Microbial Analysis of Subgingival Plaque Samples Compared to That of Whole Saliva in Patients With Periodontitis

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Background: The detection of special bacterial species in patients with periodontitis is considered to be useful for clinical diagnosis and treatment. The collection of subgingival plaque samples is the common way for the determination of periodontopathic bacteria. However, recently, salivary analysis has been discussed as an advantageous future diagnostic method for periodontitis because it offers simple quantitative sampling and the possibility to assess various bacteria. The aim of this cross-sectional study is to investigate whether there is a correlation between the results of different bacterial species in saliva and subgingival plaque samples from individuals with aggressive periodontitis (AgP) and chronic periodontitis (CP).

Methods: Whole saliva and subgingival plaque samples from the deepest pocket of each quadrant were collected from 43 patients with CP and 33 patients with AgP. Twenty different bacterial species from both samplings were determined by the 16S ribosomal RNA-based polymerase chain reaction with microarray technique.

Results: All bacterial species were detected in salivary and subgingival plaque samples. For *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, as well as *Actinomyces viscosus*, *Campylobacter rectus/showae*, *Prevotella intermedia*, *Parvimonas micra*, *Eubacterium nodatum*, and *Campylobacter gracilis*, a significant positive correlation between salivary and subgingival plaque samples was detected in patients with both types of periodontitis. There were no significant differences in bacteria in salivary and subgingival plaque samples between AgP and CP.

Conclusion: Salivary analysis might be discussed as a potential alternative to subgingival plaque sampling for microbiologic analysis in both AgP and CP. *J Periodontol 2014;85:819-828.*

KEY WORDS

Bacteria; dental plaque; periodontitis; polymerase chain reaction; saliva.

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Periodontitis is an inflammatory disease mainly caused by specific bacteria, which lead to the breakdown of the periodontium and, ultimately, to the loss of the affected teeth. It is regarded as an infectious disease in which, under the influence of some risk factors, specific periodontopathic pathogens lead to the onset of periodontitis in certain susceptible individuals. These bacteria may be acquired by oral transmission of close family members.[1] These microorganisms in a biofilm trigger an excessive immune response, which plays an important role in the pathogenesis of periodontal disease. As part of the human oral microbiome, periodontopathic bacteria exist together with hundreds of other bacterial types in the oral cavity, and research into the numbers and varieties of pathogenic species is ongoing.[2] In recent decades, some specific bacteria, such as *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Treponema denticola* (*Td*), and *Tannerella forsythia* (*Tf*), have been identified to be strongly related to periodontitis.[3] Meanwhile, >1,000 bacterial species have been detected in the oral cavity, but their potential pathogenicity to periodontal tissues is still unclear.[4]

Periodontopathic bacteria can be detected by different methods, such as culture,[5] light microscopy,[6] electron microscopy in combination with in situ hybridization,[7] checkerboard DNA–DNA

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hybridization,[8] polymerase chain reaction (PCR),[9] or real-time PCR.[10] For the dental practitioner, semiquantitative analysis is considered to be sufficient for the interpretation of the microbial load, and therefore a common method to determine bacteria is currently by paper-point sampling of subgingival plaque and PCR analysis.[11] Recently, commercially available microarray assays for the oral cavity were regarded to be advantageous because of their high sensitivity, which allows for the detection of subtle differences compared to other assays.[12]

Until now, the cluster model of Socransky et al.[3] has been regarded as valid, and the main focus is on the red and orange complexes that are strongly associated with periodontitis. On the one hand, the mere presence of these bacteria does not instigate the development of periodontitis because greater amounts of such bacteria and host and risk factors play crucial roles in the pathogenesis of this disease.[13,14] On the other hand, periodontal disease severity was discussed to be associated with salivary levels of periodontal pathogens.[15] It has also been shown that, after a successful periodontal treatment, periodontopathic bacteria were diminished or even eradicated, and treatment failure was associated with bacteria that invaded tissues, such as *Aa* or *Pg*.[16] Destroying the biofilm by removal of subgingival plaque deposits and infected root cementum is the generally accepted treatment for periodontitis.[17] Mechanical therapy of periodontitis alone is not able to eliminate bacteria, such as *Aa*, *Pg*, *Tf*, *Td*, *Prevotella intermedia* (*Pi*), or *Fusobacterium nucleatum* (*Fn*).[18] In addition to this mechanical approach, antibiotics are prescribed in some cases of aggressive (AgP) or severe chronic (CP) periodontitis. Inappropriate antibiotic treatment may affect human microbial ecology in a negative way and favors resistance development among serious pathogens; thus, the choice of the optimal antibiotic regimen should be based on microbial analysis.[19] Therefore, it is highly recommended that the microbial flora be assessed by taking subgingival plaque samples before adequate antibiotics are chosen as an adjunct to mechanical periodontal therapy.

