J Clin Periodontol 2002: 29: 638–644
Printed in Denmark . All rights reserved

Journal of Clinical Periodontology
0303-6979

Comparison of microbial cultivation and a commercial PCR based method for detection of periodontopathogenic species in subgingival plaque samples

Sigrun Eick and Wolfgang Pfister

Department of Microbiological Microbiology, University Hospital of Jena, Germany

Eick S, Pfister W: Comparison of microbial cultivation and a commercial nucleic acid based method for detection of periodontopathogenic species in subgingival plaque samples. J Clin Periodontol 2002; 29: 638–644. © Blackwell Munksgaard, 2002.

Abstract

Objectives: Microbiological laboratory procedures are involved in diagnosis and therapy control of progressive and refractory forms of periodontitis. In recent years techniques have been developed based on the detection of nucleic acids. The purpose of this study was to validate the commercially available microDent® test which employs probes for *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola*.

Methods: 122 plaque samples obtained from periodontal pockets with various depths from 33 early onset periodontitis (EOP) patients and 15 periodontally healthy subjects were analysed by cultivation and the microDent® kit.

Results: Both cultivation and the nucleic acid based assay showed a positive correlation of pocket depth with the frequency and quantity of periodontopathogenic species. *T. denticola* was found only in pockets >4 mm in EOP patients. Comparison of the two methods revealed that the microDent® kit identified both *P. gingivalis* and *B. forsythus* more often than did the cultivation method.

Conclusions: Nucleic acid techniques should replace cultivation methods as gold standard in microbiological diagnosis of progressive periodontitis. The microDent® kit can be recommended for microbiological laboratories analysing subgingival plaque samples.

Key words: periodontopathogenic species; microbiological diagnosis; nucleic acid based detection

Accepted for publication 31 July 2001

Periodontal infections are caused by certain bacteria. A consensus report concerning periodontal diseases and microbial etiology designated *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Bacteroides forsythus* as the most important species (Genco et al. 1996). In addition, bacterial species such as *Prevotella intermedia*, *Eikenella corrodens*, *Campylobacter rectus* and spirochetes are considered to be closely related to periodontitis (Genco et al. 1996). Therefore microbiological laboratory procedures are involved in diagnosis and therapy control of progressive and refractory forms of periodontitis (Mombelli 1994). Microbiological findings are important prognostic markers predicting whether attachment gain will be stable or not (Nieminen et al. 1996). For a long period of time, culture techniques have been generally used for the detection of bacteria. However, anaerobic and capnophilic cultivation is time-consuming and labour-intensive, and needs viable bacteria.

In recent years, techniques based on the detection of nucleic acids have been commercially available. Many laboratories use self-made tests (Ashimoto et al. 1995, Riggio et al. 1996) including multiplex-PCR based methods. Garcia et al. (1998) described the simultaneous detection of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. A combination with *B. forsythus* instead of *P. intermedia* was presented by Tran & Rudney (1999). For some years the DMDx® test (Omnigene, Cambridge, MA, USA) has been made commercially available. Only a few laboratories, such as ANAWA (Wangen, Switzerland), are allowed to offer this test to the periodontist. Recently Hain Diagnostika Ltd. (Nehren, Germany) has developed the microDent® kit, which can be used in each microbiological laboratory involved in the diagnosis of periodontopathogenic species. In this test the multiplex PCR of 16s rDNA is followed by a simultaneous reverse hybridization for the species *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *Treponema denticola*.

The purpose of this study was to validate the microDent® test in comparison with conventional procedures such as microaerophilic and capnophilic cultivation. The comparison of the two methods should also demonstrate opportunities and limitations of each method.

Materials and methods

Study population and sampling sites

In this study 33 patients with early onset periodontitis (EOP) and 15 periodontally healthy subjects were included. The participants were 18–35 years of age, otherwise healthy and non-smokers. They had at least 20 natural teeth and had not undergone any periodontal or antibiotic therapy for at least 3 months. The EOP patients had at least four sites with a pocket depth of ≥ 5 mm and a attachment loss of ≥ 4 mm. The 15 controls had no clinical evidence of gingivitis, no radiographic evidence of bone loss, or pocket depth of > 3 mm.

Subgingival plaque was sampled from 33 EOP patients from several teeth with different probing depth. Altogether 32 pockets with a depth of ≤ 4 mm, 33 pockets with a depth of 4.5–6 mm, 26 pockets with a depth of 6.5–9 mm and 16 pockets with a depth of > 9 mm were analysed. Additionally, 15 supragingival plaque samples from periodontally healthy subjects were tested. Two paper points were inserted in each pocket for 20 s. Subsequently, they were placed in 2 mL of a transport medium (reduced buffered saline). After vigorous mixing for 30 s the samples were divided into two parts. One part was immediately cultivated, and the other one was stored at -20°C until analysed by PCR.

