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Detection of periodontopathogenic bacteria with two different microbiological tests



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Microbiological tests have become a useful device in periodontal diagnostics. Knowledge of the grouping and quantity of specific bacteria in subgingival biofilms helps clinicians to expand their image of periodontal diseases beyond clinical findings and to evaluate treatment outcomes. Even if several microbiological diagnostic tests are available, there is little information on the advantages and disadvantages of specific test kits. The present research report deals with the comparison of the results of two different microbiological test kits.

■ Introduction

The oral cavity is inhabited by a plethora of bacterial species, stated in literature to be between 300 and 1000 in number¹⁻⁴. The spread of this estimate might be related to the method used for identification and bacterial taxonomy. Commonly applied methods in microbiological diagnostics comprise cultivation, several microscopic techniques, enzymatic and immunological assays and polymerase chain reaction (PCR) *inter alia*⁵⁻⁸. With regard to accuracy, economics, expenditure of time and reliability, every method has its advantages as well as its drawbacks. Cultivation, for example, which is still the gold standard for identification of bacteria, is an approved microbiological tool, but there are difficulties in its application to several bacterial species and in addition it is a high cost, time consuming procedure^{9,10}. However, cultivation has one advantage over other microbiological identification methods: it reveals information about antibiotic sensitivity¹⁰. In

contrast, genotypic technologies have the advantages of versatility, speed and precision, but initially lacked accuracy. This gap has been closed by the development of real-time PCR and quantitative DNA-DNA hybridisation^{7,8,11,12}.

Despite the variety of oral microflora, few bacterial species have been found to be strongly related to the pathogenesis of periodontal diseases¹³. Once it became known that Gram-negative microorganisms prevail in deep dental pockets¹⁴, *Porphyromonas gingivalis* (P.g.), *Bacteroides forsythus* (reclassified as *Tannerella forsythensis*¹⁵ [T.f.]) and *Actinobacillus actinomycetemcomitans* (reclassified as *Aggregatibacter actinomycetemcomitans*¹⁶ [A.a.]) were classed as the three periodontal pathogens¹⁷. Furthermore the concept of dental plaque as a biofilm in which different bacterial species live in an organoid community has been established. Building up communities is beneficial for single bacterial species, as they gain access to ecological niches that are unavailable to them when solitary^{18,19}.

On the basis of cluster analyses of subgingival plaque samples, the subgingival microflora has been subdivided into microbial complexes^{20,21}. In particular, the members of the red complex, namely *P.g.*, *T.f.*, *Treponema denticola* (*T.d.*) and *A.a.* show a highly virulent potential against periodontal tissues^{22,23}.

The supportive use of antibiotics in the treatment of periodontal diseases has been proved to enhance clinical outcomes^{4,24,25}. Nevertheless, general use of antibiotics should be avoided, as there is growing evidence that the number of antibiotic resistant microorganisms has dramatically increased in recent years²⁶⁻²⁹. The use and the choice of antibiotics in the treatment of periodontitis should be based on microbiological testing and be limited to certain disease patterns, such as severe forms of chronic periodontitis (CP), aggressive periodontitis (AgP), refractory periodontitis or recurrent periodontitis³⁰⁻³².

Nowadays a multitude of microbiological diagnostic tests is commercially available to clinicians. The purpose of the present investigation was to compare the results of two test kits, both of which are extolled to detect and quantify specific oral bacteria, but differ in the laboratory methodology on which they are based.

■ Study design

■ Subjects

A total of 12 patients (5 females and 7 males) with a diagnosis of CP (n=1) or AgP (n=11), according to current classification criteria³³, were recruited. Inclusion criteria were at least one site of each quadrant with a pocket probing depth (PPD) of >4 mm, a clinical attachment level (CAL) of ≥5 mm, radiographically evidenced bone loss, and absence of pus. Exclusion criteria were scaling and root planing or surgical periodontal therapy during the last 3 months and systemic application of antibiotics or topical application of antibiotics at periodontal sites during the last 6 months.

Subgingival plaque samples were collected and analysed. Periodontal pockets were treated with quadrant-wise scaling and root planing and with application of systemic antibiotics according to the results of the microbiological tests. At the next

follow-up, further subgingival plaque samples were taken for analysis at the same sites.

