0.001
Prevotella intermedia	77.6	82.8	88.7	68.6	0.64	<0.001
Parvimonas micra	44.8	91.6	84.1	62.4	0.53	<0.001
F. nucleatum	97.9	18.9	79.2	73.7	0.59	<0.001
Campylobacter rectus	57.2	82.0	86.9	48.0	0.63	<0.001
Eikenella corrodens	81.4	68.3	80.1	70.1	0.59	<0.001
Eubacterium nodatum	40.9	97.3	85.7	80.5	0.59	<0.001
Capnocytophaga sp.	49.1	69.0	80.9	33.7	0.33	<0.001

[a] Sensitivity of real-time PCR 1000–10,000 bacteria per sample, sensitivity microIDent® 1000 (*A. actinomycetemcomitans*)–10,000 (other species) per sample, checked by *Porphyromonas gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, *A. actinomycetemcomitans* ATCC 33384, *Treponema denticola* ATCC 35405, *F. nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611, *Campylobacter rectus* ATCC 33238, *Eikenella corrodens* ATCC 23834, *Eubacterium nodatum* ATCC 33099, *Parvimonas micra* ATCC 33270, *Capnocytophaga gingivalis* ATCC 33624, *Capnocytophaga ochracea* ATCC 27872, *Capnocytophaga sputigena* ATCC 33612, and clinical isolates.

sampling times. Only 3 months after treatment, an increase of that species was found. Contrary, the bacterial loads of the other major pathogens *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* differed clearly between time points. Compared to baseline, bacterial counts were reduced at each follow-up. Analysis of *F. nucleatum* and *Eikenella corrodens* resulted in significant differences between the sampling times. Lower numbers of *Eikenella*

