Periodontal pathogens: A quantitative comparison of anaerobic culture and real-time PCR

Khalil Boutaga a, Arie Jan van Winkelhoff a, Christina M.J.E. Vandenbroucke-Grauls b, Paul H.M. Savelkoul b,∗

a Department of Oral Microbiology, Academic Center for Dentistry Amsterdam, VU University Medical Center Amsterdam, Amsterdam, The Netherlands
b Universiteit van Amsterdam and Vrije Universiteit, Department of Medical Microbiology and Infection Control, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands

Received 1 February 2005; received in revised form 21 March 2005; accepted 25 March 2005
First published online 5 May 2005

Abstract
Periodontitis is a multi-factorial chronic inflammatory and destructive disease of the tooth-supporting tissues. Quantitative anaerobic culture techniques have been used for microbial diagnosis of the different forms of the disease. The aim of this study was to compare real-time PCR with quantitative anaerobic culture for detection and quantification of 5 prominent periodontal pathogens. Real-time PCR assays with the 16s rRNA genes of Actinobacillus actinomycetemcomitans, Prevotella intermedia, Tannerella forsythensis, Peptostreptococcus micros and Fusobacterium spp. were developed. The PCR was validated on pure cultures of various bacterial strains. Subsequently, subgingival plaque samples from 259 adult patients with periodontitis were analyzed with quantitative anaerobic culture and real-time PCR. A standard curve for DNA quantification was created for each primer-probe set based on colony-forming units equivalents.
All bacterial species were correctly identified. The lower limits of detection by PCR varied between 1–50 colony-forming units equivalents depending on the species. No cross-reactivities with heterologous DNA of other bacterial species were observed.
Real-time PCR results showed a high degree of agreement with anaerobic culture results. Real-time PCR is a reliable alternative for diagnostic quantitative anaerobic culture of subgingival plaque samples.
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Keywords: Periodontitis; Subgingival plaque; Quantitative real-time PCR; Anaerobic culture

1. Introduction
The human oral cavity is colonized by at least 300 different bacterial species [1,2], most of which are innocuous. Colonization into the subgingival plaque by certain species can lead to infection of the periodontium resulting in gingivitis and periodontitis [3,4]. The subgingival microflora in deepened periodontal pockets is dominated by Gram-negative anaerobic rods and spirochetes [1,3,5]. Actinobacillus actinomycetemcomitans [6], Porphyromonas gingivalis [7–9], Tannerella forsythensis [10,11], Prevotella intermedia [12,13] and Peptostreptococcus micros [14–16] are strong markers of periodontitis in adults and these species have been linked to progression of the disease. Fusobacterium spp. are associated with chronic and acute gingivitis and occur in high prevalence in periodontitis. Fusobacteria show pathogenic synergism with other bacteria [17] and they are able to aggregate with other pathogens in the
periodontal pocket [18]. Strict anaerobic bacteria most often require special techniques for detection and identification due to their stringent growth requirements.

As technology advances, more sensitive techniques based on DNA-amplification such as the Polymerase Chain Reaction, have been developed. Recently, we compared real-time PCR and anaerobic culture for the detection and quantification of P. gingivalis in patients suffering from periodontitis [19]. Real-time PCR confirms the results obtained with quantitative culture of P. gingivalis but offers significant advantages with respect to rapidity and sensitivity.

The aim of the present study was to investigate the prevalence and numbers of several prominent oral pathogens including A. actinomycetemcomitans, P. intermedia, T. forsythensis, P. micros and Fusobacterium spp. in adult patients with periodontitis by real-time PCR and compare these by using anaerobic culture.

2. Materials and methods

2.1. Study population and sample collection

Subgingival plaque samples from 259 adult patients with periodontitis were collected. Patients were >25 years old, had periodontal pockets >5 mm (mean pockets depth = 6.97 ± 1.18 mm) that showed bleeding upon pocket probing. Patients had not used antibiotics in the past 3 months.

Samples were obtained from the deepest periodontal pocket in each quadrant of the dentition by using sterile paper points [20]. The samples were pooled in 1.5 ml Reduced Transport Fluid (RTF) [21] and processed for anaerobic cultivation within 4 h after sampling. Upon arrival samples were vortexed for 2 min and divided: 100 µl was used to prepare 10-fold serial dilutions in sterile phosphate-buffered saline (PBS) solution for culturing, and 100 µl was used for real-time PCR testing. The remaining 1.3 ml was stored at −20 °C.