The utility of microbiologic testing for disease diagnostics or as an indicator of healing and disease, however, has not been proven conclusively by available evidence.[20] Most commonly, pooled paper-point sampling of the four deepest periodontal pockets is performed, and the presence of the red and orange complexes as well as *Aa* is investigated by semiquantitative PCR. Previous methods involved bacterial culture or the use of checkerboard DNA–DNA hybridization but lack applicability to daily clinical practice. Salivary sampling seems to be more advantageous because it does not require a patient being in the dental chair, and it can be easily collected at home. Moreover, periodontopathic bacteria in saliva have been successfully determined,[15] and a positive relationship of the presence of some species in whole saliva compared to that in periodontal pocket samples has already been revealed.[21] Previous studies used mostly culture techniques,[5] checkerboard DNA–DNA hybridization,[22] or real-time PCR,[23,24] which are disadvantageous for everyday clinical application. The aim of this study is to evaluate whether 20 different periodontopathic and periodontitis-related bacteria in saliva samples could be detected equally well by site-specific sampling and microarray-based PCR in AgP and CP. This could simplify the sampling procedure and the applicability of microbial testing in the dental practice.

MATERIALS AND METHODS

Study Population

The study population consisted of 43 patients with CP and 33 patients with AgP (48 males and 28 females [25 smokers and 51 non-smokers], aged 20 to 64 years; mean age: 42.20 ± 9.46 years). For periodontal diagnostics, probing depth (PD), clinical attachment loss (AL), and bleeding on probing (BOP) were recorded at six sites per tooth by experienced periodontists (HH, KB), using a periodontal probe,# from February 2010 to July 2012. Clinical data for the patients are given in Table 1. Bone loss was evaluated with intraoral and panoramic radiographs. Periodontitis was classified according to the American Academy of Periodontology classification 1999,[25] with age and pattern of disease as the main criteria for the AgP group. All patients had ≥20 teeth and suffered from a severe form of periodontal disease (supporting bone loss ≥30%),[25] with at least six teeth having PD ≥5 mm, had no periodontal or antibiotic treatment within the preceding 3 months, and had no injuries, bleeding, or any infection in the oral cavity. This study was approved by an amended ethics protocol, and all participants gave written consent for participation (Medical University of Vienna, EK623/2007).

Sampling

Salivary and subgingival plaque sampling was performed from 8:00 am to 11:00 am. Participants were required to refrain from eating, drinking, smoking, or brushing their teeth after midnight on the day of sampling. First, participants were seated and given a saliva extraction solution** for 2 minutes to actively rinse their mouth for stimulated whole saliva collection. After saliva collection, the individuals rinsed their mouth with water, and the deepest pocket

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Table 1.

Descriptive Statistics and Clinical Data of the Study Population Consisting of Individuals With AgP and Those With CP

Variable	AgP	CP	Total
N	33	43	76
Females (n)	13	15	28
Smoker (%)	27	37	33
Age (years [mean ± SD])	34.21 ± 6.20	48.33 ± 6.50	42.20 ± 9.46
Number of teeth (mean ± SD)	28.64 ± 2.60	27.21 ± 2.76	27.83 ± 2.76
Number of teeth with PD ≥5 mm (mean ± SD)	20.18 ± 7.61	19.93 ± 5.83	20.04 ± 6.61
PD (mm [mean ± SD])	3.87 ± 0.91	4.01 ± 0.93	3.95 ± 0.92
PD of sampling sites (mm [mean ± SD])	7.52 ± 1.13	7.19 ± 1.12	7.33 ± 1.13
AL (mm [mean ± SD])	4.39 ± 0.95	4.54 ± 1.21	4.48 ± 1.10
BOP (% [mean ± SD])	46.22 ± 24.82	40.82 ± 23.64	43.16 ± 24.15

of each quadrant was chosen for subgingival plaque sampling. The sites of collection were isolated with cotton rolls, and supragingival plaque was gently removed with curets and dried with air. Subgingival plaque was taken by the insertion of sterile paper points for 15 seconds, and the samples were pooled in a tube and frozen at –40°C before analysis.