■ Sampling of subgingival plaque

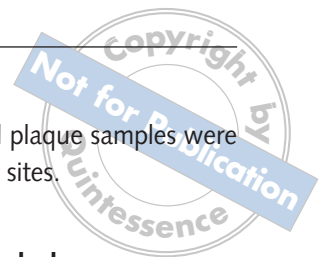
The deepest periodontal pocket without pus in each quadrant was selected for sampling. Saliva was excluded by applying cotton rolls and the tooth surfaces were gently dried with air. Supragingival plaque was carefully removed using sterile curesttes. Two sterile paper points ISO 35 (Coltène/Whaledent, Langenau, Germany) were simultaneously inserted to the bottom of the pocket and remained there for 20s. Immediately after sampling, one of the two simultaneously applied paper points was dipped into the collection tube of IAI PadoTest 4.5 (Institut für Angewandte Immunologie [IAI], Zuchwil, Switzerland), while the other paper point was dipped into the collection tube of Meridol® Perio Diagnostics (GABA, Therwil, Germany). The samples were immediately sent to the institute's laboratories for analysis.

■ IAI PadoTest 4.5

The following operation protocol was a kind gift from the IAI. Collection tubes of IAI PadoTest 4.5 contain 100µl guanidine thiocyanate buffer for stabilisation of the samples. After arrival in the laboratory, the collection tubes are heated to 70°C for 10s, thoroughly vortexed and stored frozen until analysis. Just before analysis, the samples are further processed to make them suitable for membrane-dotting. Aliquots of each are then applied to five nylon membranes mounted in dot-blot vacuum manifolds. The membranes are processed by standard procedures, and each is hybridised to one of five ³²P-labelled, specific probes for the small subunit ribosomal RNAs (ssrRNAs) of *A.a.*, *T.f.*, *P.g.*, *T.d.* and a universal probe (UP).

Blots are quantified by direct counting in a Trace-96 system (Inotech). No calibration curves are necessary, as counts per minute (cpm) readings are linear from 2x10⁴ to 600x10⁶ bacteria. Autoradiograms of the membranes are produced and inspected, after counting, to check the whole procedure.

On each membrane, denaturated reference standards are applied for each of the four bacteria





along with the samples. A processing control is provided by applying a constructed sample of *Escherichia coli* on each membrane. The values of each bacterial species are computed by comparison with the homologous standard. For the determination of total bacteria (UP), a combination of the four standards is used. The standards are calibrated dilutions of plasmids containing a cloned cDNA copy of the *ssrRNA* of each of the four bacterial species. The crude measure is the number of ribosomes in the sample for each of the four bacterial species and for all bacteria. The results are given as a number of bacteria by assuming that one bacterium possesses 10^4 copies of *ssrRNA*.

Additionally, a comparison with the results of a field study conducted in Switzerland is provided for each bacterium in the sample. The contents of the sample for the bacterium are ranked according to its position relative to the 60, 90 and 95 percentiles of the distribution in this field study.

According to the manufacturer's information, the lower limits of detection are 5×10^3 bacteria for *A.a.* and 10^4 for the other species.

Meridol® Perio Diagnostics

This test is based on the technology of real-time PCR. Bacterial DNA is isolated and purified. For every target sequence of DNA, specific primers are used. One of the two primers (the reporter) is fluorescently marked, and the other (the quencher) is able to quench the fluorescence signal of the reporter if both primers are within a defined distance from each other. A further specific probe (TaqMan® probe) targeting a sequence between the reporter and the quencher is used to increase the test's specificity. During DNA amplification, the exonuclease activity of the Taq polymerase splits off and destroys the TaqMan® probe. This process causes a fluorescence signal that, like all the other fluorescence events, is recorded by detectors in real time. The intensity of the fluorescence signal is a measure for the reaction product and directly proportional to the number of the bacterial species in the plaque sample.

According to manufacturer's information, the range of confident measurement is between 10^2 and 10^6 bacteria for all tested species.

Statistical analysis

McNemar test was performed for each species before and after the treatment procedure.