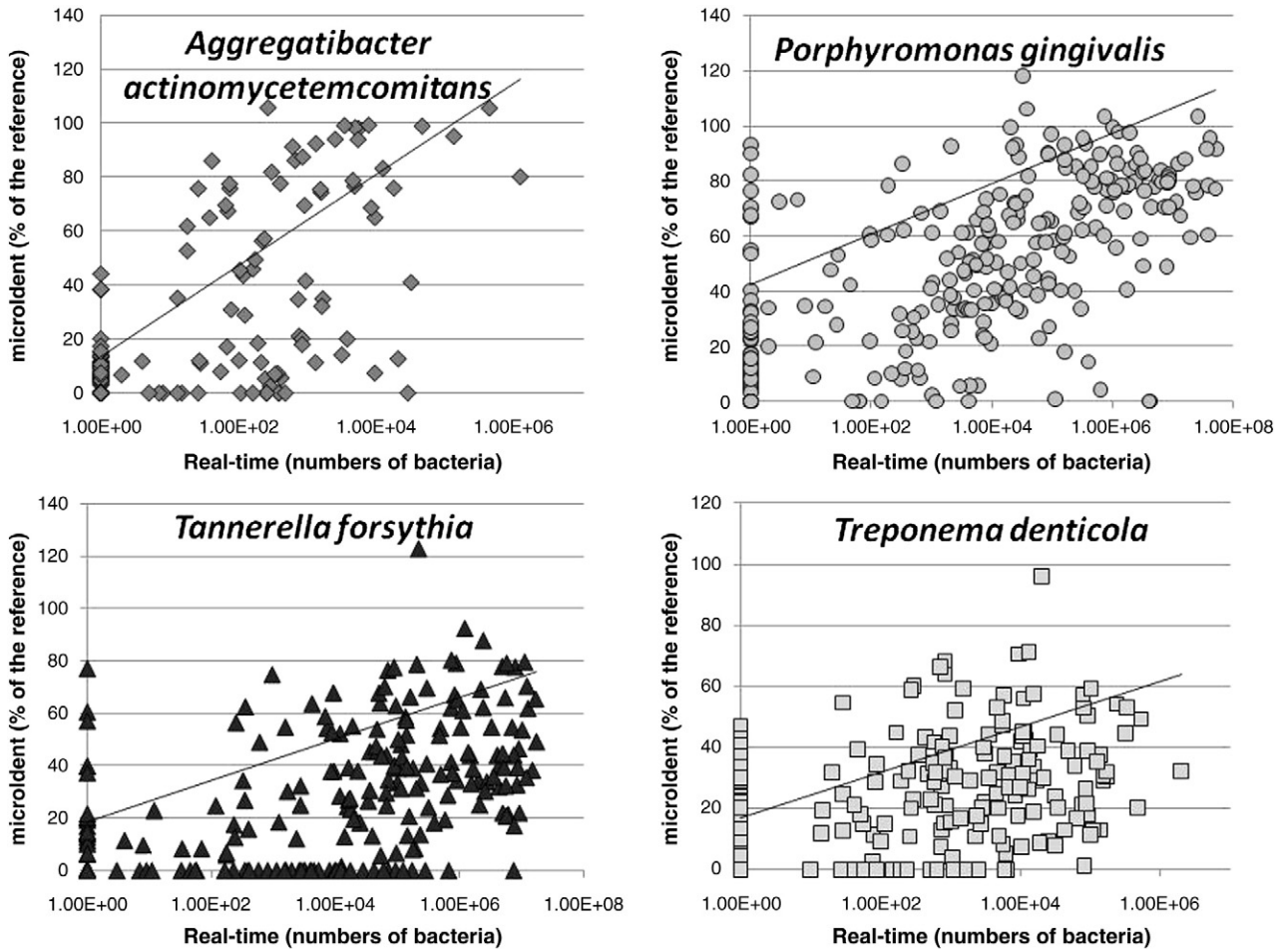

Fig. 1. Comparison of the loads of the major periodontopathogenic bacteria determined by real-time PCR (numbers of bacteria within plaque sample) and by DNA-strip technology (percentage of the densitometry reference). *P* and *R* values are shown in Table 1.

Table 2
Microbiological evaluation of treatment 3 months (t2), 6 months (t3), and 12 months (t4) after baseline (t1) by real-time PCR[a]

	Friedman test	Wilcoxon test (each compared with baseline t1)		
		t2	t3	t4
A. actinomycetemcomitans	0.132	**0.016**[b]	0.717	0.802
Porphyromonas gingivalis	**<0.001**	**0.007**	**<0.001**	**<0.001**
Tannerella forsythia	**<0.001**	**0.006**	**<0.001**	**<0.001**
Treponema denticola	**<0.001**	**0.001**	**<0.001**	**<0.001**
Prevotella intermedia	0.084	0.196	0.064	**0.001**
Parvimonas micra	0.591	0.159	**0.044**	0.088
F. nucleatum	**<0.001**	**0.001**	**0.012**	0.102
Campylobacter rectus	0.120	**0.033**	**0.002**	0.073
Eikenella corrodens	**0.026**	**0.024**	**0.012**	**0.004**
Eubacterium nodatum	0.080	**0.020**	**0.000**	**0.021**
Capnocytophaga sp.	0.422	0.067	0.118	0.773

[a] *P* values—subject-based (significant differences are in bold) obtained from 25 patients at each time point; results of the first 4 species are presented in Fig. 2.
[b] Increased numbers were found after treatment compared to baseline; all others are lowered.

corrodens and *Eubacterium nodatum* were counted up to 12 months after baseline; decreases of counts of *Parvimonas micra*, *F. nucleatum*, and *Campylobacter rectus* were only temporary. *Prevotella intermedia* was found to be reduced only 12 months after baseline. The summarized numbers of *Capnocytophaga* did not change at any time (statistics, Table 2; results of the major pathogens, Fig. 2). Analyzing the species of *Capnocytophaga* separately, the levels of *Capnocytophaga gingivalis* were lower 3 and 6 months after treatment ($P = 0.034$ and $P = 0.021$, respectively), and those of *Capnocytophaga ochracea* at the 6 months follow-up ($P = 0.001$) (data not shown).