Whole saliva was immediately transferred to tubes containing sodium azide against microbial growth and centrifuged (3,220 rpm, 4°C). Approximately 2 mL whole saliva was left in the transfer tube and frozen at –40°C until microbial analysis.

Microbiologic Analyses

The paper points were analyzed in the collection tube. Saliva in the transfer tubes was centrifuged, and the supernatant was transferred into 2-mL tubes. DNA was extracted by means of a DNA extraction kit.††

Protein kinase K, buffer solution, and EtOH were added, and tubes were centrifuged, vortexed, and heated at 95°C. Part of the 16S ribosomal RNA (rRNA) gene was amplified by a highly conserved primer pair flanking the diversity box of each 16S rRNA gene. A chip‡‡ was used for hybridization, followed by washes at 50°C and drying by centrifugation. Finally, the DNA chip was analyzed by a scanner,§§ and the signals were scored as: –, (+), +, ++, or +++, depending on the signal/noise ratio provided by the scanner. The designations (+), +, ++, and +++ are of a semiquantitative nature describing the bacterial load in the sample. The signals were analyzed by specific analytic software,‖‖ and graphs were drawn using data visualization software.[26]

Statistical Analyses

Descriptive statistics were applied for the characterization of the study population. Agreement of overall positive semiquantitative and negative results of bacteria between subgingival plaque and saliva were analyzed by sensitivity, specificity, and κ tests. Sensitivity and specificity were calculated for the presence and absence of bacteria in salivary and subgingival plaque samples, with the conventional paper-point sampling technique as the gold standard. For each bacterium, a logistic model was calculated to analyze the effect of mean PD on disagreement versus agreement of results in saliva and the periodontal pocket, adjusted for potential confounders (smoking, age, sex, diagnosis, number of teeth, number of teeth with PD ≥5 mm). *P* values <0.05 after adjustment for multiple testing by the Holm method were considered significant. All computations were done with statistical software.¶¶

RESULTS

All bacterial species were detected in saliva and subgingival plaque with an agreement of >90% between the two sampling methods for certain bacteria (*Td*, *Tf*, *Parvimonas micra* [*Pm*], *Fn*). For the major periodontal pathogens *Aa*, *Pg*, *Td*, and *Tf*, the sensitivity of detection in saliva compared to subgingival plaque ranged from 68% to 96%, and the specificity

†† ParoCheck, Greiner Bio-One.
‡‡ ParoCheckChip, Greiner Bio-One.
§§ CheckScanner, Greiner Bio-One.
‖‖ Check Report Software v.4.0.2, Greiner Bio-One.
¶¶ R.2.15.2, R Core Team, R Foundation for Statistical Computing, Vienna, Austria.

ranged from 82% to 100%. For the other species, there was great variability in sensitivity and specificity of detection. The analysis of *Aa*, *Pg*, *Tf*, and *Td*, as well as *Actinomyces viscosus* (*Av*), *Campylobacter rectus* (*Cr*)/*showae* (*Cs*), *Pi*, *Pm*, *Eubacterium nodatum* (*En*) and *Campylobacter gracilis* (*Cg*) showed a fair-to-moderate agreement between salivary and subgingival plaque samples according to the κ coefficient but was statistically significant (Table 2).

The total sum of the frequency of detection (pocket versus saliva) of *Td* (AgP, 97% versus 91%; CP, 100% versus 95%), *Tf* (AgP, 97% versus 91%; CP, 100% versus 98%), *Cr, Cs* (AgP, 85% versus 73%; CP, 84% versus 77%), and *Cg* (AgP, 55% versus 43%; CP, 77% versus 58%) was slightly higher in subgingival plaque samples compared to that in the salivary samples. For the detection of the other 16 bacterial species, saliva was superior to subgingival plaque. Interestingly, *Streptococcus mitis* (*Sm*), *Veillonella parvula* (*Vp*), and *Actinomyces odontolyticus* (*Ao*) were found in high amounts in saliva in patients with AgP and patients with CP (Figs. 1 through 4). The agreement of bacteria in salivary and subgingival plaque samples for *Tf* and *Td*, as well as *En* was significantly influenced by mean PD in unadjusted *P* values ($P<0.05$), but adjustment for multiple testing leaves this as only a trend toward significance.

There was no significant difference in the presence or absence of bacterial species among patients with AgP and those with CP in salivary and subgingival plaque samples.

DISCUSSION

Salivary analysis is a promising diagnostic tool for periodontal disease because of the ease of saliva collection and the possible detection of bacteria in

Table 2.

