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Summary

The present clinical study aimed to investigate if there are differences in microbiological results dependent on the subgingival biofilm collection method. Subgingival biofilm samples were collected from the four deepest pockets (>5 mm) of 17 patients with aggressive periodontitis (AgP) and 33 patients with chronic periodontitis (CP), firstly by paper point and thereafter by curette. Samples obtained with the same method were pooled together from each patient and forwarded for molecular microbiological analysis by a commercially available assay (IAI Pado Test 4.5) that estimates total bacterial load and levels of *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*. Data analysis included frequency of detection, quantification and correlation of detection levels between the two sampling methods. *P. gingivalis*, *T. forsythia*, *T. denticola* were detected in >90 % of the samples, and their detection levels exhibited strong correlation between sampling methods. The detection consistency of *A. actinomycetemcomitans* was 56% between the two sampling methods. *A. actinomycetemcomitans* was more readily detected by paper point compared with curette collection with a stronger correlation between the two methods in AgP. Subgingival biofilm sampling by curette or paper point do not yield differences in the detection of the three "red complex" species. However, *A. actinomycetemcomitans* was more consistently detected by means of paper point collection, which can be crucial in the decision to administer antibiotics as an adjunctive periodontal treatment.

Keywords: Biofilm, periodontal microbiota, periodontitis, molecular diagnostics, sampling

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Introduction

The microbial aetiology of periodontitis is well established. It is highlighted by the involvement of Gram-negative species, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* (1,2). The presence of these bacteria in elevated numbers may constitute an indication for prescribing antibiotics as an adjunctive modality to the treatment plan (3). The decision to use systemic antibiotics as part of periodontal therapy has to be made cautiously. Apart from the risk of adverse reactions, extensive use of systemic antibiotics may contribute to the ever increasing incidence of antibiotic-resistant strains, which is a major risk to public health (4). Hence, antibiotics should be administered only after proper patient evaluation, which may include microbiological analysis, particularly of periodontal sites with residual disease activity following conventional periodontal treatment (5,6). The variety of periodontal pathogens still harboured in those sites may increase the risk of site re-infection and the resurge of disease activity. Systemic antibiotics may be particularly beneficial when *A. actinomycetemcomitans* is detected in high levels, as this pathogen has the capacity to invade or colonise periodontal tissues or colonize extra-dental domains from where they may translocate to periodontal sites (7).

Crucial considerations for microbial diagnostics in periodontology are the sampling procedures as well as the detection methodologies employed. The most widely utilized collection methods of subgingival biofilms from the periodontal pocket are by means of curettes or paper points (8-10). Molecular methodologies have been developed for the detection of periodontal pathogens and oral microbiota in general, including polymerase chain reaction (PCR)-based techniques, oligonucleotide hybridization techniques and high-throughput genomic sequence platforms (11). Based on these, commercially available tests have also been developed that would assist periodontal diagnostics. One such is the 16S rRNA-based IAI Pado Test 4.5 (Zuchwil, Switzerland) (12-14). The test enables a quantitative

analysis of subgingival biofilm samples for total bacterial loads, *A. actinomycetemcomitans* and the three "red complex" (15) species, namely of *P. gingivalis, T. denticola* and *T. forsythia*.

The primary aim of the present study was to investigate differences between two subgingival biofilm sampling techniques, namely collection by curettes or paper points, both evaluated by the IAI Pado Test 4.5.

Material and methods

Study population and clinical examination

This study included 50 consecutive subjects who were diagnosed and treated from April 2011 to August 2011 as part of the postgraduate programme in Periodontology of the Clinic of Preventive Dentistry, Periodontology and Cariology, Center of Dental Medicine, University of Zürich, Switzerland. The study was conducted in accordance to the Helsinki Declaration and each subject provided informed consent for the use of their data for research or teaching purposes. Data was analyzed anonymously.

The patients were diagnosed with either chronic periodontitis (CP), or aggressive periodontitis (AgP), scheduled for a systematic periodontal treatment. The diagnostic criteria of CP or AgP were based on the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (16). None of the patients had received antibiotics or periodontal treatment for a period of at least six months prior to sampling.

