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Reproducibility of a multiplex PCR-based method for the detection and semi-quantification of periodontopathogenic species in subgingival plaque samples



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Objective: The aim of the present study was to evaluate the reproducibility of a commercial kit for the detection and semi-quantification of five periodontopathogenic species.

Background: A commercial multiplex PCR-based method has recently been validated for the detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Prevotella intermedia*.

Study design: Sixty-five untreated sites with different probing pocket depths in 20 chronic periodontitis patients were sampled twice by simultaneously applying two sterile paper points. All samples were coded and separately analysed for the detection and semi-quantification of five periodontopathogenic species.

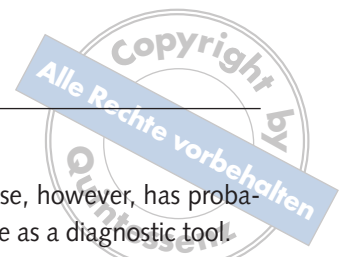
Results: The present study indicated acceptable reproducibility of the commercial test for *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis*: agreement varied between 70% and 79% in species-positive sites. In contrast, low agreement was found for *Actinobacillus actinomycetemcomitans* (47%) and *Prevotella intermedia* (57%). Data analysis indicated, however, that low agreement for these species might have been a consequence of the border line of the detection limit.

Conclusion: The results of this reproducibility study support the use of this multiplex PCR-based method for the detection and semi-quantification of periodontopathogens in subgingival plaque samples.

■ Introduction

Independent sources have reported that mainly Gram-negative anaerobes from the subgingival microflora represent the key aetiological factor of chronic and aggressive periodontitis¹⁻³. Based on the fulfilment of a number of criteria suggested by

Socransky⁴, *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) have been designated as true periodontopathogens⁵. In addition, moderately strong evidence of periodontal pathogenicity has been described for *Prevotella intermedia* (Pi), *Prevotella nigrescens*, *Peptostreptococcus micros*, *Fusobac-*



terium nucleatum, *Campylobacter rectus*, *Eubacterium nodatum* and various spirochetes such as *Treponema denticola* (*Td*)⁵. Their aetiological role is less evident since most of them are members of the resident oral microflora, being present in healthy as well as in diseased subjects. Other species, such as *Eikenella corrodens*, *Capnocytophaga* species, enteric rods, *Pseudomonas* species, *Staphylococcus* species and yeasts, have been suggested as possible periodontopathogens⁵. However, more research is needed to elucidate their true role in the aetiopathogenesis of periodontitis.

Different methods have been developed for the detection of periodontopathogenic species in subgingival plaque samples. Bacterial culturing is still considered as the gold standard when determining the value of a new microbial detection method. Indeed, culturing enables relative and absolute counts of cultured species to be obtained and furthermore, it is the only proper method to assess their antibiotic susceptibility⁵⁻⁷. However, culture techniques are labour-intensive, time-consuming and expensive. Moreover, only viable bacteria can be grown, making sampling and transport conditions crucial. In addition, sensitivity may be low with detection limits near 10⁴ cells⁸.

In an attempt to overcome some of these drawbacks, several culture-independent techniques have been proposed, essentially comprising immunological assays (enzyme-linked immunosorbent assay (ELISA), (in)direct immunofluorescent microscopy assays, membrane assays etc.), enzymatic methods (colour reaction based on the hydrolysis of *N*-benzoyl-DL-arginine-2-naphthylamide) and molecular methods of bacterial identification⁸. The principle of the latter resides in the recognition of specific bacterial DNA or RNA fragments. Whole-genomic nucleic acid probes for the detection of *Aa*, *Pg*, *Pi* and *Td* have become commercially available (DMDx[®], Omnigene, Cambridge, MA, USA). However, because of cross-reactivity with non-target bacteria leading to low sensitivity and specificity of this diagnostic test, oligonucleotide probes are currently preferred⁸. A unique DNA probe technique developed by Socransky et al⁹, particularly suitable for a rapid enumeration of large numbers of samples for up to 40 different species, is checkerboard DNA-DNA hybridisation¹⁰⁻¹³. The need for sophisticated labora-

tory equipment and expertise, however, has probably prevented its general use as a diagnostic tool.

