Clin Oral Invest (2010) 14:533–541
DOI 10.1007/s00784-009-0333-x

ORIGINAL ARTICLE

Quantification of periodontal pathogens by paper point sampling from the coronal and apical aspect of periodontal lesions by real-time PCR

Pia-Merete Jervøe-Storm · Hazem AlAhdab · Max Koltzscher · Rolf Fimmers · Søren Jepsen

Received: 19 February 2009 / Accepted: 30 July 2009 / Published online: 20 August 2009

Abstract The present study compared the recovery of six periodontal pathogens by paper point samples from two different aspects of periodontal lesions by quantitative real-time polymerase chain reaction (PCR). Twenty patients with untreated chronic periodontitis were randomized into two groups. Before subgingival instrumentation and after 10 weeks samples in group A were taken first with a paper point half length (HP) of the probing depth, then with a paper point full length (FP) at the same site. In group B sampling sequence was reversed. Analysis by real-time PCR enabled quantification of six bacteria as well as total bacterial count (TBC). Statistical analysis included *t* test, Kappa, and Spearman's correlations. Higher TBC could be harvested by use of FP than by HP (mean of differences of ln-transformed counts before therapy: −0.791, CI [−1.515, −0.068], SD 0.770, p=0.034; after therapy: −0.563, CI [−1.151, 0.024], SD 0.625, p=0.059). The plaque composition regarding total target pathogens was similar for both samples. Both, for TBC as well as for single target bacteria a strong positive correlation was found between HP and FP (Kappa, Spearman correlation: *Aggregatibacter actinomycetemcomitans* 0.807, 0.778; *Fusobacterium nucleatum* 0.573, 0.772; *Porphyromonas gingivalis* 0.733, 0.824; *Prevotella intermedia* 0.480, 0.756; *Treponema denticola* 0.807, 0.814; and *Tannerella forsythia* 0.692, 0.695). The recovery of target pathogens was similar following sampling at various depths of the periodontal lesion.

Keywords Periodontal pathogens · Chronic periodontitis · Diagnosis · Sampling · Paper points · Quantitative polymerase chain reaction

P.-M. Jervøe-Storm (✉) · H. AlAhdab · S. Jepsen
Department of Periodontology,
Operative and Preventive Dentistry, University of Bonn,
Welschnonnenstraße 17,
53111 Bonn, Germany
e-mail: storm@uni-bonn.de

M. Koltzscher
Carpegen GmbH,
Münster, Germany

R. Fimmers
Institute for Medical Biometry, Informatics and Epidemiology,
University of Bonn,
Bonn, Germany

Introduction

Subgingival plaque bacteria of the periodontal pocket function as a coordinated spatially organized and metabolically integrated microbial community [1]. Histological sections of human subgingival plaque suggest a complex organization of attached microorganisms with distinct tooth-associated and epithelial cell-associated biofilms. Between these two more dense layers a third layer of presumably planktonic bacteria may be observed [2]. It is speculated that the different parts of the biofilm contain variable amounts of the periodontopathogenic bacteria [1]. On the basis of analysis of a large number of subgingival plaque samples, the paradigm of bacterial complexes has been developed [3]. It was suggested that the microbial complexes are found in specified locations in the subgingival plaque [4–8]. Since the tooth-associated biofilm is regarded as an extension of the biofilm found at the gingival margin, it may be quite similar in its composition [2]. The epithelial cell-associated biofilm contains large numbers of *Treponema denticola* and *Porphyromonas gingivalis.* It is strongly suspected that these regions differ markedly in their microbial composition, physiological

state, and response to different therapies [2]. It may be speculated that apically located microbiota may be more significant to the disease process than more coronally located microorganisms.

An elevated level of pathogenic bacteria in the periodontal crevice can initiate or increase periodontal breakdown. Earlier evaluations of the content of the periodontal pocket were limited by microbiological identification methods, that would preclude quantification and/or sensitive and specific identification of target pathogens. An analysis of the samples based on the method of quantitative real-time polymerase chain reaction (PCR) might possibly overcome these limitations and shortcomings. The evaluation with real-time PCR enables not only the identification of putative periodontopathogenic species, but also the calculation of their relative proportion of the total bacterial load [9, 10]. Identification of periodontopathogenic bacteria is important for further diagnosis and planning of adjunctive therapy [11].