Cultivation

The plaque samples were serially diluted up to 10^{-5}. Aliquots of 0.1 mL were plated on Schaedler-agar (Oxoid, Basingstoke, UK) supplemented with 8% sheep blood without antibiotics, on the same agar plates with with 7.5 mg/L vancomycin or 100 mg kanamicin, on Columbia-agar (Oxoid) supplemented with 8% sheep blood and Tryptic soy-serum-bactitracin-vancomycin (TSBV)-agar plates (Slots 1982). The Columbia-agar plates and TSBV-agar plates were incubated in a 10% CO_2 atmosphere and the Schaedler-agar plates anaerobically at 37°C. The incubation time was 7 d except for the Columbia-agar plates (48 h). After incubation a quantitative analysis was performed for the species *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia/nigrescens*, *C. rectus*, *Fusobacterium nucleatum*, *E. corrodens*, *B. forsythus* and *Streptococcus intermedius/constellatus*. For comparison with PCR results a score system was used: 0 denotes no colony forming units (cfu) of the species; 1 denotes $\leq 10^3$ cfu/plaque sample; 2 denotes $\geq 10^4$ cfu/plaque sample. Identification of the species was based on colony and cellular morphology, Gram-staining, respiratory requirements, trypsin-like activity determined by N-Benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) reaction (Grenier & Turgeon 1994) and biochemical reactions by using ID 32 A, ID 32 Strep (bioMerieux, Marcy l'Etoile, France). Spirochetes were identified by spinning 0.4 mL of the vortexed plaque sample through a Cytospin® column (Shandon) at 500 r.p.m. for 15 min. and staining with Gram dye. Spirochaetes were subsequently documented at 1000× magnification according to the following score system: 0 – no detection; 1 – not visible in every microscopy field; 2 – visible in every microscopy field. Finally, primers described by Ashimoto et al. (1995) were used for exact differentiation of the species.

PCR and hybridization

The second aliquot of the plaque samples was used for performing the microDent® test which is able to identify five periodontopathogenic bacterial species of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* in a single run. First the solution was warmed up to room temperature and centrifuged at 10000***g*** for 20 min. The supernatant was removed and the DNA was extracted by using the High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany) according to the recommendations of Hain Diagnostika. In short, 200 µL of tissue lysis buffer and 40 µL of proteinase K solution were added to the sediment and the mixture was incubated with shaking at 72°C for 10 min. After adding 200 µL of binding buffer and only after mixing the sample was incubated at 95°C for 5 min.

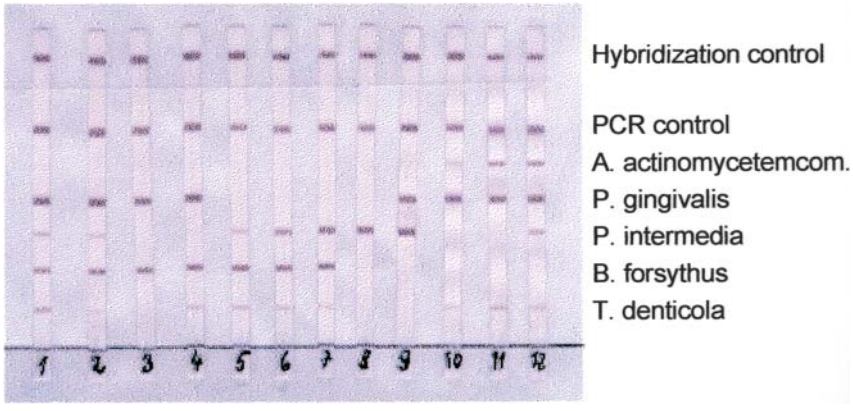

Fig. 1. Reverse hybridization of subgingival plaque samples by the microDent® test.

PCR amplification was carried out in a reaction volume of 50 µL consisting of 5 µL of template DNA and 45 µL of reaction mixture containing 35 µL of primer–nucleotide mix (microDent®), 5 µL of 10 × PCR buffer, 5 µL of 25-mM $MgCl_2$ and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). PCR cycling was carried out in a TRIO-Thermoblock thermal cycler (Biometra, Gottingen, 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. Negative and positive controls were included in each batch of samples. The positive control consisted of 5 µL of genomic DNA (about 50 ng) of the strain *P. intermedia* ATCC 25611, and the negative control was 5 µL of sterile water, each added to 45 µL of reaction mixture.