Collection of microbiological samples

The collection of subgingival biofilm samples and microbiological analysis was part of the standard clinical examination and treatment planning. For each subject, subgingival biofilm samples were collected from the four deepest sites (pocket depth ≥5 mm) that did not exhibit pus secretion as observed by prior clinical examination. The subgingival biofilm samples for

microbiological analyses were collected by two different methods, namely the paper point and the curette collection method. Before sampling, the teeth were isolated from the cheek and tongue with cotton rolls and the supragingival surface was cleaned with rubber cups and polishing paste. Care was taken not to provoke any bleeding in the adjacent tissues. First, a paper point was quickly inserted down to the base of the pocket and left there for ten seconds before subsequent removal. Afterwards, a Gracey curette was gently inserted into the same site of the pocket and further subgingival biofilm was collected with a single stroke. The adhering material was wiped off on another sterile paper point. All samples collected by the same method were pooled together for each patient and shipped in separate transport test tubes, each containing 100 µL of guanidine buffer. All participating clinicians were trained in advance to perform the sample collection in a standardized manner.

Microbiological analysis

On the day of sampling the tubes containing subgingival biofilms were sent for microbiological analysis (Institut für Angewandte Immunologie IAI AG, Zuchwil, Switzerland). An RNA-based assay (IAI Pado Test 4.5) was applied that allows the detection and quantification of bacterial ribosomal 16S rRNA. This enables the quantification of total bacterial load using complementary universal bacterial DNA-probes as well as the specific quantification of *A. actinomycetemcomitans, T. forsythia, P. gingivalis* and *T. denticola* using taxon-specific probes. A reliable estimate of the viable bacterial cells is achieved based on the fact that, in contrast to DNA, RNA is rapidly degradable. The values provided are estimates of bacterial numbers, based on the estimated ribosome content per bacterial cell.

Statistical analysis

As a "pooled" analysis was chosen for both sampling methods, a site-specific analysis was not further possible. The mean differences between the sampling groups (paper point versus curette) were compared with the non-parametric Wilcoxon matched pairs test, for each of the taxa considered and the total bacterial load. The correlation between bacterial numbers obtained by the two sampling methods was investigated by the Spearman's correlation analysis, and the r correlation coefficient is provided. Statistically significant differences were considered at $P<0.05$.

Results

Thirty three patients with CP (18 male, 15 female) and 17 patients with AgP (8 male, 9 female) were included in this study. As a first step, the frequency of detection of the four key species was investigated. The descriptive statistics (Table 1) indicate that the three "red complex" species were detected in the majority of samples (92%) irrespective of the sampling method. There was a detection consistency of 96% between the two methods. Interestingly, this was not the case for *A. actinomycetemcomitans*, for which consistency was only 56% (36% positive and 20% negative by both methods).

The average bacterial counts obtained by paper point or curette sampling method did not show significant differences being at the range of 8×10^7 (Table 2). Likewise, no significant differences were detected in the numbers of *A. actinomycetemcomitans* with the two sampling methods. However, each one of the three "red complex" species were detected at significantly higher levels by the paper point compared to the curette sampling, but numerically the difference was moderate (30% to 42%). Of note, *A. actinomycetemcomitans* was detected at lower levels compared to the other three tested taxa.

There was no significant correlation between total bacterial numbers obtained by the two sampling methods (Table 3), but there was a strong positive correction between the numbers of each of the "red complex" species. For *A. actinomycetemcomitans* numbers, there was only a marginal correlation between the methods ($P = 0.046$).

The correlation of *A. actinomycetemcomitans* levels between the two sampling methods was further investigated, taking the clinical diagnosis into consideration (Table 4). For samples obtained from CP patients no correlation was found ($r = 0.26$, $P = 0.14$) whereas it reached statistical significance for AgP patients ($r = 0.49$, $P = 0.04$). Moreover, *A. actinomycetemcomitans* was detected at higher levels in AgP compared to CP with a 2.3-fold difference for curette sampling and a 4.4-fold difference for paper point sampling, respectively (Table 4).

Discussion

The present study used a commercially available molecular detection assay in order to evaluate the agreement of the microbiological findings obtained by the two traditional subgingival biofilm sampling methods, namely curette and paper point sampling. Of note, this assay is routinely used in Switzerland for periodontal microbiological diagnostics and it can help filter the indication for antibiotics usage in periodontal treatment, if not supported by the microbiological findings. Earlier studies using conventional bacterial culture and phase contrast microscopy have reported significantly higher total numbers of colony forming units and spirochetes by collection with paper point compared to collection by curette (17). Molecular detection methods, such as real-time PCR, demonstrate a good agreement with conventional bacterial cultures in the detection of putative periodontal pathogens, such as *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* (18). Subgingival sampling by curette is more likely to achieve an efficient collection of bacteria from the tooth-adherent biofilm,