A promising molecular method of bacterial identification introduced in 1985 is PCR (polymerase chain reaction) technology. Although conventional PCR (qualitative PCR) is a sensitive and specific technique, it has a number of limitations, especially when studying small quantities of DNA or rare DNA sequences^{6,14}. Furthermore, it is not suitable for the quantification of periodontopathogens¹⁵. However, the number of bacteria might be of clinical relevance because an association was described between bacterial counts above certain critical levels and disease recurrence^{16,17}. Needless to say, the mere presence of a periodontopathogen as determined by qualitative PCR holds, therefore, little clinical and therapeutic importance. In order to overcome some of these limitations, (real-time) quantitative PCR and multiplex PCR-based methods have been developed. Real-time quantitative PCR technology has been described as a reliable alternative for diagnostic anaerobic culture of subgingival plaque samples. Indeed, a number of studies showed a high level of agreement between these methods^{18,19}. Eick and Pfister²⁰ published similar results for a commercial multiplex PCR-based test (microDent[®] kit, Hain Lifescience GmbH, Nehren, Germany). In this diagnostic test the multiplex PCR of 16S rDNA is followed by a simultaneous reverse hybridisation for *Aa*, *Pg*, *Tf*, *Td* and *Pi*.

The goal of the present study was to evaluate the reproducibility of this commercial kit for the detection and semi-quantification of these five species.

■ Study design

■ Patient selection

Twenty chronic periodontitis patients (5 males and 15 females), otherwise systemically healthy subjects, aged between 33 and 71 years, were included in this study. Each patient had at least one bleeding pocket on probing per quadrant with a probing pocket depth (PPD) of ≥ 6 mm and clinical attachment loss of ≥ 3 mm. In addition, there was radiographic evidence of extended bone loss ($\geq 1/3$ of the root length). None of the volunteers had undergone any



form of periodontal treatment in the past. In addition, none of the participants had taken antibiotics for at least 4 months. Four smokers were enrolled.

■ Microbial sampling

Sixty-five experimental sites were sampled: 15 shallow pockets (PPD \leq 3 mm), 25 medium-deep pockets (4–6 mm PPD) and 25 deep pockets (PPD \geq 7 mm) were analysed. In each patient at least one medium-deep pocket and one deep pocket were sampled.

Following meticulous removal of supragingival calculus and plaque using sterile standard periodontal curettes and sterile cotton pellets, each experimental site was dried and isolated from water and saliva using cotton rolls. Subsequently, two sterile paper points (#50, 6163/45, Hain Lifescience GmbH, Nehren, Germany) were simultaneously inserted and left in place for 20 s. Thereupon, each sample was placed in a separate sterile vial and stored at 4°C. All 130 samples were delivered to the laboratory for analysis. Special attention was paid to blind the laboratory personnel by coding all vials. In addition, all analyses were performed without information on patient's demographics or clinical parameters.

■ Multiplex PCR and hybridisation

The method, as reported by Eick and Pfister²⁰, was used to identify *Aa*, *Pg*, *Tf*, *Td* and *Pi* in subgingival plaque samples. In short, DNA was extracted by using the High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany). Following incubation and centrifugation during 35 min, 5 μ l of DNA sample solution was isolated for PCR amplification. The latter was performed in a thermoblock using 45 μ l of a mixture containing 35 μ l of the microDent[®] primer-nucleotide mix, 5 μ l of PCR buffer, 5 μ l of 25 mM MgCl₂ and 1 U Taq polymerase. Following initial denaturation at 95°C during 5 min, amplification was conducted, comprising 10 cycles at 95°C for 30 s and at 60°C for 2 min, followed by 20 cycles at 95°C for 10 s, at 55°C for 30 s and at 72°C for 30 s. The final cycle was carried out at 72°C for 10 min. Thereupon, reverse hybridisation was performed: biotinylated amplicons were denatured and incubated at 45°C with hybridisation buffer and strips

with two control lines and five species-specific probes. Following the binding of these probes with the PCR products, any unspecifically bound DNA was removed. After streptavidin-conjugated alkaline phosphatase had been added, samples were washed. A substrate for alkaline phosphatase was added to visualise hybridisation products. Thereupon, the strips were left to dry between absorbing papers. Validated samples were used as controls.

The results were obtained on the basis of developed bands: for each subgingival plaque sample, the presence or absence of each of the five species was determined with a cut-off of 10³ genome equivalents. In case of detection, the test provided an estimation of bacterial counts (10³–10⁴ genome equivalents; 10⁵–10⁶ genome equivalents).

■ Statistical analysis

Data analysis was performed on a site level. Based on dichotomised data (0 = no detection of the pathogen; 1 = detection of the pathogen irrespective of bacterial counts), overall detection frequencies were calculated for each of the five periodontopathogens. Subsequently, the data were split up by their origin (shallow pockets, medium-deep pockets and deep pockets) and detection frequencies were once again calculated. In order to evaluate the level of agreement between subgingival plaque samples and their duplicates for the detection and semi-quantification of each of the pathogenic species, crosstabs were constructed using 3 scores: 0 = no detection of the pathogen; 1 = detection of the pathogen in low numbers (10³–10⁴ genome equivalents); 2 = detection of the pathogen in high numbers (10⁵–10⁶ genome equivalents). Agreement was expressed on a percentage scale. In addition, weighted kappa scores were calculated. Percentage agreement was also calculated in species-positive pockets.