To be able to evaluate the results of microbiological analysis reliable sampling methods are needed. Sampling of subgingival plaque is performed with various methods, of which curettes and paper points are the most commonly used [11, 12]. In a recent study, the absorbing ability of the paper point was compared with the "Pocket-out-method", based on collecting saliva, cell debris, bacteria, fungi, and viruses from the supra- and subgingival area by means of a swab [13]. Both methods gave similar results, demonstrating that paper points are mainly absorptive. Paper points are supposed to collect plaque from the outer layer of the plaque, which may contain more pathogens than the tooth-associated plaque [1].

The aim of the present study was to compare paper point samples from coronal and apical aspects of periodontal lesions to reveal potential differences in the recovery of six putative periodontal pathogens by quantitative real-time PCR.

Materials and methods

Patients

Twenty patients (53 years±9 of age; 10 females) with untreated chronic periodontitis were referred for treatment in the Department of Periodontology, Operative and Preventive Dentistry at the University of Bonn, Germany. Informed consent by the patients and a positive approval by the international ethics committee (Freiburg, Germany) had been obtained.

Inclusion criteria were (1) age between 18 and 70 years, (2) good general health, (3) at least one single rooted tooth with probing pocket depth≥6 mm and, (4) a low plaque score (PI≤20%; O'Leary et al. [14]). Exclusion criteria were (1) antibiotic therapy in the past 6 months, (2) periodontal therapy within the last 6 months, or (3) tooth extraction during the study.

Sampling procedure

The paper point sampling sites were determined at baseline 2 weeks before first samples were taken prior to deep scaling and root planing without use of antiseptics. Six weeks later a second clinical assessment was performed, followed by the same sampling procedure 2 weeks later.

The patients were randomized with a computer generated list into two groups (A and B) each with 10 patients. In group A probing pocket depths showed a mean of 7.1± 0.72 mm before therapy (baseline), after therapy (10th week) a reduction was obtained (5.4±1.23 mm). In group B mean probing pocket depth was 7.4±0.94 mm before therapy and 4.6±1.05 mm after therapy.

Sampling was performed in a standardized way. Briefly, selected lesions and the adjacent teeth were isolated with cotton rolls. Supragingival plaque was carefully removed with a sterile scaler to prevent the contamination of the samples. Sterile paper points ISO #40 (Co. Roeko, Langenau, Germany) were inserted for 20 s in the pocket and then immediately transferred into a sterile transport tube. In group A the first sample was taken using one sterile paper point, which was inserted to half length of the probing depth. The second sample was harvested from the same site as the first one, however, inserted to full length of the probing depth. In group B patients samples were taken in the opposite sequence. Plaque sampling was performed immediately before instrumentation and 8 weeks after instrumentation. All samples were obtained by the same dentist in order to standardize the sampling procedure.

Samples were sent to a specialized laboratory (Carpegen GmbH, Münster, Germany) for evaluation by real-time PCR (meridol®Perio Diagnostics, GABA International, Münchenstein, Switzerland) for detection and quantification of *Aggregatibacter* (former *Actinobacillus*) *actinomycetemcomitans*, *Fusobacterium nucleatum*, *P. gingivalis*, *Prevotella intermedia*, *T. denticola*, and *Tannerella forsythia* as well as total bacterial counts (TBC), as previously described [10].

The real-time PCR used in this study is DNA-probe based (TaqMan® -MGB probes, Applied Biosystems, Foster City, CA, USA), which allows highly specific identification of the respective target sequences. Specificity was validated with purified genomic DNA from several bacterial and fungal species as well as with human DNA. Even closely related species, such as *P. intermedia* and *Prevotella nigrescens*, did not show any cross-reactivity. Additionally, real-time PCR results were confirmed by DNA-sequencing of amplicons obtained from reactions with complex templates (e.g. patient

samples). Absolute quantification was done for each analysis-run by means of well-characterized plasmid standards and was verified by analyzing bacterial cultures of the six pathogens with defined cell numbers at different concentrations. These validation controls are repeated regularly, to approve a coefficient of variation for quantification below 15%. Putative inhibitions of the PCR reactions (e.g. due to poor template quality or purity) are controlled by internal control reactions to avoid false negative or downwardly deviating results.

Statistical analysis

A cross-over design was performed for plaque sampling before as well as after therapy randomly allocating to possible sequences: half length paper point–full length paper point and full length paper point–half length paper point to equally sized groups of patients. Bacterial counts were transformed to natural logarithms before analysis adding one to each count before transformation to avoid problems with zero counts. Differences between the bacterial counts with half and full length paper point sampling were analyzed with standard techniques for cross-over analysis [15]. The comparison between the two sampling techniques was made by a two sample t test applied to the period differences divided by two, compared between the two sequence groups (half length paper point minus full length paper point). Point estimators for the difference between both techniques and confidence limits were also revealed from this test. Carry-over effect was checked by comparing the period averages (sums) between both patient groups also using a two sample t test. In case of a carry-over effect, the estimator of the difference may be biased. The correlation between bacterial counts obtained with the two methods was analyzed using Spearman correlation coefficient. For measuring the agreement of the qualitative detection of different bacteria with both sampling techniques Cohen's Kappa was used. The significance level was set at $p<0.05$.