5 µL of each reaction product was loaded on a 2% agarose gel to control the PCR. The subsequent reverse hybridization was performed according to the microDent® kit. In short, the biotinilated amplicons were denatured and incubated at 45°C with hybridization buffer and strips coated with two control lines and five species-specific probes. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any unspecifically bound DNA. Streptavidin conjugated alkaline phosphatase was added, the samples were washed and hybridization products were visualized by adding a substrate for alkaline phosphatase. Results could be obtained after approx. 5 h; the 'hands-on-time' was about 1.5 h.

Developed bands were categorized as follows: score 2 represents a clear band, while score 1 denotes a weak band. According to the manufacturer the cut-off of the test is set to 10^3-10^4 genome equivalents. An example of a test assay (agarose gel and hybridization) is shown in Fig. 1.

All positive microDent® results (both score 1 and score 2) were used for determining sensitivity and specificity of the kit as compared with conventional procedures.

Results

Conventional procedures

When comparing plaque samples of periodontally healthy subjects and shal-

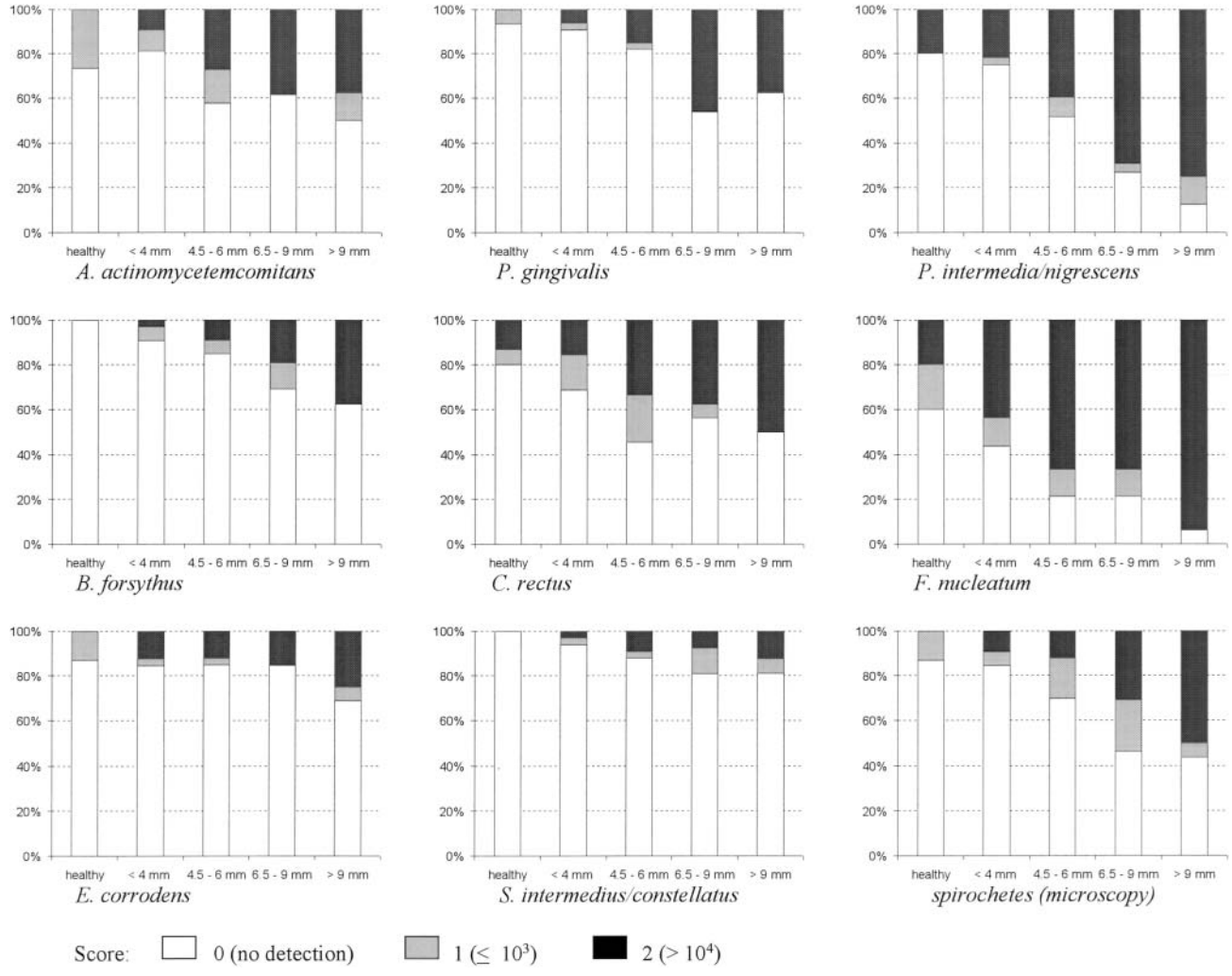

Fig. 2. Results of cultivation of 122 plaque samples obtained from 33 EOP patients with different pocket depths and 15 periodontally healthy subjects.