■ Results

■ Detection frequencies

Table 1 shows the detection frequencies for each of the five periodontopathogens. Overall, *Aa* and *Pi* were infrequently detected (in 23% and 26% of the

**Table 1** Detection frequencies.

Pathogen	Overall detection frequency (%) ^a	Detection frequency in shallow pockets (≤ 3 mm) (%) ^b	Detection frequency in medium-deep pockets (4 to 6 mm) (%) ^c	Detection frequency in deep pockets (≥ 7 mm) (%) ^c
<i>Aa</i>	23	13	24	28
<i>Pg</i>	51	0	52	80
<i>Tf</i>	65	0	80	88
<i>Td</i>	57	7	64	80
<i>Pi</i>	26	0	32	36

^a Number of pockets = 65^b Number of pockets = 15^c Number of pockets = 25**Table 2** Reproducibility of oligonucleotide probe for the detection of *Aa*.

Detection by oligonucleotide probe		Detection by duplicate oligonucleotide probe			
		Score			Total
Score	0	0	1	2	
	0	50	4	0	54
	1	2	3	1	6
	2	0	1	4	5
	Total	52	8	5	65

Overall agreement = 88%; weighted kappa score = 0.72 ($p < 0.001$)

Agreement in case of detection = 47%

Table 3 Reproducibility of oligonucleotide probe for the detection of *Pg*.

Detection by oligonucleotide probe		Detection by duplicate oligonucleotide probe			
		Score			Total
Score	0	0	1	2	
	0	32	3	0	35
	1	2	3	1	6
	2	0	1	23	24
	Total	34	7	24	65

Overall agreement = 89%; weighted kappa score = 0.89 ($p < 0.001$)

Agreement in case of detection = 79%

sites respectively), whereas the other species were detected in more than half of the pockets. Scrutinising the data revealed that all pathogens were most frequently encountered in deep pockets and most rarely in shallow pockets. *Pg*, *Tf*, and *Pi* were not detected in the latter.

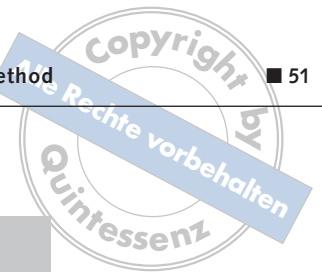
■ Reproducibility analysis

Tables 2, 3, 4, 5 and 6 show crosstabs for each of the periodontopathogens. The overall percentage agreements, ranging from 82% for *Tf* to 89% for *Pg* and *Pi*, and the weighted kappa scores, ranging from 0.72 for *Aa* to 0.89 for *Pg*, indicated high to excel-

lent agreement. However, when only considering species-positive sites, agreement levels were considerably lower. For *Pg* good agreement was still found (79%). For *Td* and *Tf* fair agreement was shown as percentage agreements were 70% and 71% respectively. There was low agreement for *Aa* (47%) and *Pi* (59%).

■ Discussion

The diagnostic and/or therapeutic additional value of microbiological testing for the management of patients with periodontitis remains controversial⁸.

**Table 4** Reproducibility of oligonucleotide probe for the detection of *Tf*.

Detection by oligonucleotide probe		Detection by duplicate oligonucleotide probe			Total
		Score			
Score	0	0	1	2	
	0	23	2	0	25
	1	2	9	4	15
	2	0	4	21	25
Total		25	15	15	65

Overall agreement = 82%; weighted kappa score = 0.81 ($p < 0.001$)

Agreement in case of detection = 71%

Table 5 Reproducibility of oligonucleotide probe for the detection of *Td*.

Detection by oligonucleotide probe		Detection by duplicate oligonucleotide probe			Total
		Score			
Score	0	0	1	2	
	0	28	2	0	30
	1	1	21	5	27
	2	0	3	5	8
Total		29	26	10	65

Overall agreement = 83%; weighted kappa score = 0.77 ($p < 0.001$)

Agreement in case of detection = 70%

Table 6 Reproducibility of oligonucleotide probe for the detection of *Pi*.