2.2. Microbiological procedures

2.2.1. Identification of anaerobic isolates

For anaerobic culture 100 µl of appropriate 10-fold dilutions were plated on blood agar plates (Oxoid no. 2, Basingstoke, UK) that were supplemented with horse blood (5% v/v), hemin (5 mg l⁻¹) and menadione (1 mg l⁻¹) and incubated in 80% N₂, 10% H₂ and 10% CO₂, at 37 °C for 7 up to 14 days. P. intermedia, P. micros and Fusobacterium spp. were identified on the basis of colony morphology (stereo microscopy 4-6x), black pigment, Gram-stain, anaerobic growth, the inability to ferment glucose, indole production as well as the production of a set of metabolic enzymes (Rapid ID kit 32A) [22,23]. T. forsythensis was identified based on biochemical testing for α-glucosidase, β-glucosidase, sialidase activities and negative indole production as described by Brahman et al. [24]. A. actinomycetemcomitans was grown on trypticase soy-serum-bacitracin-vancomycin (TSBV) plates and incubated at 37 °C in air + 5% CO₂ for 3 days [25]. The identification of A. actinomycetemcomitans was based on its characteristic colony morphology (star-like inner nucleus structure) and a positive catalase reaction with 3% hydrogen peroxide. The total number of colony forming units (CFU) per sample was determined.

2.2.2. Growth of reference strains

Reference strains were grown as recommended by the American Type Culture Collection (ATCC). Determination of the number of CFU per milliliter of the bacterial suspensions was made by growing the bacteria 2–3 days in Brain Heart Infusion (BHI) supplemented with 5 mg l⁻¹ hemin and 5 mg l⁻¹ menadione, and plating serial dilutions as described above. The strains used in this study were: P. gingivalis (W83), T. forsythensis (clinical isolate), A. actinomycetemcomitans (NCTC 9710), P. intermedia (ATCC 25611), P. nigrescens (clinical isolate), P. micros (clinical isolate), Streptococcus sanguinis (clinical isolate), Bacteroides fragilis (ATCC 25285), Prevotella melaninogenica (ATCC 25845), Prevotella denticola (clinical isolate), Prevotella nigrescens (NCTC 9338), Porphyromonas endodontalis (clinical isolate), Bacteroides asaccharolyticus (clinical isolate), Prevotella oralis (clinical isolate), Haemophilus influenzae (clinical isolate), Haemophilus aphrophilus (clinical isolate), Haemophilus parainfluenzae (clinical isolate), Haemophilus paraphrophilus (clinical isolate), Fusobacterium nucleatum (clinical isolate), Fusobacterium polymorphum (FDC 397) and 5 additional clinical isolates of Fusobacterium spp.

2.3. DNA isolation from plaque samples and bacterial reference cultures

From plaque samples and bacterial culture dilutions 100 µl was used for automated DNA extraction and purification with the MagNA Pure DNA Isolation Kit III (Bacteria, Fungi) (Roche Molecular Diagnostics, Almere, The Netherlands). The protocol included 1-h pretreatment with proteinase K (20 mg ml⁻¹) at 56 °C. After isolation DNA was eluted in 100 µl elution buffer.

The efficacy of the MagNA Pure DNA extraction was tested as described previously [19].

2.4. Validation of the real-time PCR

2.4.1. Design of PCR primers and probes

Table 1 shows the sequences of the primer/probe sets. The primer/probe set for T. forsythensis [26] and for
A.	extit{actinomycetemcomitans},

\textit{P. intermedia},

\textit{P. micros} and

\textit{Fusobacterium} spp. were designed as described before [19]. Selected primers and probes were checked by blast search for homology with unrelated sequences, NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/) [27].

2.4.2. Validation of the primer/probe sets for real-time PCR

The optimal concentrations of the forward and the reverse primers and the probe in the PCR volume were 300, 300 and 100 nM, respectively, for \textit{A. actinomycetemcomitans}; 300, 900 and 50 nM, respectively, for \textit{T. forsythensis}; 300, 900 and 200 nM, respectively, for \textit{P. intermedia}, 300, 900 and 100 nM, respectively, for \textit{P. micros}; 300, 300 and 300 nM, respectively, for \textit{Fusobacterium} spp.

The specificity of the probe and primer sets for their target DNA was tested with purified genomic DNA from the different bacterial reference strains.

The analytical sensitivity of each of the 5 real-time PCR sets was determined in triplicate with DNA isolated from \textit{A. actinomycetemcomitans}, \textit{T. forsythensis}, \textit{P. intermedia}, \textit{P. micros} and \textit{Fusobacterium} spp., respectively. Serial 10-fold dilutions of homologous DNA were used as standard curves.

PCR amplification was performed as described previously [19]. For quantification, the results from unknown plaque samples were projected on the standard curves of the target bacteria.