Agreement, Sensitivity, and Specificity of Bacterial Species Detected in Salivary and Subgingival Plaque Samples in All Patients (κ Test)

Bacterial Species	Agreement (%)	Sensitivity	Specificity	κ	*P* Value
Aa	78.95	68.42	82.46	0.4754	<0.001
Pg	86.84	88.14	82.35	0.6504	<0.001
Td	94.74	94.67	100	0.3184	<0.001
Tf	96.05	96	100	0.3871	<0.001
Av	76.32	85	43.75	0.2875	0.0488
Cr, Cs	85.53	85.94	83.33	0.5600	<0.001
Ec	48.68	93.75	15.91	0.0841	0.4955
Pi	85.53	90.74	72.73	0.6433	<0.001
Pm	92.11	98.51	44.44	0.5318	<0.001
Fn	98.68	100	0	0.0000	1
Ao	71.05	100	4.35	0.0596	0.3795
Capnocytophaga spp.	71.05	96.36	4.76	0.0153	1
Campylobacter concisus	42.11	90.91	33.85	0.0957	0.3443
En	72.37	86.84	57.89	0.4474	<0.001
Streptococcus constellatus group	31.58	78.95	15.79	−0.0297	1
Cg	76.32	70.59	88	0.5220	<0.001
Sm group	68.42	100	0	0.0000	—
Prevotella nigrescens	67.11	54.05	79.49	0.3375	1
Streptococcus gordonii group	50.00	5.13	97.3	0.0237	0.7715
Vp	78.95	100	0	0.0000	—

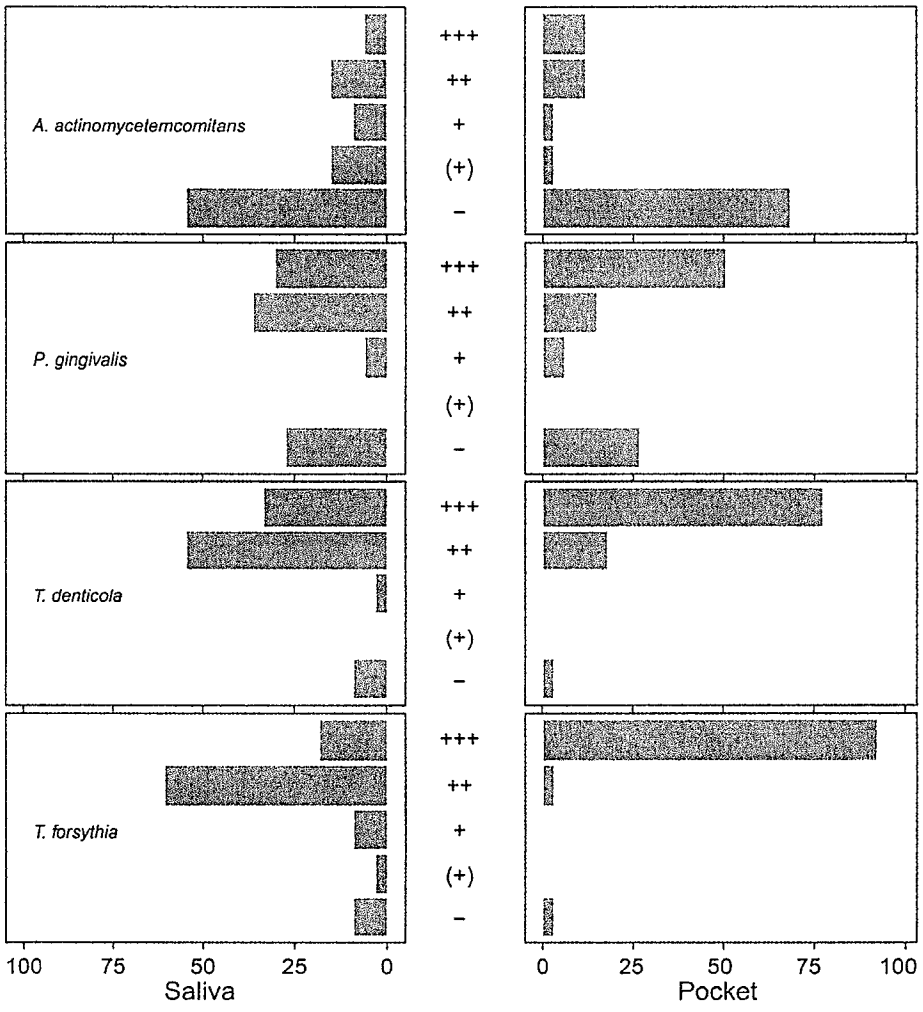

Figure 1.
Percentage of detection levels in saliva versus periodontal pockets in AgP (n = 33) of the major periodontopathic bacteria.