Results

Subgingival plaque samples were collected from 45 periodontal pockets before and after conventional periodontal therapy with deep scaling (DS). The three missing samples were not processed, as no deep pockets were present in the respective quadrant (PPD < 4 mm). The distribution of PPDs (mean = 7.93 mm) before periodontal treatment is shown in Fig 1. Bleeding on probing (BOP) was assessed at 42 of the 45 sites. Thirty-nine (92.9%) sites showed BOP (Fig 2). In 11 of 12 cases, DS together with an adjuvant systemic application of amoxicillin 500 mg t.i.d. and metronidazole 400 mg t.i.d. was performed for 7 days. In one case, mechanical therapy was performed without application of antibiotics.

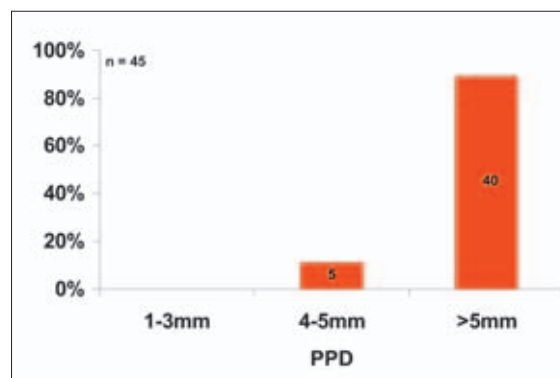


Fig 1 Distribution of pocket probing depth (PPD) before treatment procedure.

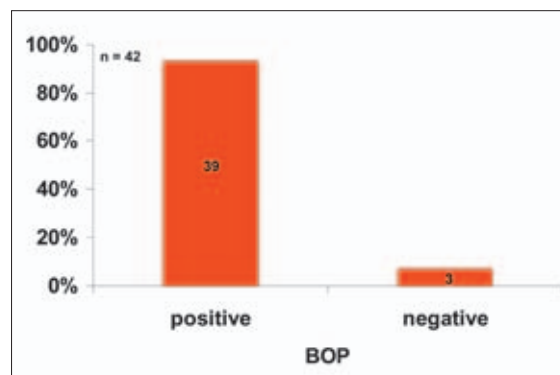


Fig 2 Distribution of bleeding on probing (BOP) before treatment procedure.

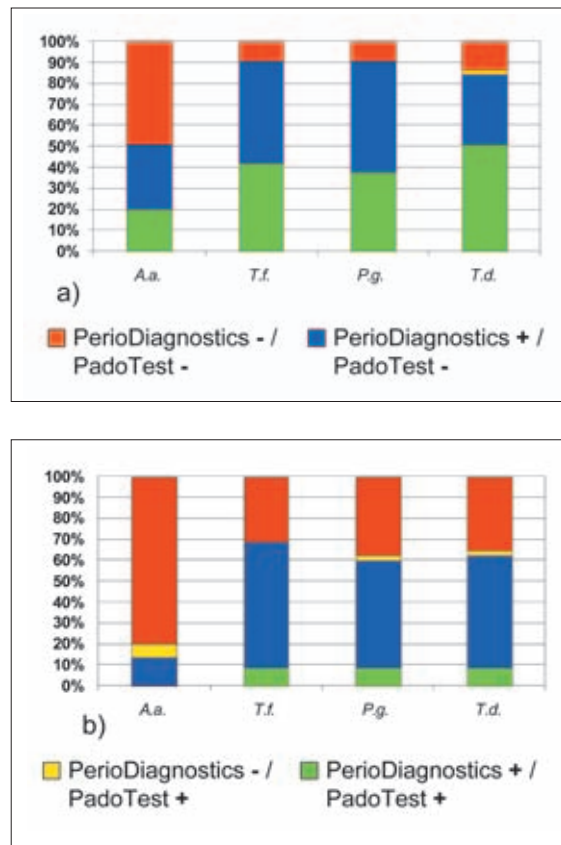


Fig 3 Qualitative results of microbiological tests (a) before and (b) after periodontal treatment. Green indicates positive results for both tests; red indicates negative results for both tests; blue indicates that the result of Meridol® Perio Diagnostics was positive and the result of IAI PadoTest 4.5 was negative; yellow indicates the opposite situation.

The distribution of positive and negative test results of both microbiological tests for *A.a.*, *T.f.*, *P.g.* and *T.d.* is shown before (Fig 3a) and after periodontal treatment (Fig 3b). The distribution of positive and negative test results of both microbiological tests is shown before (Fig 4a) and after periodontal treatment (Fig 4b).