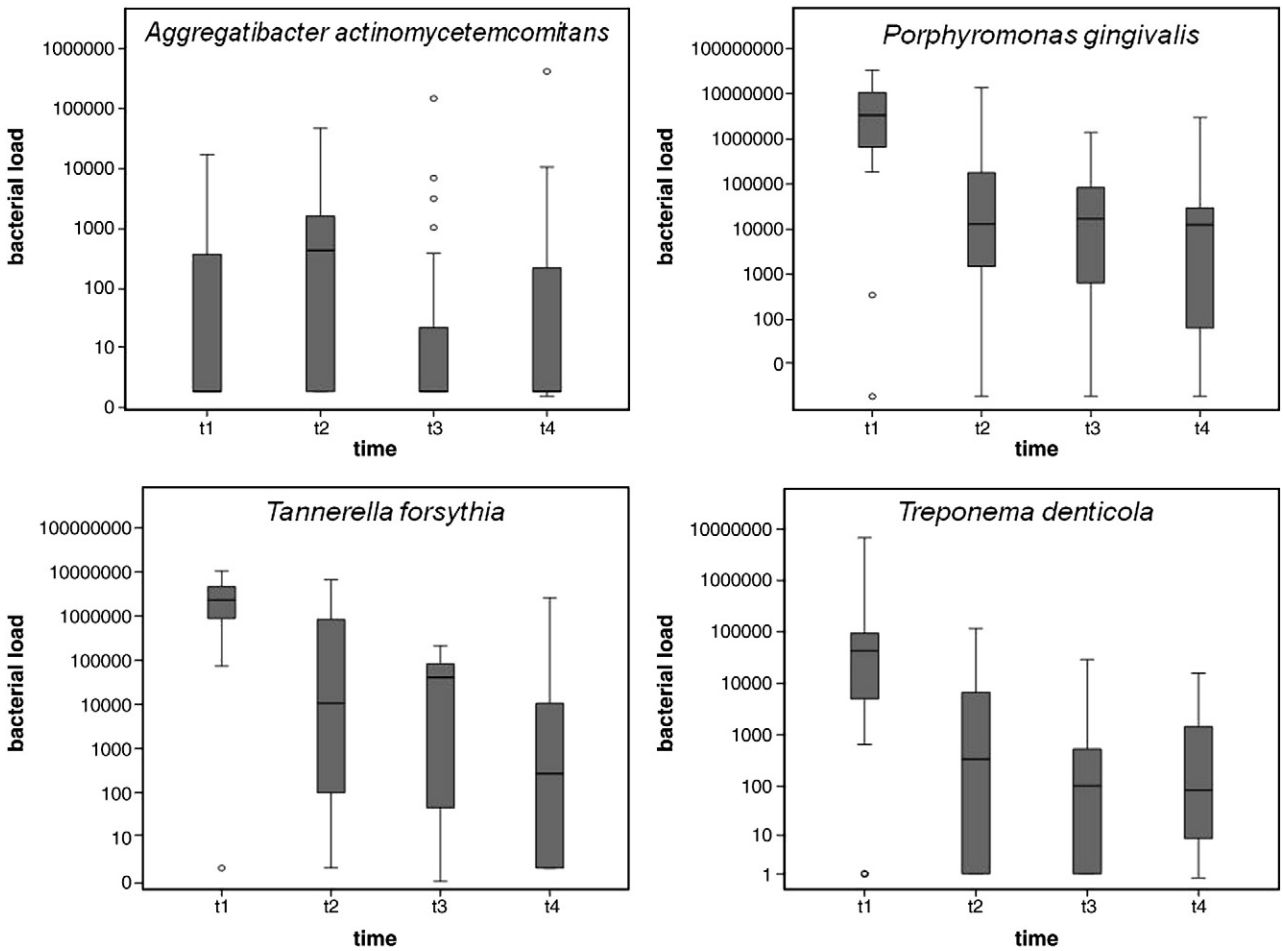

Fig. 2. Numbers of the major periodontopathogens within plaque samples (expressed as median including quartiles) determined by real-time PCR at different times of periodontitis treatment (t1: baseline, t2: 3 months, t3: 6 months, t4: 12 months after treatment; each time 75 samples). *P* values are shown in Table 2.