this biologic fluid. Former studies have already shown that bacterial testing in saliva is effective and may replace other well-established but more intricate methods of microbial analysis in periodontal disease.[21]

In the present study, 20 different bacterial species are measured in both salivary and subgingival plaque samples by means of a microarray-based 16S rRNA-based PCR. This semiquantitative determination of bacteria by PCR was shown to be more available for diagnosis, treatment schedule, and control of patients with periodontitis compared to real-time PCR.[27] It was also reported that PCR-based chip technology seems more advantageous than conventional microbial culture techniques because of the high sensitivity of detection and the simplicity and convenience of clinical application.[5] Concerning the detection of periodontopathic bacteria in saliva, cell culture, checkerboard DNA–DNA hybridization technique, as well as semiquantitative and quantitative PCR have been used in recent years. Von Troil-Lindén et al.[5] showed that *Aa*, *Pg*, *Pi*, *Pm*, and *Cr* could be determined in the saliva of patients with periodontitis by cell culture methods. Furthermore, Darout et al.[28] reported that 12 different periodontitis-associated bacteria and 15 cariogenic and dental-health-related species were detected in the saliva of the study population by checkerboard DNA–DNA hybridization, with a range of detection from 10^5 to 10^6 cells per probe. Sakamoto et al.[29] demonstrated that by real-time PCR, *Aa*, *Pg*, and *Tf* were detectable in the saliva of healthy individuals and those with periodontitis, with a detection level from 10^8 to 10^9 cells per probe. The advantage of microarray-based PCR used in the present study compared to previously described methods is its high sensitivity, with a detection limit of 1 to 5×10^2 cells per probe.[30]

For the assessment of bacterial species in salivary and subgingival plaque samples, some studies found that *Aa*, *Pg*, *Td*, *Tf*, *Fn*, *Pm*, *Cr*, *Vp*, *Streptococcus mutans*, *Streptococcus anginosus*, *Streptococcus salivarius*, *Lactobacillus acidophilus*, *Prevotella* species, *Leptotrichia* species, *Capnocytophaga* species, *Bacteroides gracilis*, *Prevotella loescheii*, spirochetes, and Gram-positive and -negative cocci and rods were present in both salivary and subgingival plaque samples.[21-24,31-35] However, the findings in these studies were heterogeneous in the quantitative and qualitative analyses of bacteria in both samplings because different techniques were used, such as culture, arbitrarily primed PCR, conventional PCR, real-time PCR, or checkerboard DNA–DNA hybridization. Other reasons for the discrepancies of results were probably attributable to the different study populations and different methods of saliva sampling.

In the present study, by using microarray-based PCR, 20 of the major periodontopathic and periodontitis-related pathogens in both salivary and subgingival