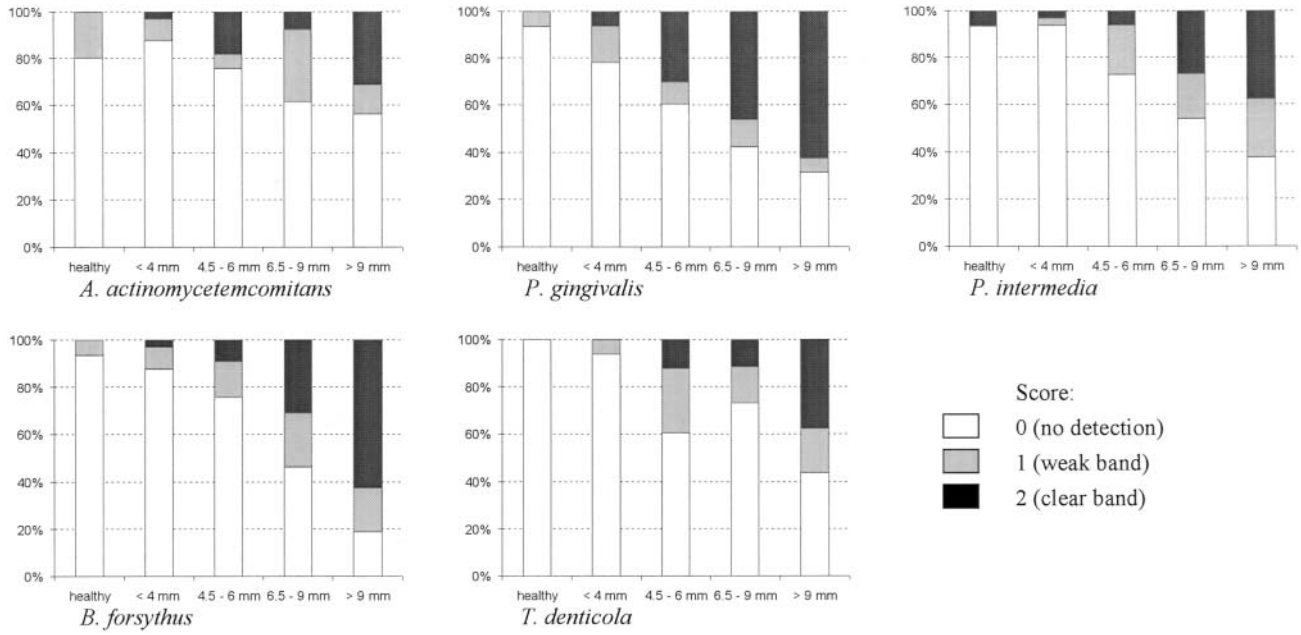

Fig. 3. Results of nucleic acid based method (microDent® test) of 122 plaque samples obtained from 33 EOP patients with different pocket depths and 15 periodontally healthy subjects.

low pockets of EOP patients, only a few differences were found. *F. nucleatum* and *C. rectus* were found to be more frequent in cases of periodontitis. The composition of subgingival microbiota among the different pockets of EOP patients was striking. Deep pockets contained more *P. intermedia/nigrescens*, *B. forsythus*, *C. rectus*, *F. nucleatum* and spirochetes than did shallow pockets. *A. actinomycetemcomitans* was detectable in similar quantities in all pockets of ≥4.5 mm. Fewer than 20% of the samples contained *E. corrodens* or *S. intermedius/constellatus*. Score 1 (cfu ≤ 10^3/sample) was very rarely found in pockets deeper than 6 mm (Fig. 2).

MicroDent® kit

Differences were seen between sulci of periodontally healthy subjects and pockets of ≤4 mm of the EOP patients for *P. gingivalis*. Positive correlations between pocket depth and the frequency and quantity of periodontopathogenic bacteria were detected for *P. gingivalis*, *B. forsythus* and *P. intermedia*. *T. denticola* was found only in pockets deeper than 4 mm (Fig. 3).