Detection by oligonucleotide probe		Detection by duplicate oligonucleotide probe			Total
		Score			
Score	0	0	1	2	
	0	48	3	0	51
	1	1	8	2	11
	2	0	1	2	3
Total		49	12	4	65

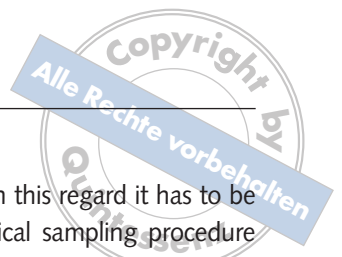
Overall agreement = 89%; weighted kappa score = 0.76 ($p < 0.001$)

Agreement in case of detection = 59%

However, three species (*Aa*, *Pg* and *Tf*) have been strongly associated with periodontal disease status, progression and unsuccessful therapy^{5,21}. *Pg*, *Tf* and *Td*, members of the red complex according to Socransky and co-workers²¹, were considered the most significant complex in periodontal disease progression²². These findings justify the need for accurate methods of bacterial identification and quantification.

The results of the present study relate to a commercial microbiological test based on multiplex PCR technology for the detection of five periodontopathogenic species (*Aa*, *Pg*, *Tf*, *Td* and *Pi*). This test has recently been validated, detecting two of the three

true periodontopathogens (*Pg* and *Tf*) more often than bacterial culturing. *Aa* was detected in similar numbers of samples by both methods²⁰. The objective of the present study was to evaluate reproducibility of this commercial kit for the detection and semi-quantification of periodontopathogenic species in subgingival plaque samples. Needless to say, reproducibility should be considered an important issue since it determines the reliability of test results. Knowing the kit is applied in European countries as an adjunctive diagnostic tool in daily clinical practice may further reinforce the importance of this paper. In addition, the test has recently been used in a study to examine the early microbial colonisation of dental



implants in patients with a history of aggressive periodontitis²³.

The results of this study indicated a positive correlation between the frequency of bacterial detection and PPD, which is in agreement with previous findings^{13,19,20,24–26}. Even though *Pg*, *Tf* and *Pi* were not identified in shallow pockets in this study, *Aa* and *Td* were detected. This is not surprising, considering that periodontopathogens may be frequently encountered, even in periodontally healthy subjects^{13,20,25}. In comparison with the study by Eick and Pfister²⁰, we detected less frequently *Aa* and *Pi* in deep pockets. This finding might be related to a difference in study population: in this report chronic periodontitis patients (33–71 years old) were selected, whereas in the study by Eick and Pfister²⁰, aggressive periodontitis patients aged between 18 and 35 years were studied. It is difficult to compare our data with those of other studies because there are a number of microbiological tests using PCR technology with different cut-offs.

With respect to the reproducibility of the microDent® kit, good to excellent overall agreement was found for all pathogens (at least 82%, weighted kappa 0.72). More important, however, is the reproducibility at species-positive sites, which was considerably lower. For *Aa* and *Pi* the results corresponded in only 47% and 59% of the cases respectively. These data may seem problematic; however, it has to be anticipated that these species were infrequently encountered, and in the case of detection it was mostly in low numbers. That is, *Aa* was only detected in 23% of the experimental sites, of which 58% were detected in 10³–10⁴ genome equivalents. Similarly, *Pi* was detected in 26% of the pockets, of which 77% were detected in low numbers. In contrast, *Pg* and *Tf* were identified in more than half of the pockets, mostly in high numbers (79% for *Pg* and 63% for *Tf*). These data indicate that the low agreement for *Aa* and *Pi* in pockets positive for these species may be a consequence of the borderline of the detection limit. Although a low cut-off for *Aa* was previously suggested because of its high pathogenicity^{27,28}, clinicians should be aware of these possible borderline cases when interpreting the results of the microDent® kit.

In order to ensure blinding of the laboratory personnel, two paper points were simultaneously inserted

into experimental pockets. In this regard it has to be acknowledged that the clinical sampling procedure itself using two paper points was a possible source of variation, which in turn may have negatively affected the results. Nonetheless, the fair to good levels of agreement between subgingival plaque samples and their duplicates for the detection and semi-quantification of periodontopathogens are indicative of acceptable reproducibility. A study by Aass and co-workers²⁹ showed less promising results for cultivation, indirect immunofluorescence and two commercial tests (EvaluSite®, Eastman Kodak, Rochester, NY, USA; and DMDx®, Omnigene, Cambridge, MA, USA), describing low reproducibility for all methods. However, it has to be anticipated that these results were based on double sample analysis with an interval of one week between both sampling procedures.

In conclusion, the results of the present study indicate acceptable reproducibility of a multiplex PCR-based method for the detection and semi-quantification of five periodontopathogenic species in subgingival plaque samples.

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