2.4.3. Inhibition

Inhibition of the PCR was tested by comparing the results of four separate reactions: (a) unspiked plaque sample, (b) plaque sample spiked with the target oral bacterium in quantities close to the threshold line, (c) plaque sample spiked with 1000 CFU of \textit{Escherichia coli}, and (d) 1000 CFU of \textit{E. coli} culture.

2.5. Genome sequence analysis

PCR amplification products were sequenced with the Big Dye terminator sequencing kit (Applied Biosystems, Foster city, CA, USA) on an ABI Prism 3100 sequencer (Applied Biosystems) following the manufacturer’s instructions. Alignment of the sequences was performed with the single-stranded rRNA sequences deposited in GenBank.

2.6. Statistics

Specificity, sensitivity, positive and negative predictive values of the real-time PCR assays were calculated with the anaerobic culture as the reference standard. Differences between the real-time PCR and the culture were tested by the Chi-square test. \(P\) values <0.05 were considered significant.

3. Results

3.1. Sensitivity and specificity of TaqMan assay

To determine the analytical sensitivity of the primer-probe set, serial dilutions of cultures were used. The sensitivity of the PCR for \textit{A. actinomycetemcomitans}, \textit{P. intermedia}, \textit{T. forsythensis}, \textit{P. micros} and \textit{Fusobacterium} was 1, 6, 1, 50 and 8 CFU, respectively. The correlation coefficient for the mean \(C_T\) values was \(R^2 \geq 0.999\).

All five TaqMan assays proved to be highly specific, amplifying only DNA extracted from...
3.4. Real-time PCR versus anaerobic culture

Table 2 shows the prevalence of the tested bacterial species in subgingival plaque samples from periodontitis patients as determined by culture and by PCR. The results of PCR and culture were congruent in 90.3%, 76.8%, 90.7%, 93.8% and 97.4% of the samples for A. actinomycetemcomitans, P. intermedia, T. forsythensis, P. micros and Fusobacterium spp., respectively. The main discrepancy between culture and PCR were culture-negative/PCR-positive samples which amounted to 7% for A. actinomycetemcomitans, 20% for P. intermedia, 7% for T. forsythensis, 6% for P. micros, and 0.8% for Fusobacterium spp. Culture-positive/PCR-negative results were observed in <3% of the subjects for A. actinomycetemcomitans, T. forsythensis, P. micros and Fusobacterium spp., and in 1.9% of the samples for P. intermedia.

PCR positive and culture negative results were further analysed. A number of samples contained <10⁴ CFU ml⁻¹ of the target species by PCR. This is under the detection limit of the culture, since every plaque sample was plated from a 10⁵ dilution, except for A. actinomycetemcomitans, for which undiluted plaque samples were plated.

For P. intermedia, 29 samples were positive by culture, but proved negative by PCR. We did not differentiate between P. intermedia and P. nigrescens in the culture assay. For P. intermedia, there was no cross-reaction with the related black-pigmented species P. nigrescens based on sequence alignments and PCR results. Oligonucleotides specific for P. nigrescens [28] were used to test the 29 samples. Twenty-four of the 29 samples produced a specific P. nigrescens amplicon of the predicted size (detected on agarose gel) (data not shown), while 5 samples remained negative. To gain more information about these 5 samples, a PCR with Prevotella genus primers [29,30] and eubacterial 16s rRNA oligonucleotides was performed and the products were sequenced. This was done to determine whether other species were present in the subgingival plaque samples. Sequencing identified 4 other

### Table 2

Comparison of detection of five periodontal pathogens in 259 periodontitis patients