Table 3
Microbiological evaluation of treatment by DNA-strip technology (semiquantitative analysis) 3 months (t2), 6 months (t3), and 12 months (t4) after baseline (t1)[a]

	Friedman test	Wilcoxon test (each compared with baseline t1)		
		t2	t3	t4
A. actinomycetemcomitans	**0.004**	**0.007**[b]	0.536	0.138
Porphyromonas gingivalis	**<0.001**	**0.012**	**<0.001**	**<0.001**
Tannerella forsythia	**<0.001**	**0.007**	**0.001**	**<0.001**
Treponema denticola	**0.005**	0.227	**0.001**	**0.001**
Prevotella intermedia	0.071	0.924	**0.021**	**0.020**
Parvimonas micra	**0.001**	**0.011**	**0.005**	**0.000**
F. nucleatum	**0.003**	**0.001**	**0.001**	**0.001**
Campylobacter rectus	**0.001**	**0.001**	**<0.001**	**0.001**
Eikenella corrodens	**0.006**	0.716	**0.020**	**0.002**
Eubacterium nodatum	**0.010**	**0.015**	**0.002**	**0.001**
Capnocytophaga sp.	**0.048**	**0.027**	**0.024**	**0.002**

[a] *P* values—subject-based (significant differences are in bold) obtained from 25 patients at each time point; results of the first 4 species are presented in Fig. 3.
[b] Increased load was found after treatment compared to baseline; all others are lowered.

3.3. Semiquantitative strip technology in treatment of periodontitis

Analysis of semiquantitative microIDent® results showed significant differences between all time points for all species except for *Prevotella intermedia*. Compared to baseline, the bacterial loads were lower for *Porphyromonas gingivalis*, *Tannerella forsythia*, *Parvimonas micra*, *F. nucleatum*/*periodonticum*, *Campylobacter rectus*, *Eubacterium nodatum,* and *Capnocytophaga* sp. at all follow-ups. As in real-time results, *A. actinomycetemcomitans* was elevated 3 months after baseline. *Treponema denticola*, *Prevotella intermedia*, and *Eikenella corrodens* were found to be at lower loads at