Results

Total bacterial counts

The distribution of TBC of half length and full length paper point samples in both groups is shown as box plots (Fig. 1). Full length paper points in both groups sampled always more total bacteria than half length paper points. In group A median counts of TBC at first sampling (before therapy) were higher than at the second sampling (after therapy) with both lengths of paper points. The same result was found for group B.

Selected target bacteria

A. actinomycetemcomitans

The median counts of *A. actinomycetemcomitans* were low due to the small number of positive sites found (Table 1).

F. nucleatum

The median counts of *F. nucleatum* in group A were reduced after therapy. In group B an increase in the median counts for *F. nucleatum* after therapy with both lengths of paper points was found. Before therapy as well in group A as in B, the relative proportions and the number of *F. nucleatum* positive sites was found higher with the first chosen length of paper point. After therapy the relative proportions were still found higher with the first chosen length of paper point; however, the same number of *F. nucleatum* positive sites was found in both subgroups (Table 1).

P. gingivalis

Median counts of *P. gingivalis* in group A were reduced after therapy; in group B an increase could be noticed. The relative proportions of *P. gingivalis* were always higher with full length paper points. After therapy the number of *P. gingivalis* positive sites was unchanged in group A and elevated in group B (Table 1).

P. intermedia

Median counts of *P. intermedia* in group A were reduced after therapy; in group B an increase could be noticed. Full length paper point findings of the relative proportions of *P. intermedia* in group A were elevated after therapy; in group B no change was seen. Half length paper point results were in both groups reduced after therapy, though more pronounced in group B. No changes of the number of positive sites were found in both groups (Table 1).

T. denticola

Median counts of *T. denticola* in group A and B were reduced after therapy. Before therapy in group A full length paper point samples harvested higher amounts of *T. denticola* than half length, after therapy the opposite was found. In group B full length paper point always sampled more bacteria than half length paper point. The same was recorded in terms of the relative proportions of *T. denticola*. No obvious difference of the number of positive sites was registered for the two lengths of paper points in both groups (Table 1).

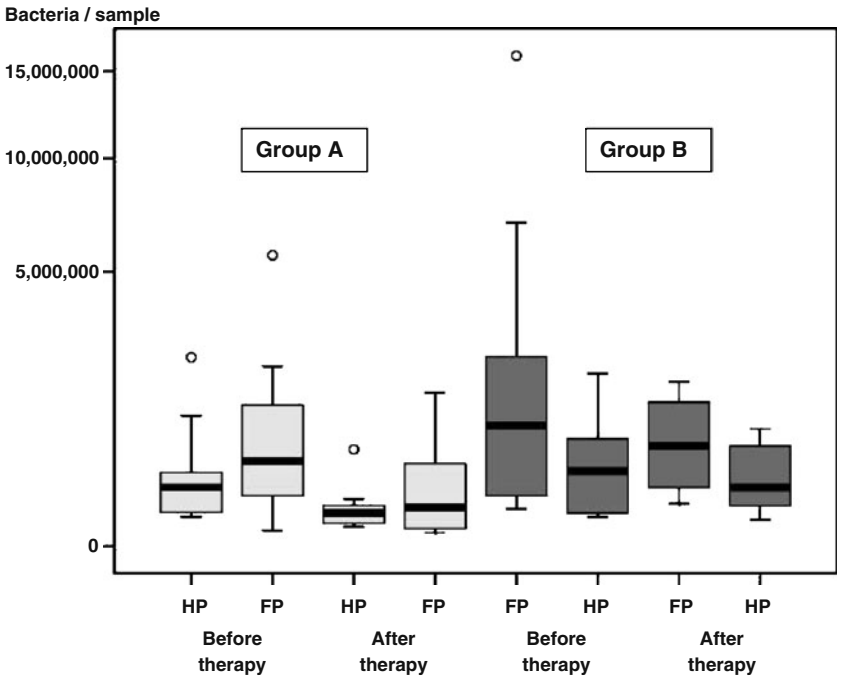

Fig. 1 Total bacterial counts (TBC) of half (*HP*) and full length paper point (*FP*) samples before and after therapy in groups A and B. *Box-plot* shows median, interquartile range between 25th and 75th percentile, *whiskers* indicate maximum and minimum, outliers are shown as *circles*, $n=10$ in each group

T. forsythia

Median counts of *T. forsythia* in group A and B were reduced after therapy. No greater difference of the number of positive sites was registered for the two lengths of paper points in both groups. The therapy seemed to have no influence on the proportions of *T. forsythia* in group A when sampling with half length paper point. Otherwise, the proportions of the bacterium harvested with full length paper point in both groups were reduced (Table 1).