<table>
<thead>
<tr>
<th>Anaerobic culture and PCR detected</th>
<th>No. of samples (%)</th>
<th>PCR (+)</th>
<th>Culture (+)⁵</th>
<th>Culture (−)</th>
<th>PCR (−)</th>
<th>Culture (+)</th>
<th>Culture (−)</th>
<th>Detection frequency (%) of the bacteria by culture</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. actinomycetemcomitans</td>
<td>51 (19.7)</td>
<td>51 (19.7)</td>
<td>20 (7.7)</td>
<td>1 (0.4)</td>
<td>5 (1.9)</td>
<td>183 (70.6)</td>
<td>5 (1.9)</td>
<td>21.6</td>
<td>27.4</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>160 (61.8)</td>
<td>119 (62.3)</td>
<td>55 (21.2)</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>39 (15)</td>
<td>183 (70.6)</td>
<td>63.7</td>
<td>83</td>
</tr>
<tr>
<td>T. forsythensis</td>
<td>211 (81.5)</td>
<td>119 (62.3)</td>
<td>20 (7.7)</td>
<td>4 (1.5)</td>
<td>5 (1.9)</td>
<td>24 (9.3)</td>
<td>183 (70.6)</td>
<td>83</td>
<td>89.2</td>
</tr>
<tr>
<td>P. micros</td>
<td>237 (91.5)</td>
<td>237 (91.5)</td>
<td>15 (5.8)</td>
<td>1 (0.4)</td>
<td>5 (1.9)</td>
<td>6 (2.3)</td>
<td>183 (70.6)</td>
<td>91.9</td>
<td>97.3</td>
</tr>
<tr>
<td>Fusobacterium spp.</td>
<td>248 (95.5)</td>
<td>248 (95.5)</td>
<td>4 (1.5)</td>
<td>2 (0.8)</td>
<td>5 (1.9)</td>
<td>24 (9.3)</td>
<td>183 (70.6)</td>
<td>96.5</td>
<td>97.3</td>
</tr>
</tbody>
</table>

⁵ Includes Prevotella intermedia and Prevotella nigrescens.

6 Five of the 29 samples which were PCR negative/culture positive.
**Prevention** species and one *Porphyromonas* species. This may suggest that culture is not always accurate in identifying the bacteria.

The upper part of Table 3 shows the sensitivity, specificity, positive and negative predictive values when culture was considered as the reference standard. For *A. actinomycetemcomitans, P. intermedia, T. forsythen- sis, P. micros* and *Fusobacterium* spp. the sensitivity was almost 100% (between 91% and 99%), the specificity between 28% and 90%, the positive predictive values were between 71% and 98% and the negative predictive values were between 71% and 97%. Considering real-time PCR as the reference standard, the sensitivity decreased in case of Aa and Pi, while staying almost the same for the other pathogens. The specificity increased and resulted in values between 71% and 97%, the positive predictive values were between 91% and 99% and the negative predictive values decreased and became between 28% and 90%.

Fig. 1 shows the relationship between the number of CFU and CFU-equivalents for the five bacterial species as determined by anaerobic culture and by real-time PCR. In 76%, 61%, 70%, 73% and 61% of the samples there was a difference <1-log between PCR and culture for respectively *A. actinomycetemcomitans, P. intermedia, T. forsythen- sis, P. micros* and *Fusobacterium* spp. In 20% to 30% of the samples, a 1- to 2-log difference in cell number was found. A difference of more than 2-log CFU’s was found in 2.8% to 15% of the samples.

Fig. 2 shows the correlation between all positive and negative results by both techniques. There was a reasonably good correlation of the positive and negative results between the PCR and culture technique. Comparison of the quantitative results only for samples positive by cul-
Identification of these organisms by culture and by PCR is difficult due to a high degree of genetic and phylogenetic similarity with other species. For example,
A. actinomycetemcomitans is genetically very closely related to Haemophilus aphrophilus, which shows cross-reactivity with some of the biochemical tests used to identify A. actinomycetemcomitans. P. intermedia and P. nigrescens are both endogenous to the oral cavity and are difficult to distinguish by biochemical tests; they show only 6% difference in their 16S rRNA genes. Despite this high degree of similarity, unique regions for application of real-time PCR were identified to detect the five species, without cross-reactivity.

Anaerobic culture has limitations compared to the PCR: it is time consuming and laborious, and it has a relatively low level of sensitivity.

In 20 samples, A. actinomycetemcomitans was detected by PCR and not by culture. This may be due to the lower analytical sensitivity of culture and the detection of DNA of both viable and non-viable bacteria by PCR. On the other hand, 5 samples were positive by culture but negative by PCR. These strains were not routinely stored so further analysis was not possible but it is most likely that the discrepancies may be due to incorrect biochemical identification of cultured colonies A. actinomycetemcomitans.

The prevalence of A. actinomycetemcomitans in this study was 22% by culture and 27% by PCR. Lee et al. [31] detected A. actinomycetemcomitans by PCR in 75% of aggressive periodontitis sites. However, Kamma et al. [32] and Mullanly et al. [33] detected A. actinomycetemcomitans only in 18.8% and 13.8% of 32 and 84 aggressive periodontitis sites, respectively. This variation in detection frequency may in part be explained by the use of different oligonucleotides and/or PCR conditions. In addition, variation in populations and other factors such as sample number, sample site and sample size may influence the detection frequency.