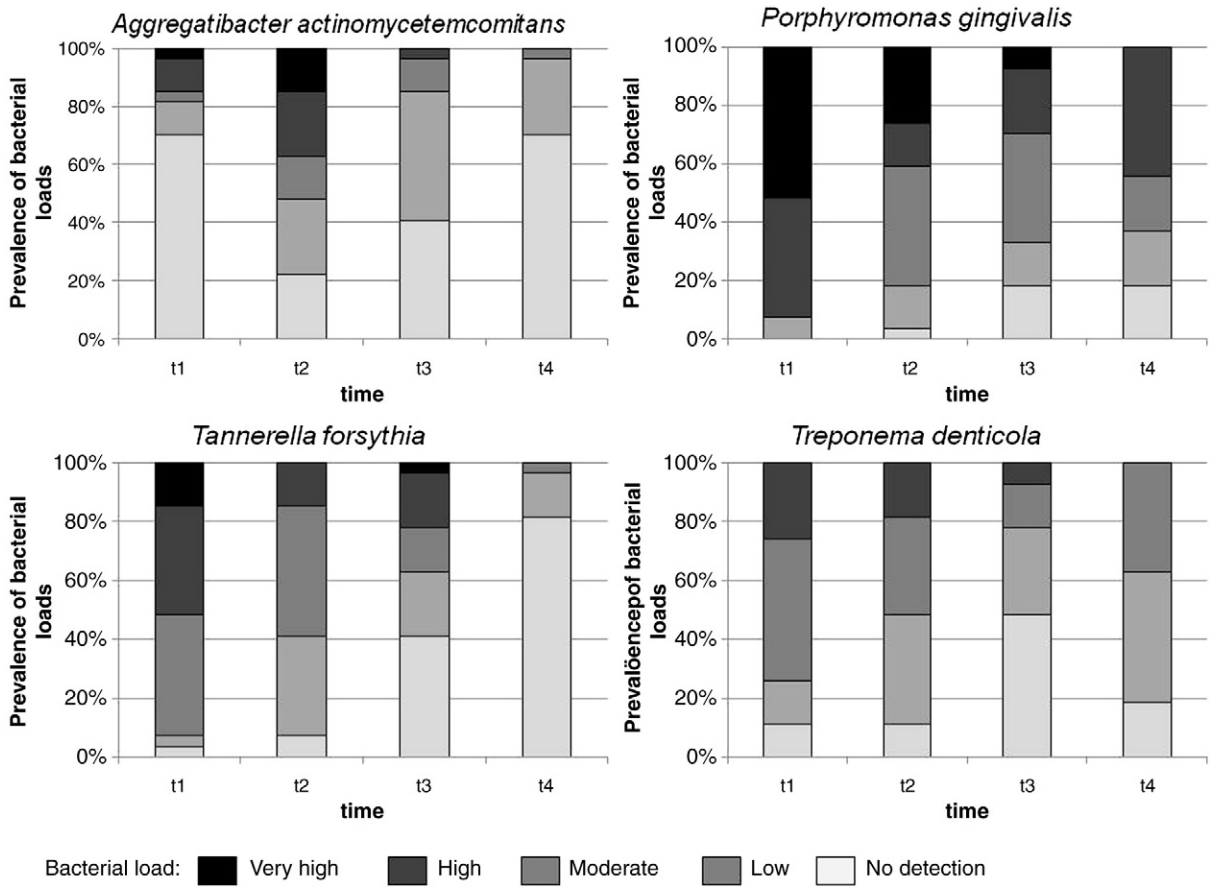

Fig. 3. Semiquantitative load (determined by percentage of relative of densitometry controls; low: 0.01–9.99%, moderate: 10.00–39.99%, high: 40–69.99%, very high: ≥70.00%) of periodontopathogens determined by DNA-strip technology at different times of periodontitis treatment (t1: baseline, t2: 3 months, t3: 6 months, t4: 12 months after treatment; each time 75 samples). *P* values are shown in Table 2.

the 6- and 12-month follow-ups (statistics Table 3, results of the major pathogens Fig. 3).

4. Discussion

Subgingival microflora consists of more than 300 different species that can be detected by techniques such as quantitative 16S rDNA cloning and sequencing (Kumar et al., 2006) as well as by microarrays (Colombo et al., 2009). Checkerboard hybridization technique using DNA from subgingival plaque and crossing with whole genomic DNA probes for mostly 40 species were also used (Haffajee et al., 2008; Socransky et al., 1994; Socransky et al., 1998; Socransky et al., 2004). Because of the methodology, cross-reactions cannot be completely excluded between closely related taxa (Socransky et al., 2004). These methods are useful to understand complexity and shifts in microbial profiles according to disease, but it is still difficult to validate these data for a single patient. These techniques present limitations in detecting all colonizers of a periodontal pocket (Shaddox and Walker, 2009) as it might be difficult to validate sensitivity and specificity of microbial analysis for clinical signs of periodontitis. Enumeration of well-known pathogens helps to characterize the patient's form of disease. In clinical trials, identification of *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola,* and so on, is used to analyze periodontal therapy (Christodoulides et al., 2008; Guentsch et al., 2008). Microbiological findings are considered to be important prognostic markers predicting whether attachment gain will be stable or not (Nieminen et al., 1996).

A few studies compared different nucleic acid-based methods with culture. In general, the PCR-based methods showed a higher prevalence of relevant species (Jervoe-Storm et al., 2005; Verner et al., 2006). Nevertheless, the PCR technique is not without any limitation; different molecules found in periodontal pockets such as hemoglobin, lactoferrin, immunoglobulin G, and collagen are known to inhibit PCR (Hanioka et al., 2005; Jentsch et al., 2004; Radstrom et al., 2004). Recently, mutational-altered DNA taq polymerase was synthesized to overcome the problem (Kermekchiev et al., 2009).

There was a significant correlation between microIDent® and real-time PCR; the accordance of the results reached a maximal coefficient (R) of 0.74. This is higher than in another study that reached a coefficient of 0.58 when they compared checkerboard and microIDent® in 350 samples (Haffajee et al., 2009). In our study, the same DNA was used for 2 methods that allowed direct comparison. Nevertheless, the correlation was not close to 1.00. Our in-house real-time PCR uses Sybr Green I, which binds nonspecifically to double-stranded DNA. This cheap, widely used method has limitations, such as limited dye stability as well as dye-dependent PCR inhibition (Monis et al., 2005). To prevent dye instability, we added the dye directly to the PCR buffer; thus, inhibition of the PCR cannot be excluded in several cases. Samples showing a low signal in all real-time PCRs compared to the microIDent® results have led to the conclusion that constituents of the subgingival plaque such as host proteins interact with Sybr Green I. A later control of these samples by spiking them with *A. actinomycetemcomitans* ATCC 33384 to inhibit PCR did not support this suggestion. Contrary, in very few cases, major periodontopathogens such as *A. actinomycetemcomitans* and *Porphyromonas gingivalis* were not detected by means of the microIDent® tests, which were found in high numbers in real-time PCRs. Mostly, other samples of the same patient were positive, suggesting that the problem was sample-specific and might be associated with the multiplex PCR, which is made in microIDent® analysis first.