Comparison of cultivation and nucleic acid based methods

The microDent® kit detected more *P. gingivalis* positive samples than did cultivation procedures. Only 21 of 34 samples positive for *P. gingivalis* (score 2) by the microDent® kit were also positive for this species by culture. This finding would result in a sensitivity of 86% and a specificity of only 76% of the microDent® kit when compared with data obtained by culturing. It should be remarked, however, that setting the cultivation method as the gold standard could be problematic because of the difficulties of detecting *P. gingivalis* by cultivation when many other bacteria are simultaneously present in a plaque sample (Table 1).

Comparison of the results for *P. intermedia* showed a sensitivity of 52% and a specificity of 95% for the nucleic based assay when compared with cultivation results. The low sensitivity, however, was due to the fact that no differentiation between *P. intermedia* and *P. nigrescens* is possible by conventional methods, as confirmed by the additionally performed PCR according to Ashimoto et al. (1995) (Table 2).

A first version of the microDent® kit identified only 50% of the samples that scored positive for *A. actinomycetemcomitans* by cultivation. The specificity was 88%. Subsequently, a new version of the microDent® kit with improved primers/probe for *A. actinomycetemcomitans* was available. Repeated tests with the new kit version resulted in a sensitivity of 76%. Now samples containing a high number (≥ 10^5) of *A. actinomycetemcomitans* scored positive. The results for samples with fewer bacteria, however, remained negative (Table 3).

Table 1. Comparison of detection of A. actinomycetemcomitans in 107 plaque samples of 33 EOP patients and in 15 samples of 15 periodontally heathy subjects by oligonucleotide method and anaerobic cultivation

		Detection by oligonucleotide probe Score			
Detection by culture		0	1	2	Total
Score	0	**69**	8	3	80
	1	9 (7*)	**5 (7*)**	0	14
	2	12 (3*)	5 (9*)	**11 (16*)**	28
	Total	90 (79*)	18 (24*)	14 (19*)	122

*Results obtained by repeated testing with the improved kit.
Agreement between the scores of the two techniques used was 70% (85/122).

Table 2. Comparison of detection of P. gingivalis in 107 plaque samples of 33 EOP patients and in 15 samples of 15 periodontally heathy subjects by oligonucleotide method and anaerobic cultivation

		Detection by oligonucleotide probe Score			
Detection by culture		0	1	2	Total
Score	0	**71**	10	13	94
	1	2	**1**	0	3
	2	2	2	**21**	25
	Total	75	13	34	122

Agreement between the scores of the two techniques used was 76% (93/122).

Table 3. Comparison of detection of P. intermedia in 107 plaque samples of 33 EOP patients and in 15 samples of 15 periodontally heathy subjects by oligonucleotide method and anaerobic cultivation

		Detection by oligonucleotide probe (*P. intermedia*) Score		
Detection by culture (*P. intermedia/nigrescens*)	0	1	2	Total
Score	0	**59**	2	1	62
	1	4	**2**	1	7
	2	25	13	**15**	53
	Total	88	17	17	122

Agreement between the scores of the two techniques used was 62% (76/122).

Table 4. Comparison of detection of B. forsythus in 107 plaque samples of 33 EOP patients and in 15 samples of 15 periodontally heathy subjects by oligonucleotide method and anaerobic cultivation

		Detection by oligonucleotide probe Score			
Detection by culture		0	1	2	Total
Score	0	**81**	12	7	100
	1	1	**4**	2	7
	2	0	2	**13**	15
	Total	82	18	22	122

Agreement between the scores of the two techniques used was 80% (98/122).

Table 5. Comparison of detection of T. denticola in 107 plaque samples of 33 EOP patients and in 15 samples of 15 periodontally heathy subjects by oligonucleotide method and microscopy

		Detection by oligonucleotide probe (*T. denticola*) Score			
Detection by microscopy (spirochetes)		0	1	2	Total
Score	0	**81**	1	0	82
	1	6	**6**	5	17
	2	4	11	**8**	23
	Total	91	18	13	122

Agreement between the scores of the two techniques used was 78% (95/122).

Cultivation of *B. forsythus* is complicated. Therefore it seems reasonable that this species was more often detected by PCR-based method compared with cultivation (Table 4). Finally, in nearly all cases where samples were scored positive for *T. denticola* by the PCR-based test, spirochetes were enumerated by microscopy, but sometimes spirochetes were seen without a positive result in the microDent® test (Table 5).

Discussion

In our study we analysed plaque samples from EOP patients obtained from pockets with various depths. The frequency of detection of the test species correlated with periodontal pocket depth. Comparison of our microbiological results with those of other studies is complicated by the fact that different methods and cut-offs were applied in each study.