and an overall representation of the pocket microbiota. On the other hand, collection by paper point is more likely to provide a better representation of the outer biofilm layer or the "free-floating" bacteria in the pocket (19). The present findings indicate a very high frequency of detection (≥96%) of all three "red complex" species, and a strong positive correlation between values obtained with the two methods. Although quantitative differences were found for these bacterial species when the two sampling methods were compared, they were within a range of 30% to 42%. Higher levels were consistently detected in samples collected by paper point compared to curette. A likely explanation is that the three "red-complex" species are late colonizers, and hence located at the outermost layer of the biofilm (20), or even potentially detached from it, making them more readily collected by paper point rather than by curette[19]. In this sense, detection of *P. gingivalis*, *T. forsythia* and *T. denticola* in a periodontal pocket is not dependent on the sampling method. Another study of the two sampling methods has led to the same conclusion applying quantitative real-time PCR to a range of bacterial species including *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, and *T. forsythia* (8). In a more recent study using quantitative real-time PCR, bacterial loads were detected at similar levels in paper points and paper strips, although the latter was more optimal for the joint determination of microbiological and immunological parameters (9). Collectively, the findings from these studies are in line with a study using checkerboard DNA-DNA hybridization, demonstrating that consecutive subgingival biofilm samples from the same pockets exhibit similar microbiological profiles (21).

The present study also demonstrates that *A. actinomycetemcomitans* is detectable at lower levels than the other three taxa. Moreover, the detection consistency of *A. actinomycetemcomitans* after sampling the same pocket by curette or paper point was 56%, with a marginally significant correlation of levels detected with the two methods. The limited consistency of findings between the two sampling methods could be attributed to levels of *A. actinomycetemcomitans* close the detection limits of the diagnostic method (10^4 copies per

sample). Collectively, these findings may indicate that the presence of the three "red complex" species can be predictably defined by both sampling methods, but this is less likely to be the case with *A. actinomycetemcomitans*. Nevertheless, when the clinical diagnosis was taken into account, levels of *A. actinomycetemcomitans* significantly correlated between the curette and paper sampling for patients with AgP, but not for patients with CP. This may not be surprising, since *A. actinomycetemcomitans* is generally found at higher levels in AgP (7). This finding was also confirmed in the present study.

In conclusion, the present study indicates that sampling by curette or paper point do not yield considerable differences in the detection of the three "red complex" species. However, collection by paper point method can detect the presence of *A. actinomyceteomcomitans* more consistently, which can be crucial in the decision to administer antibiotics as adjunctive periodontal treatment.

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References

1. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998 25: 134-44.

2. Socransky SS, Haffajee AD, Dzink JL. Relationship of subgingival microbial complexes to clinical features at the sampled sites. J Clin Periodontol 1988 15: 440-4.

3. Herrera D, Alonso B, Leon R, Roldan S, Sanz M. Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm. J Clin Periodontol 2008 35(8 Suppl): 45-66.

4. Holmes NE, Howden BP. The rise of antimicrobial resistance: a clear and present danger. Expert review of anti-infective therapy 2011 9: 645-8.

5. Slots J. Primer for antimicrobial periodontal therapy. J Periodontal Res 2000 35: 108-4.

6. Slots J. Selection of antimicrobial agents in periodontal therapy. J Periodontal Res 2002 37: 389-98.

7. Slots J, Ting M. Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment. Periodontol 2000 1999 20:82-121.

8. Jervoe-Storm PM, Alahdab H, Koltzscher M, Fimmers R, Jepsen S. Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. J Periodontol 2007 78: 909-17.

9. Guentsch A, Kramesberger M, Sroka A, Pfister W, Potempa J, Eick S. Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. J Periodontol 2011 82: 1051-60.

10. Zambon JJ. Principles of evaluation of the diagnostic value of subgingival bacteria. Ann Periodontol 1997 2: 138-48.

11. Paster BJ, Dewhirst FE. Molecular microbial diagnosis. Periodontol 2000 2009 51:38-44.

12. Cosgarea R, Baumer A, Pretzl B, Zehaczek S, Kim TS. Comparison of two different microbiological test kits for detection of periodontal pathogens. Acta Odontol Scand 2010 68:115-21.

13. Mettraux G, Husler J. [Implementation of transgingival antibacterial photodynamic therapy (PDT) supplementary to scaling and root planing. A controlled clinical proof-of-principle study]. Schweiz Monatsschr Zahnmed 2011 121 :53-67.

14. Luterbacher S, Mayfield L, Bragger U, Lang NP. Diagnostic characteristics of clinical and microbiological tests for monitoring periodontal and peri-implant mucosal tissue conditions during supportive periodontal therapy (SPT). Clin Oral Implants Res 2000 11: 521-9.