A. actinomycetemcomitans is cultivable using selective trypic soy-serum-bacitracin-vancomycin (TSBV) agar; the accordance of culture with PCR was found to be good in a few reports (Eick and Pfister, 2002; Riggio et al., 1996). Others described more discrepancies, so a study comparing checkerboard with culture found only 20% of the detected cultured *A. actinomycetemcomitans* by checkerboard and 26% of the detected *A. actinomycetemcomitans* using checkerboard by culture (Papapanou et al., 1997). If *A. actinomycetemcomitans* is detected in severe cases of aggressive periodontitis, an adjunctive usage of an antibiotic along with mechanical debridement is recommended (Walker and Karpinia, 2002). High levels of that species before SRP might be a negative predictor of treatment outcome (Fujise et al., 2002). Therefore, correct identification of that species is essential for treatment schedule and evaluation. In our study, the general positive accordance between our used methods may indicate this. Nevertheless, it should be noted that an increase of that species after SRP was found in the study population. *A. actinomycetemcomitans* is more frequently prevalent in aggressive periodontitis (Schacher et al., 2007).

Also, a good correlation for *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* was found between microIDent® and real-time PCR. Numbers of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* exist concomitantly in subgingival biofilms and are increased in sites with higher probing depths and bleeding on probing (Mineoka et al., 2008). Residual coexistence of *Porphyromonas gingivalis* and *Tannerella forsythia* and high levels of *Porphyromonas gingivalis* are more frequent in sites with less reduction and bleeding on probing after SRP (Fujise et al., 2002). Combination of high salivary MMP-8 and *Treponema denticola* in subgingival plaque displays a robust characteristic in predicting periodontal disease severity (Ramseier et al., 2009). Thus, these species belong to the major pathogens, which should be detected in each microbial analysis of periodontitis.

In addition, the correlation coefficient was found to be >0.5 between the 2 used nucleic acid-based methods for all other analyzed species except for that of *Capnocytophaga*

sp. Mostly, a clear decrease of the bacterial loads was observed after treatment. *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens* as well as *F. nucleatum*, *Prevotella intermedia*, and *Parvimonas micra* (Edwardsson et al., 1999) are known to be associated with clinical periodontal disease status (Booth et al., 2004; Edwardsson et al., 1999; Haffajee et al., 2009; Rams et al., 1996). We did not find lower levels of *Capnocytophaga* sp. using real-time PCR after treatment, resulting from the unchanged numbers of *Capnocytophaga sputigena*. Confirming the report by others (Salari and Kadkhoda, 2004), *Capnocytophaga sputigena* was present in higher numbers than *Capnocytophaga gingivalis* in subgingival plaque. This may be because microIDent® uses a mixed probe for *Capnocytophaga*, which might be more sensitive for *Capnocytophaga gingivalis*. It might be favorable to use *F. nucleatum* and *Capnocytophaga gingivalis* as representatives of fusobacteria and *Capnocytophaga* instead of mixed probes in microbial analysis of periodontitis.

Periodontopathogens are found in low numbers also in periodontally healthy subjects (Lau et al., 2004; Mineoka et al., 2008); therefore, using a threshold might be helpful. According to the relevance, microIDent® uses 10^3 for *A. actinomycetemcomitans* and 10^4 for the other species included in the test systems. Both methods showed the changed numbers of periodontopathogens after treatment. In part, the difference was more visible using microIDent® analysis probably resulting from the used threshold and the semiquantitative analysis. Semiquantification might mask small changes after treatment. Furthermore, both methods are based on conservative 16SrDNA, where an exact enumeration of bacterial numbers is impossible because each bacterial cell may contain a variable amount of this molecule, so in part search of primers based on single gene sequence is recommended (Morillo et al., 2003).