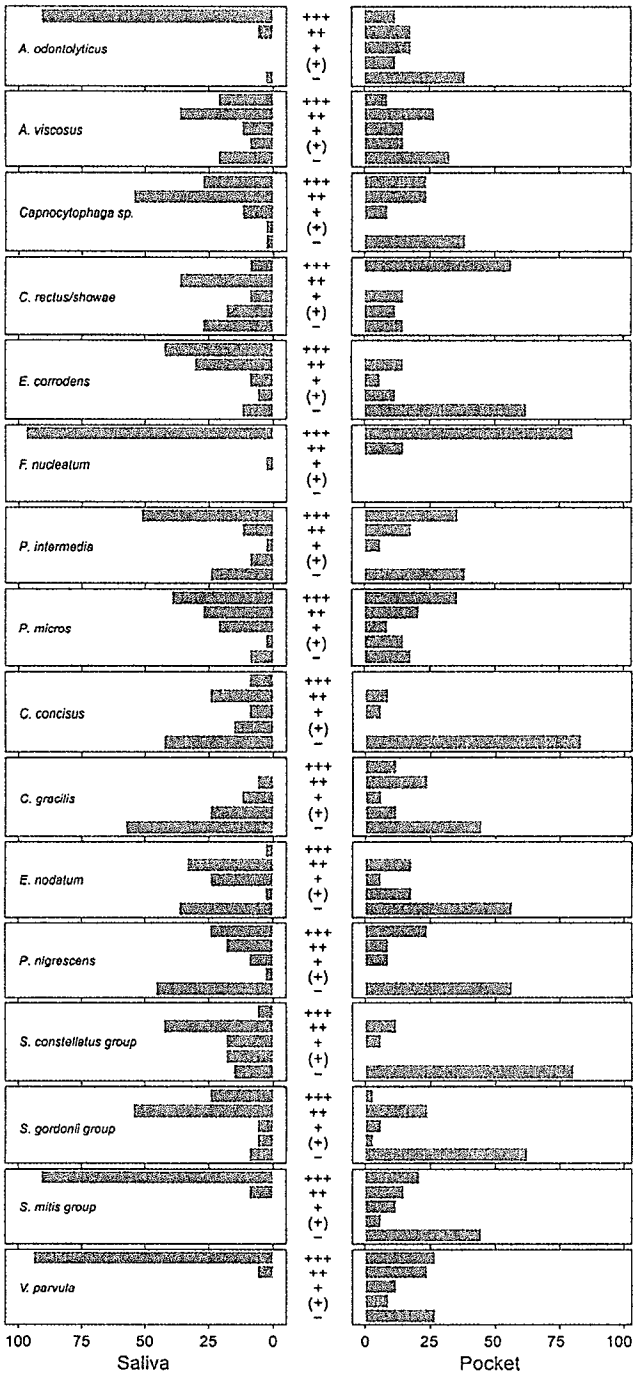

Figure 2.
Percentage of detection levels in saliva versus periodontal pockets in AgP (n = 33) of 16 other bacterial species than the major periodontopathic bacteria.

plaque samples are found, most of which were comparable with the findings of Umeda et al.,[21] Boutaga et al.,[23] or He et al.[24] However, most of these studies focused on only some periodontopathic bacteria in CP. Only two studies differentiated between AgP and CP, with the determination of a limited number of bacterial species.[21,33] Mombelli et al.[36] reviewed 11 papers comparing five bacterial species (*Aa*, *Pg*, *Tf*, *Cr*, and *Pi*) in subgingival plaque in patients with AgP and patients with CP and concluded that the presence or absence of periodontal pathogens cannot distinguish between CP and AgP. In agreement with this conclusion, the present results show that there was no significant difference in all 20 bacterial species among the patients with AgP or CP in both salivary and subgingival plaque samples.

The present study demonstrates, among 20 species, the significant correlation in salivary and subgingival plaque samplings of *Aa*, *Pg*, *Td*, and *Tf*, as well as *Av*, *Cr*, *Cs*, *Pi*, *Pm*, *En*, and *Cg*. In particular, *Aa*, which has been described to be associated with AgP,[37] was present in saliva in 45% of patients with AgP and 19% of patients with CP, as well as 30% and 21%, respectively, in the periodontal pocket. Similar results have been reported previously with a detection frequency for *Aa* of <50% in saliva and subgingival plaque of patients with periodontitis.[24,35] Regarding the correlation of *Pg*, *Tf*, *Td*, and *Pi* in saliva and subgingival plaque, the present findings support the results of previous studies.[21,23,24,32,33] In contrast to findings from Umeda et al.,[21] however, a fairly good agreement for the concomitant presence of *Aa* or *Tf* was demonstrated.[21]

The red complex in biofilms is well known to be strongly pathogenic for periodontitis, but other periodontitis-associated bacteria have been discussed recently. Lovegrove[38] found that, in addition to the red complex, *Cr*, *En*, *Pm*, and *Streptococcus intermedius* in subgingival plaque samples were also associated with periodontal disease. In patients with periodontitis, not only red complex but also other bacteria, such as *Fn*, were found in 100% of samples of both saliva and subgingival plaque. With coaggregation properties and an immunosuppressive role, it seems to be a key species in the periodontopathic biofilm.[39] In addition, a high percentage (85%) of *Eikenella corrodens* (*Ec*) in the saliva of patients with periodontitis was shown.