Prior to treatment, test results of Meridol® Perio Diagnostics and IAI PadoTest 4.5 were positive for *A.a.* at 9 sites (20%), for *T.f.* at 19 sites (42.22%), for *P.g.* at 17 sites (37.78%) and for *T.d.* at 23 sites (51.11%). Taken together, both tests revealed positive results for at least one of the four bacterial species at 31 sites (68.89%). Meridol® Perio Diagnostics alone showed positive results for *A.a.* at 14 sites (31.11%), for *T.f.* at 22 sites (48.89%), for *P.g.* at 24 sites (53.33%) and for *T.d.* at 15 sites

(33.33%). Both tests were negative for *A.a.* at 22 sites (48.89%), for *T.f.* at 4 sites (8.89%), for *P.g.* at 4 sites (8.89%) and for *T.d.* at 6 sites (13.33%). In total, at 14 sites (31.11%) only Meridol® Perio Diagnostics showed positive results for at least one of the four bacterial species. IAI PadoTest 4.5 alone was positive for *T.d.* at 1 site (2.22%). There was no case in which IAI PadoTest 4.5 solely indicated the presence of *A.a.*, *T.f.* or *P.g.*

After treatment, test results of Meridol® Perio Diagnostics and IAI PadoTest 4.5 were positive for *T.f.* at 4 sites (8.89%), for *P.g.* at 4 sites (8.89%) and also for *T.d.* at 4 sites (8.89%). At no site was *A.a.* detected by Meridol® Perio Diagnostics and IAI PadoTest 4.5. Taken together, both tests revealed positive results for at least one of the four bacterial species at 9 sites (20%). Meridol® Perio Diagnostics alone showed positive results for *A.a.* at 6 sites (13.33%), for *T.f.* at 27 sites (60%), for *P.g.* at 23 sites (51.11%) and for *T.d.* at 24 sites (53.33%). Altogether, Meridol® Perio Diagnostics solely revealed positive results for at least one of the four bacterial species at 27 sites (60%). Both tests were negative for *A.a.* at 36 sites (80%), for *T.f.* at 14 sites (31.11%), for *P.g.* at 17 sites (37.78%) and for *T.d.* at 16 sites (35.56%). In summary, Meridol® Perio Diagnostics and IAI PadoTest 4.5 revealed negative results for at least one of the four bacterial species at 8 sites (17.78%).

IAI PadoTest 4.5 alone was positive for *A.a.* at 3 sites (6.67%), for *P.g.* at 1 site (2.22%) and for *T.d.* at 1 site (2.22%). There was no case in which IAI PadoTest 4.5 solely indicated the presence of *A.a.* In summary, IAI PadoTest 4.5 solely revealed positive results for at least one of the four bacterial species at 1 site (2.22%).

The chi-squared test demonstrated significant differences between the results of both microbiological tests in all cases ($P < 0.05$), except for the detection of *A.a.* after treatment ($P > 0.05$).

■ Discussion and conclusions

The present study is a comparison of two different microbiological tests for the detection of periodontopathogenic bacteria. As the counts of bacterial species (results not shown) varied too much between the two tests for each site (i.e. at the same

site IAI PadoTest 4.5 detected no bacteria whereas Meridol® Perio Diagnostics detected 8.8×10^6 bacteria), it was not reasonable to perform statistical analyses with absolute numbers. This situation raises the question of whether absolute counts of a specific microbiological test are reliable to differentiate between healthy, diseased, active or inactive periodontal sites. Average counts of bacteria in human gingival crevices range from 10^3 in physiological sites to 10^8 in deep periodontal pockets⁴. Haffajee and Socransky³⁴ performed risk assessment analyses for new attachment loss >2mm in the following 2 months at sites with different threshold levels of *P.g.* or *A.a.* Their results indicated a statistically significantly higher risk for attachment loss at sites where the counts of *P.g.* were $>6 \times 10^5$ and *A.a.* $>3 \times 10^4$. The definition of threshold levels seems to be a good marker for the direction of periodontal therapy, but it is only reasonable as long as highly sensitive tools for quantification are available.