The treatment schedule did not allow any intervention depending on the microbial analysis. To include this into a study protocol would strengthen the importance of microbial diagnosis in periodontitis treatment. For example, the only application of metronidazole is suggested in periodontitis with high loads of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, but not in an *A. actinomycetemcomitans*-associated disease (Shaddox and Walker, 2009).

Using in-house real-time PCR as a cheap method should be restricted for large studies. Semiquantitative DNA-strip technology is more suitable for microbial analysis in individual diagnosis, treatment schedule, and control of periodontitis patients.

Acknowledgment

We are grateful to Claudia Ranke (University Hospital Jena) for excellent assistance in performing the assays. We are indebted to Richard Miron (University of Bern) for language corrections. This study was supported by Hain Lifescience, Nehren, Germany. The authors declare that they have no conflicts of interest.

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 Microbiol Immunol* 11:266–273.

Booth V, Downes J, Van den Berg J, Wade WG (2004) Gram-positive anaerobic bacilli in human periodontal disease. *J Periodontal Res* 39:213–220.

Christodoulides N, Nikolidakis D, Chondros P, Becker J, Schwarz F, Rossler R, Sculean A (2008) Photodynamic therapy as an adjunct to non-surgical periodontal treatment: a randomized, controlled clinical trial. *J Periodontol* 79:1638–1644.

Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, Socransky SS, Hasturk H, Van Dyke TE, Dewhirst F, Paster BJ (2009) Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol* 80:1421–1432.

Dahlen G (2006) Microbiological diagnostics in oral diseases. *Acta Odontol Scand* 64:164–168.

Edwardsson S, Bing M, Axtelius B, Lindberg B, Soderfeldt B, Attstrom R (1999) The microbiota of periodontal pockets with different depths in therapy-resistant periodontitis. *J Clin Periodontol* 26:143–152.

Eguchi T, Koshy G, Umeda M, Iwanami T, Suga J, Nomura Y, Kawanami M, Ishikawa I (2008) Microbial changes in patients with acute periodontal abscess after treatment detected by PadoTest. *Oral Dis* 14:180–184.

Eick S, Pfister W (2002) Comparison of microbial cultivation and a commercial PCR based method for detection of periodontopathogenic species in subgingival plaque samples. *J Clin Periodontol* 29:638–644.

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. *J Periodontol* 73:1253–1259.

Guentsch A, Jentsch H, Pfister W, Hoffmann T, Eick S (2008) Moxifloxacin as an adjunctive antibiotic in the treatment of severe chronic periodontitis. *J Periodontol* 79:1894–1903.

Haffajee AD, Patel M, Socransky SS (2008) Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis. *Oral Microbiol Immunol* 23:148–157.

Haffajee AD, Yaskell T, Torresyap G, Teles R, Socransky SS (2009) Comparison between polymerase chain reaction-based and checkerboard DNA hybridization techniques for microbial assessment of subgingival plaque samples. *J Clin Periodontol* 36:642–649.

Hanioka T, Matsuse R, Shigemoto Y, Ojima M, Shizukuishi S (2005) Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid. *J Periodontal Res* 40:331–338.

Holt SC, Ebersole JL (2005) *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontology* 38:72–122.

Jentsch H, Sievert Y, Gocke R (2004) Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. *J Clin Periodontol* 31:511–514.

Jervoe-Storm PM, Koltzscher M, Falk W, Dorfler A, Jepsen S (2005) Comparison of culture and real-time PCR for detection and quantification of five putative periodontopathogenic bacteria in subgingival plaque samples. *J Clin Periodontol* 32:778–783.

Kermekchiev MB, Kirilova LI, Vail EE, Barnes WM (2009) Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res* 37:e40.

Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL (2006) Changes in periodontal health status are associated with bacterial

community shifts as assessed by quantitative 16S cloning and sequencing. *J Clin Microbiol* 44:3665–3673.

Lau L, Sanz M, Herrera D, Morillo JM, Martin C, Silva A (2004) 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* 31:1061–1069.