Most periodontopathogenic species are also found in low frequencies and quantities in periodontally healthy subjects. Griffen et al. (1998) detected *P. gingivalis* in 25% of samples from healthy subjects. In the study of Tran & Rudney (1999), 55% of healthy sites were positive for *P. gingivalis*, 30% for *A. actinomycetemcomitans* and 5% for *B. forsythus*. Therefore Conrads (1999) recommended a cut-off to detect only relevant quantities of periodontopathogenic species. The cut-off of the microDent® kit is set to 10^3-10^4 genome equivalents.

In general our results are in accordance with other studies of progressive periodontitis. For example, our results for plaque samples from deep periodontal pockets obtained by a nucleic acid based method were similar to those of Ashimoto et al. (1995), and the cultivation findings for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia/nigrescens* resembled those of Van der Weijden et al. 1994). However, compared with others we detected a higher prevalence of *A. actinomycetemcomitans* (Darby et al. 2000, Kamma et al. 1994, Yano-Higuchi et al. 2000). This might be explained by the fact that the patients in our study on average were younger than those in other studies, thereby confirming a association of *A. actinomycetemcomitans* with age (Savitt & Kent 1991).

Socransky et al. (1998) also compared the microflora of pockets with different depths and found a higher prevalence of *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *B. forsythus* in deep pockets than in shallow pockets. Interestingly, we did not detect *T. denticola* in shallow pockets of EOP patients. This might indicate that this spe-

cies is only a secondary colonizer of diseased periodontal sites. Ximenez-Fyvie et al. (2000) found *T. denticola* in supragingival plaque from teeth with deep pockets.

C. rectus was determined to be more frequent in shallow pockets of EOP patients compared with healthy controls. This finding is in agreement with the study of Maiden et al. (1997), who reported that *C. rectus* and *B. forsythus* are species possibly associated with active initial periodontal lesions.

Comparison of the two methods used in this study showed differences in the detection of periodontopathogenic species. The microDent® test more often identified *P. gingivalis* and *B. forsythus* than did the cultivation method. This finding originated from the deficient selectivity of the commonly used cultivation media. In addition, anaerobiosis, indispensable for periodontopathogenic bacteria like *P. gingivalis*, is sometimes difficult to maintain during sample collection and transportation. Similarly, Van Steenbergen et al. 1996), Slots & Chen (1993) and Riggio et al. (1996) found a higher percentage of *P. gingivalis* positive samples by a PCR-based method in comparison with cultivation.

The clear differentiation between *P. intermedia* and *P. nigrescens* by conventional methods is very difficult (Cookson et al. 1996, and our own results not published yet), and differentiation by gene probes or primers based on 16S rDNA often requires very exact conditions to avoid false positive results (Conrads et al. 1997, Shah et al. 1995). However, this differentiation might be pivotal since a number of studies have pointed out that only *P. intermedia* and not *P. nigrescens* is correlated with the outcome of periodontitis and deep periodontal pockets (Gharbia et al. 1994, Teanpaisan et al. 1995, and our own results not published yet). In contrast, Umeda et al. (1998) detected both *Prevotella* species in deep periodontal pockets. The microDent® test detects only *P. intermedia*.

We encountered some initial difficulties in detecting *A. actinomycetemcomitans* with the microDent® kit. The first improved version identified more but still not all *A. actinomycetemcomitans* containing samples. Because of its high pathogenicity, a low cut-off for this bacterium is considered to be crucial (Rams et al. 1996, Renvert et al. 1996). The cut-off of the microDent® assay for *A. actinomycetemcomitans* was set to a lower level (10^3 genome equivalents), but further validation of the test is needed. In addition, an internal positive control was incorporated in the test, ensuring that negative results are not due to a failure of the PCR reaction.

Nucleic acid based methods are quicker and more convenient than anaerobic cultivation. They should be used in microbiological diagnosis of subgingival plaque samples of patients with progressive forms of periodontitis. The detection of *P. gingivalis* and *B. forsythus*, two of the three most important periodontopathogenic species (Genco et al. 1996), is remarkably better by nucleic acid techniques than by culture. *A. actinomycetemcomitans*, also a designated periodontal pathogen, was detected in similar numbers of samples by both techniques. None the less, cultivation provides detection of multiple bacterial species coincidentally as well as allowing the determination of antibiotic resistance. Therefore cultivation still plays a major role, particularly when examining cases of refractory periodontitis.

The microDent® assay has been shown to be highly sensitive and specific for the five test periodontal pathogens, and can be recommended for laboratory use to aid in microbiological diagnosis of periodontal diseases. We would like to suggest a basic kit for the detection of the three designated pathogens and the development of an additional assay for other possible pathogens such as *C. rectus*, *E. corrodens* and *S. constellatus*.