In 55 samples a positive PCR result for P. intermedia was associated with a negative culture. This was due to the detection limit of culture (<10^4) and in some cases possibly to the large amount of non-viable cells in the sample. Furthermore, 29 samples were PCR negative, but positive by culture; 24 samples yielded a positive signal with P. nigrescens specific primers. Sequence analysis determined Prevotella subspecies, Prevotella oris, Prevotella oralis and one Porphyromonas species as previously described [34]. Paster et al. reported the phylogeny of the Bacteroides subgroup and the validity of previously proposed schemes for reclassification of these bacteria, based on 16S rRNA sequences from strains of species of Bacteroides, Prevotella, Porphyromonas and related bacteria.

Techniques that do not require culturing and which differentiate between P. intermedia and P. nigrescens are available, but so far they have the disadvantage of yielding only qualitative [33,35–37], or semi-quantitative data [38,39]. Clearly, there is a need for a rapid and objectively quantitative procedure for the direct quantification of the two species in clinical samples to investigate their postulated roles in oral health and disease in more detail.

T. forsythensis was detected in 83% of the 259 subgingival plaque samples by culture and in 89% samples by PCR showing a good agreement of both techniques. This high prevalence was in agreement with earlier studies described by Leys et al. [40] and by Van Winkelhoff, et al. [23]: T. forsythensis was found in 83% and 90.5% of the tested periodontitis subjects respectively. The high prevalence shown in this study in periodontitis patients is clearly higher than the results obtained by Lau et al. [41]. In 20 samples, the PCR was positive for T. forsythensis, but negative by culture. This was completely due to the detection limit (as mentioned before) and the fastidious nature of this species.

In case of P. micros, a significant agreement was found when considering PCR-culture-positive samples. In 15 samples, the culture failed to detect P. micros, and in one occasion, P. micros could not be detected by PCR. The culture detection limit and DNA isolation efficiency respectively may play a role within these samples.

Fusobacterium spp. were more commonly detected than any other organism by both techniques (Table 2). In two cases, the PCR failed to detect and quantify the bacteria in the sample, which was due to the inhibition of the PCR.

Comparison of both techniques in this study showed a discrepancy ranging from 2% (Fusobacterium spp.) to 32.4% (P. intermedia) in favour of the PCR. With the exception of A. actinomycetemcomitans, all species were cultured on non-selective medium, which hampers the detection of small numbers of the organisms in the presence of a large number of background bacteria. The culture technique consistently resulted in a lower prevalence of the five periodontal pathogens.

PCR-positive/culture negative and PCR-negative/culture positive discrepancies negatively influence the sensitivity and specificity of the PCR for the tested oral bacteria. However <1-log difference was found between the PCR and culture in 70–78% of the samples.

When culture was used as the golden reference standard, PCR demonstrated a high sensitivity and positive predictive value in the detection of all 5 tested bacterial species. For P. micros the specificity was low, which was due to the PCR positive-culture negative results. In case of PCR is considered as the reference standard, the culture showed lower sensitivity, but high specificity and positive predictive value in the detection of the tested pathogens. These differences can be explained by the different detection limits.

In general, the differences between the PCR and culture may give new insights in the pathogenesis and the role of the periodontal bacteria in periodontitis. Since microbiological tests are used in treatment planning...
for patients who do not initially respond to conventional mechanical treatment, the results of the PCR may be a better guide for antibiotic therapy strategies for patients with severe and refractory periodontitis. From one hand to start an efficient antibiotic therapy in severe cases, from the other hand to monitor and evaluate the efficacy of the antibiotic treatment.

We found a high linear correlation between positive and negative results obtained by real-time PCR and by culture for all samples. This is in accordance with other studies [42–44]. The number of target bacteria CFU present in subgingival samples with at least $10^4$ CFU ml$^{-1}$ determined by real-time PCR correlated very well with the numbers of CFU determined by culture. This further confirms that the larger number of positive samples detected by PCR compared to the number detected by culture is due to the detection limit of the culture technique. A linear correlation calculated on the basis of the quantitative results for the positive samples by both techniques (Fig. 2) showed that there is also a quantification of the 5 periodontal pathogens tested. In general, the differences between the PCR and culture may give new insights in the pathogenesis and the role of the less-readily cultivated and uncultivable bacteria in periodontitis.

In conclusion, the real-time PCR is labour saving and well suited for rapid, specific and sensitive identification and quantification of the 5 periodontal pathogens. In general, the differences between the PCR and culture may give new insights in the pathogenesis and the role of the less-readily cultivated and uncultivable bacteria in periodontitis.

Acknowledgements

We thank Laboral Diagnostics and the Clinic for Periodontology Amsterdam for providing patient material. The present study was supported by the Interuniversity Dentistry Research School (IOT) Amsterdam.

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