Lyons SR, Griffen AL, Leys EJ (2000) Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J Clin Microbiol* 38:2362–2365.

Mineoka T, Awano S, Rikimaru T, Kurata H, Yoshida A, Ansai T, Takehara T (2008) Site-specific development of periodontal disease is associated with increased levels of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* in subgingival plaque. *J Periodontol* 79:670–676.

Mombelli A, Gmur R, Gobbi C, Lang NP (1994) *Actinobacillus actinomycetemcomitans* in adult periodontitis. II. Characterization of isolated strains and effect of mechanical periodontal treatment. *J Periodontol* 65:827–834.

Monis PT, Giglio S, Saint CP (2005) Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Anal Biochem* 340:24–34.

Morikawa M, Chiba T, Tomii N, Sato S, Takahashi Y, Konishi K, Numabe Y, Iwata K, Imai K (2008) Comparative analysis of putative periodontopathic bacteria by multiplex polymerase chain reaction. *J Periodontal Res* 43:268–274.

Morillo JM, 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*. *J Periodontal Res* 38:518–524.

Nieminen A, Asikainen S, Torkko H, Kari K, Uitto VJ, Saxen L (1996) Value of some laboratory and clinical measurements in the treatment plan for advanced periodontitis. *J Clin Periodontol* 23:572–581.

Papapanou PN, Madianos PN, Dahlen G, Sandros J (1997) "Checkerboard" versus culture: a comparison between two methods for identification of subgingival microbiota. *Eur J Oral Sci* 105:389–396.

Radstrom P, Knutsson R, Wolffs P, Lovenklev M, Lofstrom C (2004) Pre-PCR processing: strategies to generate PCR-compatible samples. *Mol Biotechnol* 26:133–146.

Rams TE, Listgarten MA, Slots J (1996) Utility of 5 major putative periodontal pathogens and selected clinical parameters to predict periodontal breakdown in patients on maintenance care. *J Clin Periodontol* 23:346–354.

Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, Shelburne CA, Rayburn LA, Tran HM, Singh AK, Giannobile WV (2009) Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol* 80:436–446.

Riggio MP, Macfarlane TW, Mackenzie D, Lennon A, Smith AJ, Kinane D (1996) Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. *J Periodontal Res* 31:496–501.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Krawetz MS, Ed. Totowa, NJ: Humana Press, pp. 365–386.

Rudney JD, Chen R, Pan Y (2003) Endpoint quantitative PCR assays for *Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*. *J Periodontal Res* 38:465–470.

Salari MH, Kadkhoda Z (2004) Rate of cultivable subgingival periodontopathogenic bacteria in chronic periodontitis. *J Oral Sci* 46: 157–161.

Savitt ED, Strzempko MN, Vaccaro KK, Peros WJ, French CK (1988) Comparison of cultural methods and DNA probe analyses for the detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, and *Bacteroides intermedius* in subgingival plaque samples. *J Periodontol* 59:431–438.

Schacher B, Baron F, Rossberg M, Wohlfeil M, Arndt R, Eickholz P (2007) *Aggregatibacter actinomycetemcomitans* as indicator for aggressive periodontitis by two analysing strategies. *J Clin Periodontol* 34:566–573.

Shaddox LM, Walker C (2009) Microbial testing in periodontics: value, limitations and future directions. *Periodontology* 50:25–38.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent Jr RL (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134–144.

Socransky SS, Haffajee AD, Smith C, Martin L, Haffajee JA, Uzel NG, Goodson JM (2004) Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. *Oral Microbiol Immunol* 19:352–362.

Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE (1994) "Checkerboard" DNA–DNA hybridization. *Biotechniques* 17:788–792.

Tran SD, Rudney JD (1999) Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. *J Clin Microbiol* 37:3504–3508.

Verner C, Lemaitre P, Daniel A, Giumelli B, Lakhssassi N, Sixou M (2006) Carpegen real-time polymerase chain reaction vs. anaerobic culture for periodontal pathogen identification. *Oral Microbiol Immunol* 21:341–346.

Walker C, Karpinia K (2002) Rationale for use of antibiotics in periodontics. *J Periodontol* 73:1188–1196.