15. Haffajee AD, Socransky SS, Patel MR, Song X. Microbial complexes in supragingival plaque. Oral Microbiol Immunol 2008 23: 196-205.

16. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999 4: 1-6.

17. Renvert S, Wikstrom M, Helmersson M, Dahlen G, Claffey N. Comparative study of subgingival microbiological sampling techniques. J Periodontol 1992;63:797-801.

18. Jervoe-Storm PM, Koltzscher M, Falk W, Dorfler A, Jepsen S. Comparison of culture and real-time PCR for detection and quantification of five putative periodontopathogenic bacteria in subgingival plaque samples. J Clin Periodontol 2005 32: 778-83.

19. Loomer PM. Microbiological diagnostic testing in the treatment of periodontal diseases. Periodontol 2000 2004 34: 49-56.

20. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000 54: 413-37.

21. Teles FR, Haffajee AD, Socransky SS. The reproducibility of curet sampling of subgingival biofilms. J Periodontol 2008 79: 705-13.

Table 1. Frequency of detection of the targeted taxa according to sampling method

Taxa	PS / Cur (+/+)	PS / Cur (+/-)	PS / Cur (-/+)	PS / Cur (-/-)
A. actinomycetemcomitans	18/50 (36 %)	15/50 (30 %)	7/50 (14 %)	10/50 (20 %)
P. gingivalis	44/50 (88 %)	0/50 (0 %)	2/50 (4 %)	4/50 (8 %)
T. denticola	47/50 (94 %)	1/50 (2 %)	0/50 (0 %)	2/50 (4 %)
T. forsythia	48/50 (96 %)	1/50 (2 %)	1/50 (2 %)	0/50 (0 %)

Samples were collected from the four deepest pockets of each patient (n = 50), first by paper points (PS) and then by a curette (Cur). The collected PS samples, or Cur samples were then pooled together for further processing and analysis by the IAI Pado Test 4.5. The frequency of detection of the different taxa among the pooled samples is provided (+: detected, -: undetected), based on the two sampling methods.

Table 2. Mean estimate bacterial levels according to sampling method (x 10^6)

Taxa	PS	Cur		*P* value
TBL	81.26 ± 32.00	76.74 ± 33.20		0.26
A. actinomycetemcomitans	0.30 ± 0.81	0.24 ± 0.79		0.23
P. gingivalis	5.53 ± 5.92	3.90 ± 4.31	*	< 0.01
T. denticola	2.43 ± 1.72	1.82 ± 1.29	*	< 0.001
T. forsythia	4.88 ± 3.19	3.76 ± 2.43	*	< 0.01

The relative bacterial counts (x 10^6), estimated by the IAI Pado Test 4.5, are presented as mean ± standard deviation (SD) of the 50 samples. Results are presented for both the paper point (PS) and curette (Cur) sampling methods. The means differences between the two groups (PS Vs Cur) are compared with the non-parametric Wilcoxon matched pairs test. Asterisk (*) represents significant difference between the two groups (P<0.05). TBL: total bacterial load.

Table 3. Relative bacterial level correlations between the two sampling methods

Taxa	Spearman r coefficient		*P* value
TBL	0.17		0.23
A. actinomycetemcomitans	0.28	*	0.04
P. gingivalis	0.79	*	< 0.0001
T. denticola	0.73	*	< 0.0001
T. forsythia	0.49	*	< 0.001

The correlation between relative bacterial counts obtained by the two different sampling methods (paper point – PS, versus curette - Cur), was investigated by the Spearman's correlation analysis. The r correlation coefficients and respective *P* values are provided for each of the five bacterial comparisons. Asterisk (*) represents significant difference between the two groups ($P<0.05$). TBL: total bacterial load.

Table 4. Relative levels of *A. actinomycetemcomitans* and correlations between the two sampling methods, based on clinical diagnosis.

Diagnosis	PS (10^6)	Cur (10^6)	r coefficient	*P* value
CP	0.13 ± 0.30	0.15 ± 0.33	0.26	0.14
AgP	0.59 ± 1.25	0.37 ± 1.25	0.49 *	0.04

The relative counts of *A. actinomycetemcomitans* detected by the two different sampling methods (paper point – PS, versus curette - Cur) are provided. Within each diagnosis group, the correlation of *A. actinomycetemcomitans* numbers was investigated by the Spearman's correlation analysis (the r correlation coefficient and respective *P* values are provided). Asterisk (*) represents significant difference between the two groups ($P<0.05$).