In recent decades, the relationship between oral infection, especially periodontitis, and systemic diseases has been addressed.[40] The presence, to a high extent, of various bacterial species in saliva could also be related to other oral and systemic diseases. For example, in the present study, *Av*, a well-known cariogenic bacterium,[41] is also detected in high amounts in both saliva and subgingival plaque of the periodontitis population. Interestingly, the overall presence of *Actinomyces* in saliva in the present study population may also imply a risk for actinomycotic bacteraemia, which was more

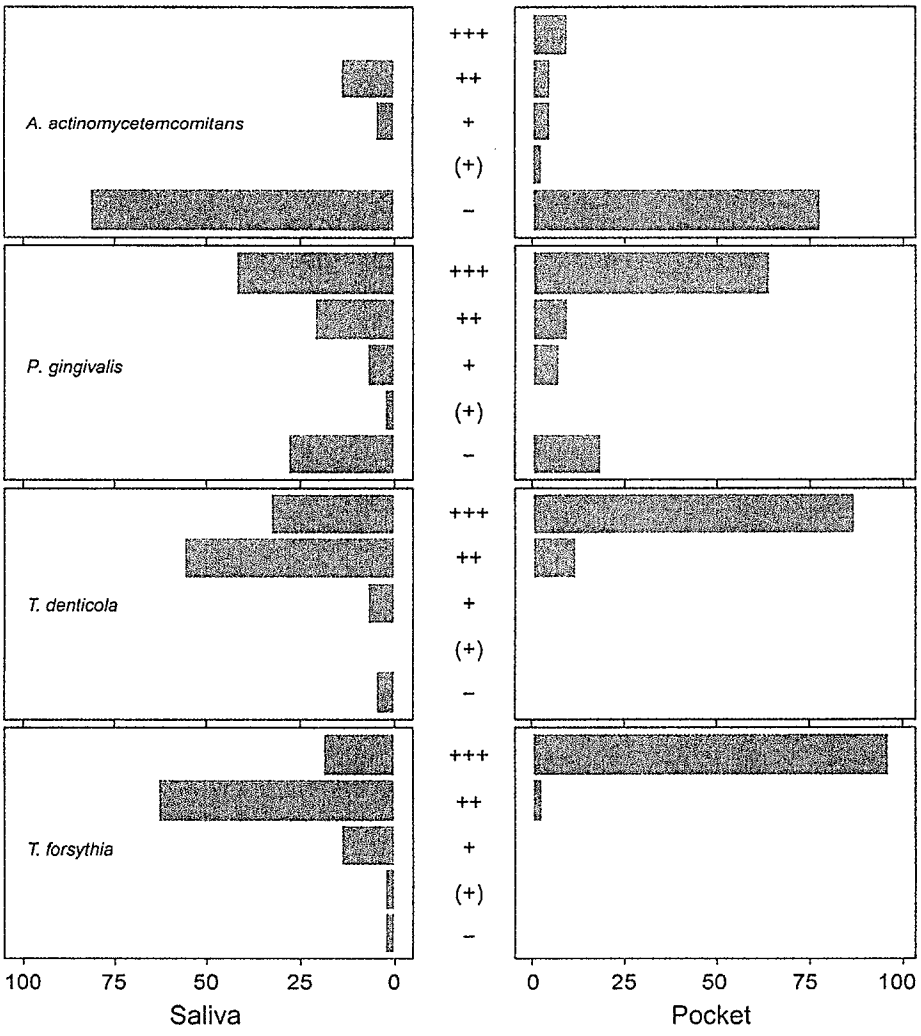

Figure 3.
Percentage of detection levels in saliva versus periodontal pockets in CP (n = 43) of the major periodontopathic bacteria.

commonly seen in patients with periodontitis.[42] It is notable that a high amount of *Sm* was detected in the saliva of patients with periodontitis, because *Streptococcus* species are related to the transport of endocarditis from the oral cavity to the heart by bacteraemia.[43]

On the one hand, it has already been shown that occurrence rates and median pathogen levels significantly differed among groups of periodontally healthy individuals and people with periodontal disease.[44] However, bacterial counts in saliva are still unable to quantify the severity of disease in an individual.[45] Neither the limited number of bacteria tested in saliva nor that in subgingival plaque can reflect the infection of the periodontium.[46] On the other hand, it is not reliable to diagnose periodontal disease only by the determination of bacteria.[47] In the present study about periodontopathic bacteria in a cohort of 44 periodontally healthy individuals, the prevalence of the red complex in saliva and gingival sulcus appears up to 25% (data not shown), which is lower than the results of patients with periodontitis.

The present study demonstrates that, among 20 bacteria, only *Tf*, *Td*, and *En* in subgingival plaque and saliva are correlated significantly with mean PD but not BOP. This supports a previous contention that a shortcoming of bacterial testing in saliva for periodontal disease screening can be attributable to the fact that the disease is chronic and progressive.[46] So far, bacterial testing in saliva may be used not primarily for diagnostic reasons but more as an indicator for an individual's risk potential to develop periodontal disease or caries.