The qualitative analyses demonstrate that Meridol® Perio Diagnostics more frequently detected the presence of periodontopathogenic bacteria. When all results before and after treatment are considered, only in five cases did IAI PadoTest 4.5 alone show positive results. One possible explanation for the greater number of positive test results of Meridol® Perio Diagnostics lies in the different ways the samples are processed. The target RNA in the samples for IAI PadoTest 4.5 is not subjected to any amplification procedure. In addition to the type of labelling complex and the material of the array system, the sensitivity of array-based expression measurements directly relies on the quantity of nucleic acids^{35,36}. Real time PCR methods offer the advantage that only small amounts of sample DNA are necessary, as they are amplified by the procedure itself^{37,38}.

The results of the present study are of great importance for the clinician, as positive results might influence the decision for prescribing antibiotics or for rescaling periodontal pockets. On the other hand, negative results might withhold clinicians from certain treatment strategies. In this context it should be mentioned that Meridol® Perio Diagnostics does not distinguish between living and dead bacteria. Even non-viable microorganisms elicit and maintain inflammatory processes and may lead to tissue damage. For example, lipopolysaccharides (LPS) on

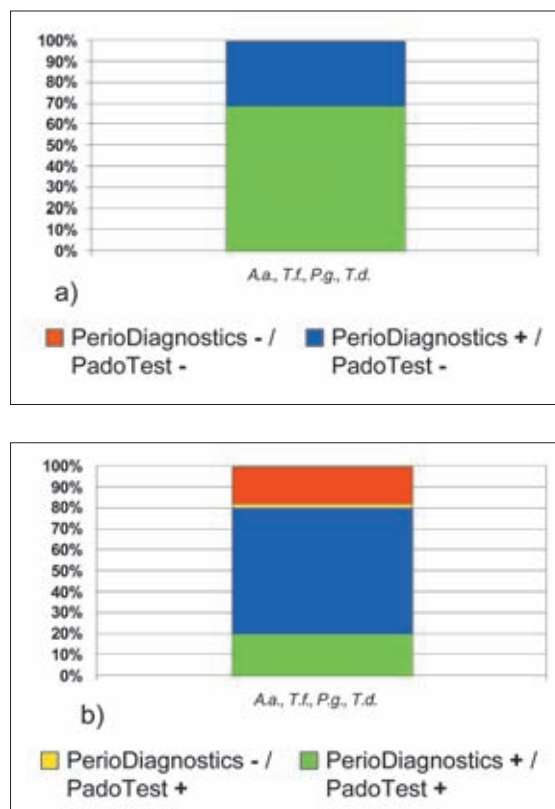


Fig 4 Qualitative results of microbiological tests (a) before and (b) after periodontal treatment for *A.a.*, *T.f.*, *P.g.* and *T.d.* Green indicates positive results for both tests; red indicates negative results for both tests; blue indicates that the result of Meridol® Perio Diagnostics was positive and the result of IAI PadoTest 4.5 was negative; yellow indicates the opposite situation.

Gram-negative cell walls activate the complement system through the alternative pathway, and destructive proteases stay active long after cell death^{39,40}. In contrast, living bacteria continuously produce virulent metabolites, grow in number, colonise ecologic niches, build communities and develop an organ-like character as a biofilm⁴¹⁻⁴³. This means that living bacteria have a more active influence on the progress of periodontal diseases. Advances have been made in the differentiation between living and dead cells with nucleic acid-based technologies. The use of ethidium monoazide (EMA) or additional RNA analyses may have the potential to broaden the information assessed by PCR-based diagnostics in the future^{44,45}. As IAI PadoTest 4.5 detects the amount of RNA, which is fixed and protected against degradation immediately after sampling, only living bacteria are detected.

As no gold standard for microbiological testing was used in this study, it is impossible to evaluate

the accuracy of Meridol® Perio Diagnostics and IAI PadoTest 4.5. Nevertheless, cultivation methods are not indisputable and over 50% of the known phylotypes in the oral cavity have not been cultivated. Furthermore, real-time PCR methods have been suggested as the new gold standard for microbiological diagnosis of periodontal bacteria^{9,11,46}. This supports the reliability of the results of Meridol® Perio Diagnostics.

In summary, the results of the present study indicate significant differences between two available microbiological diagnostic tests. The choice of a microbiological test should consider proven reliability in sensitivity, specificity and quantification. Further investigations are required to qualify different microbiological tests and to clarify their relevance for periodontal treatment.

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