Acknowledgements

This study was supported by BMBF. We thank Professor Gisela Klinger and Dr Bernd Sigusch for sampling subgingival plaque from different pockets of EOP patients and supragingival plaque from periodontally healthy subjects. Sigrid Jarema is acknowledged for her excellent technical assistance.

Zusammenfassung

Vergleich mikrobiologischer Kultivierung mit einem kommerziellen auf Nukleinsäuren basierenden Test zum Nachweis parodontalpathogener Keime aus subgingivalen Plaqueproben

Hintergrund: Mikrobiologische Labormethoden werden zur Diagnose und Therapiekontrolle aggressiver und therapierefraktärer Formen von Parodontitis verwendet. In den letzten Jahren wurden Verfahren entwickelt, die auf dem Nachweis von Nukeinsäuren basieren.

Zielsetzung: Validierung des kommerziell erhältlichen microDent®-Tests, der Sonden für *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *B. forsythus* und *T. denticola* verwendet.

Material und Methoden:122 Plaqueproben, die aus parodontalen Taschen verschiedener Tiefen bei 33 Patienten mit aggressiver Parodontitis (AP) und 15 parodontal gesunden Personen gewonnen worden waren, wurden mittels kultureller Methoden und mit dem microDent®-Testverfahren analysiert.

Ergebnisse: Sowohl die Kultur als auch der molekularbiologische Test zeigten eine positive Korrelation zwischen Taschentiefen und Häufigkeit bzw. Menge der untersuchten Parodontalpathogene. *T. denticola* wurde nur in Taschen $>$ 4 mm bei AP-Patienten nachgewiesen. Der Vergleich beider Methoden ergab, dass der microDent®-Test sowohl *P. gingivalis* als auch *B. forsythus* häufiger nachweisen konnte als die Kultur.

Schlussfolgerung: Techniken zum Nachweis von Nukleinsäuren sollten kulturelle Methoden als Goldstandard in der mikrobiologischen Diagnostik progressiver Formen der Parodontitis ablösen. Der microDent®-Test kann mikrobiologischen Laboratorien für die Analyse subgingivaler Plaqueproben empfohlen werden.

Résumé

Comparaison entre la culture microbienne et la méthode commerciale basée sur l'acide nucléique pour la détection d'espèces parodontopathogéniques dans des échantillons de plaque sous-gingivale

But: Des procédures microbiologiques de laboratoire sont utilisées dans le contrôle de diagnostic et de traitement de formes progressives et réfractaires de parodontite. Au cours des dernières années, des techniques se basant sur la détection des acides nucléiques ont été élaborées. L'objectif de cette étude était de valider le test microDent® disponible dans le commerce, permettant de détecter *A. actinomycetemcomitans, P. gingivalis, P. intermedia, B. forsythus* et *T. denticola*.

Méthodes: 122 échantillons de plaque, prélevés au niveau de poches parodontales de profondeur variable chez 33 patients atteints de parodontite sévère et chez 15 sujets au parodonte sain, ont été analysés par culture et au moyen du kit microDent®.

Résultats: La méthode de culture et le test basé sur les acides nucléiques ont tous deux révélé une corrélation positive entre la profondeur de poche et la fréquence et la quantité d'espèces parodonto-pathogéniques. *T. denticola* n'a été détecté que dans les poches $>$ 4 mm chez les patients atteints de parodontite. La comparaison des deux méthodes a montré que le kit microDent® détectait à la fois *P. gingivalis* et *B. forsythus* plus souvent que la méthode de culture.

Conclusions: Les techniques de l'acide nucléique devraient remplacer les méthodes de cul-

ture et devenir la référence en matière de diagnostic microbiologique des parodontites progressives. Le kit microDent® peut être recommandé aux laboratoires microbiologiques qui analysent des échantillons de plaque sous-gingivale.

References

Ashimoto, A., Chen, C., Bakker, I. & Slots, J. (1995) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiology and Immunology* **11**, 266–273.

Conrads, G. (1999) Molekularbiologische Verfahren in der Anaerobier-, speziell Parodontitiserreger-Diagnostik. *Mikrobiologie* **9**, 165–169.

Conrads, G., Pelz, K., Hughes, B., Seyfarth, I. & Devine, D. A. (1997) Optimized oligonucleotides for the differentiation of *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Microbiology and Immunology* **12**, 117–120.

Cookson, A. L., Wray, A., Handley, P. S. & Jacob, A. E. (1996) An investigation into the use of SDS-PAGE of cell surface extracts and proteolytic activity to differentiate *Prevotella nigrescens* and *Prevotella intermedia*. *FEMS Microbiology Letters* **136**, 109–115.

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.