Because bacteria are constantly washed out into the saliva from the periodontal pockets and their presence can be detected to a high extent in this fluid,[29] it was hypothesized that the analysis of saliva could be used conveniently in dental practice. In particular, *Aa* was detected more frequently in saliva than in the periodontal pocket, which could be attributable to the facultative anaerobic nature of *Aa*. Interestingly, *Aa* was not significantly different between AgP and CP in the present study.

The present results demonstrate that the determination of bacteria in saliva is partially representative of their presence in subgingival plaque. This is inconsistent with a previous report that the numbers and types of bacteria in saliva are less representative of the subgingival microbiota and that their proportions and prevalence are different at the two locations.[28] Testing bacteria in saliva may also have an implication for evaluating the transmission of periodontopathic bacteria through saliva and in the decision-making process for the prescription of antibiotics. Salivary microbial testing can also be advantageous in ensuring that

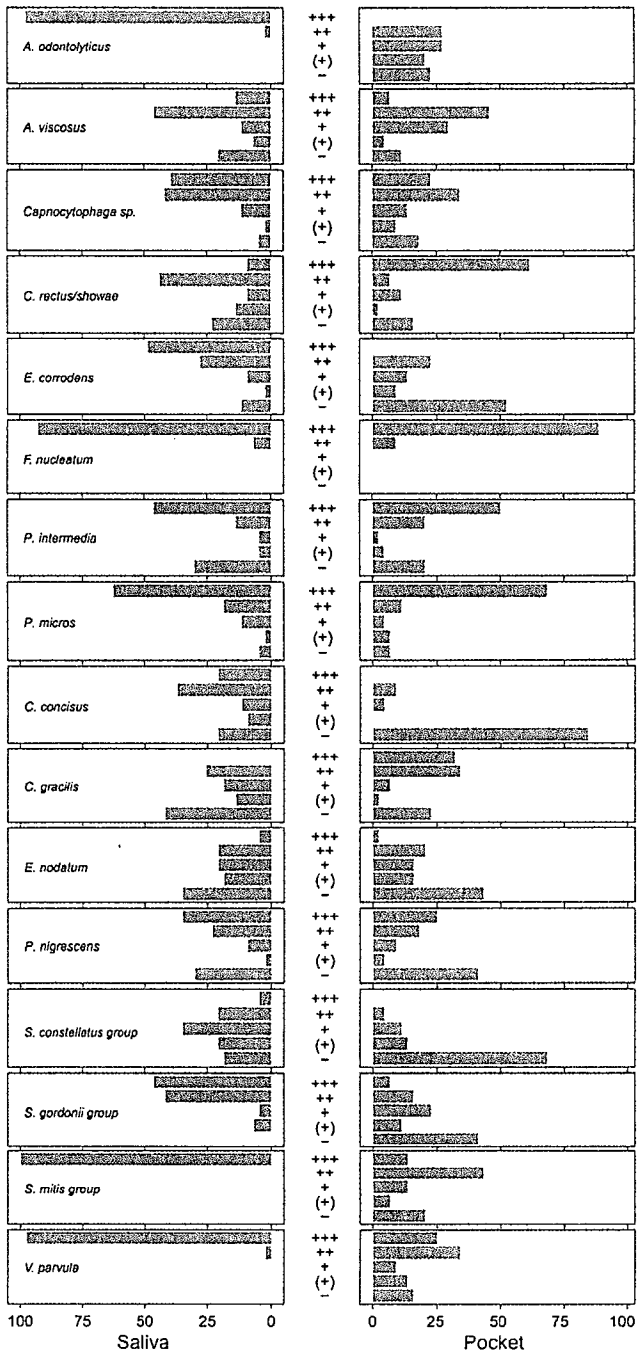

Figure 4.
Percentage of detection levels in saliva versus periodontal pockets in CP (n = 43) of 16 other bacterial species than the major periodontopathic bacteria.

periodontal pathogens have been eliminated or reduced after the completion of periodontal therapy.[48] Salivary analysis of different microbial species may provide clinicians with information about the risk and status of bacteria-related diseases, especially periodontal disease.

CONCLUSIONS

The present study indicates that the identification of the major periodontopathic bacteria as well as periodontitis-associated species in saliva by microarray-based PCR might represent an easier and more efficient method to the already established subgingival plaque–sample collection method. There were no significant differences in microbiologic findings between patients with AgP and those with CP.

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