Garcia, L., Tercero, J. C., Legido, B., Ramos, J. A., Alemany, J. & Sanz, M. (1998) Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis* by multiplex PCR. *Journal of Periodontal Research* **33**, 59–64.

Genco, R. J., Kornman, K., Williams, R., Offenbacher, S., Zambon, J. J., Ishikawa, I., Listgarten, M., Michalowicz, B., Page, R., Schenkein, H., Slots, J. & Socransky, S. (1996) Consensus report periodontal diseases: pathogenesis and microbial factors. *Annals of Periodontology* **1**, 926–932.

Gharbia, S. E., Haapasalo, M., Shah, H. N., Kotiranta, A., Lounatmaa, K., Pearce, M. A. & Deidre, A. D. (1994) Characterization of *Preveotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *Journal of Periodontology* **65**, 56–61.

Grenier, D. & Turgeon, J. (1994) Occurrence and identity of proteolytic bacteria in adult periodontitis. *Journal of Periodontal Research* **29**, 365–370.

Griffen, A. L., Becker, M. R., Lyons, S. R., Moeschberger, M. L. & Leys, E. J. (1998) Prevalence of *Porphyromonas gingivalis* and periodontal health status. *Journal of Clinical Microbiology* **36**, 3239–3242.

Kamma, J. J., Nakou, M. & Manti, F. A. (1994) Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters. *Journal of Periodontology* **65**, 1073–1078.

Maiden, M. F., Macuch, P. J., Murray, L. & Tanner, A. (1997) 'Checkerboard' DNA-probe analysis and anaerobic culture of initial periodontal lesions. *Clinical Infectious Diseases* **25**(Suppl. 2), S230–S232.

Mombelli, A. (1994) Parodontaldiagnostik. Die Rolle der Mikrobiologie. *Schweizer Monatsschr für Zahnmedizin* **194**, 54–57.

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

Rams, T. E., Listgarten, M. A. & Slots, J. (1996) Utility of 5 major putative periodontal pathogens and selected clinical parameters to predict periodontal breakdown in patients on maintenance care. *Journal of Clinical Periodontology* **23**, 346–354.

Renvert, S., Dahlen, G. & Wikstrom, M. (1996) Treatment of periodontal disease based on microbiological diagnosis. Relation between microbiological and clinical parameters during 5 years. *Journal of Periodontology* **67**, 562–571.

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.

Savitt, E. D. & Kent, R. L. (1991) Distribution of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by subject age. *Journal of Periodontology* **62**, 490–494.

Shah, H. N., Gharbia, S. E., Scully, C. & Finegold, S. M. (1995) Oligonucletide probes to the 16S ribosomal RNA: implications of sequence homology and secondary structure with particular reference to the oral species *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Diseases* **1**, 32–36.

Slots, J. (1982) Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology* **15**, 606–609.

Slots, J. & Chen, C. (1993) Detection of *Porphyromonas gingivalis* associated with periodontitis by DNA methods. *Clinical Infectious Diseases* **16**(Suppl. 4), S317–S318.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L., Jr (1998) Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* **25**, 134–144.

Teanpaisan, R., Douglas, C. W. I. & Walsh, T. F. (1995) Characterisation of black-pigmented anaerobes isolated from diseased and healthy sites. *Journal of Periodontal Research* **30**, 245–251.

Tran, S. D. & Rudney, J. D. (1999) Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. *Journal of Clinical Microbiology* **37**, 3504–3508.

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

Van der Weijden, G. A., Timmerman, M. F., Reijerse, E., Wolffe, G. N., Van Winkelhoff, A. J. & Van der Velden, U. (1994) The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in selected subjects with periodontitis. *Journal of Clinical Periodontology* **21**, 583–588.

Van Steenbergen, T. J. M., Timmerman, M. F., Mikx, F. H. M., De Quincey, G., Van der Wejden, G. A., van der Velden, U. & de Graaff, J. (1996) Discrepancy between culture and DNA probe analysis for the detection of periodontal bacteria. *Journal of Clinical Periodontology* **23**, 955–959.

Ximenez-Fyvie, L. A., Haffajee, A. D. & Socransky, S. S. (2000) Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *Journal of Clinical Periodontology* **27**, 648–657.

Yano-Higuchi, K., Takamatsu, N., He, T., Umeda, M. & Ishikawa, I. (2000) Prevalence of *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival microflora of Japanese patients with adult and rapidly progressive periodontitis. *Journal of Clinical Periodontology* **27**, 597–602.

Address:
Dr Sigrun Eick
Department of Medical Microbiology
University Hospital of Jena
Semmelweisstr. 4
D-07740 Jena
Germany

e-mail: sigrun.eick@med.uni-jena.de