Total target pathogen proportions

Group A paper point samples contained the same total target pathogen proportions (TTPP) regardless of length before therapy (both lengths, 21%). After therapy slightly

Table 1 Means of proportions in percent (frequency of sites positive for the presence of specific bacteria) in group A and B, for both sampling techniques as found before and after therapy

	A before		A after		B before		B after	
	H	F	H	F	F	H	F	H
Aa	3.62 (1)	0.66 (1)	0.06 (1)	0.15 (1)	1.91 (5)	0.18 (3)	0.65 (4)	0.20 (5)
Fn	1.49 (8)	1.35 (6)	2.06 (4)	1.70 (4)	2.44 (10)	1.29 (7)	1.74 (9)	1.24 (9)
Pg	10.96 (8)	12.54 (7)	2.34 (7)	4.94 (6)	6.92 (7)	6.66 (5)	8.98 (10)	5.63 (10)
Pi	0.64 (6)	0.35 (4)	0.25 (5)	1.89 (3)	1.94 (9)	5.96 (5)	1.89 (9)	1.30 (7)
Td	2.11 (6)	3.33 (6)	2.51 (6)	1.42 (5)	8.28 (9)	4.66 (8)	4.05 (10)	2.04 (9)
Tf	2.62 (8)	2.87 (7)	2.65 (6)	1.47 (5)	4.03 (10)	3.48 (8)	3.32 (10)	2.01 (10)
TTPP	21	21	10	12	26	22	21	12

Level of detection: 10^3 bacteria/plaque sample ($n=10$ in each group)

Aa Aggregatibacter actinomycetemcomitans, *Fn Fusobacterium nucleatum*, *Pg Porphyromonas gingivalis*, *Pi Prevotella intermedia*, *Td Treponema denticola*, *Tf Tannerella forsythia*, *A* or *B* group affiliation, *TTPP* total target pathogen proportions as rounded sum, *H* paper point half length, *F* full length

higher TTPP were found with full length paper points (half length, 10%; full length, 12%; Fig. 2). Comparing the two sampling techniques in group A the plaque composition as to target pathogens was fairly similar.

In group B full length paper points sampled always higher TTPP than half length paper points at any time point (before/after therapy: full length 26%/21%, half length 22%/12%; Fig. 3). A more pronounced reduction of TTPP was recorded with half length paper points.

Comparison of sampling techniques

Differences between full and half length paper point samples before and after therapy were calculated. Full length paper points collected significantly higher amounts of TBC than half length paper points before therapy, after therapy this difference failed to reach statistical significance (Table 2).

No significant differences for *A. actinomycetemcomitans* or *P. intermedia* were found between the two sampling methods independent of sampling time point. Full length paper point could harvest significantly higher amounts of *F. nucleatum* as well as *P. gingivalis* than half length paper points at any time point. For *T. denticola* only before therapy a statistically significant difference were observed. Full length paper points collected after therapy statistically significant higher amounts of *T. forsythia* than half length paper points (Table 2).

Agreement between sampling techniques

For the description of the agreement of quantitative results of both sampling techniques Spearman correlation coefficients were calculated. Both, for TBC as well as for single target bacteria, a strong positive correlation was found between the two paper point sampling methods (Table 3). For measuring the qualitative agreement between the two sampling techniques Kappa was used with a threshold level of detection of $\geq 10^3$ to define a sample as positive. The results showed an excellent agreement for *A. actinomycetemcomitans* and *T. denticola*, a good agreement for *P. gingivalis* and *T. forsythia* and a fair agreement for *F. nucleatum* and *P. intermedia* (Table 3).

Discussion

Median counts of total bacteria were higher before than after therapy, regardless of paper point length and group affiliation. Full length paper points sampled always more total bacteria than half length paper points, probably due to their larger sampling volume. This difference was significant for the results before therapy, after therapy this difference failed to reach statistical significance. A possible explanation for this finding could be the decrease of the probing pocket depth at the re-evaluation in the present

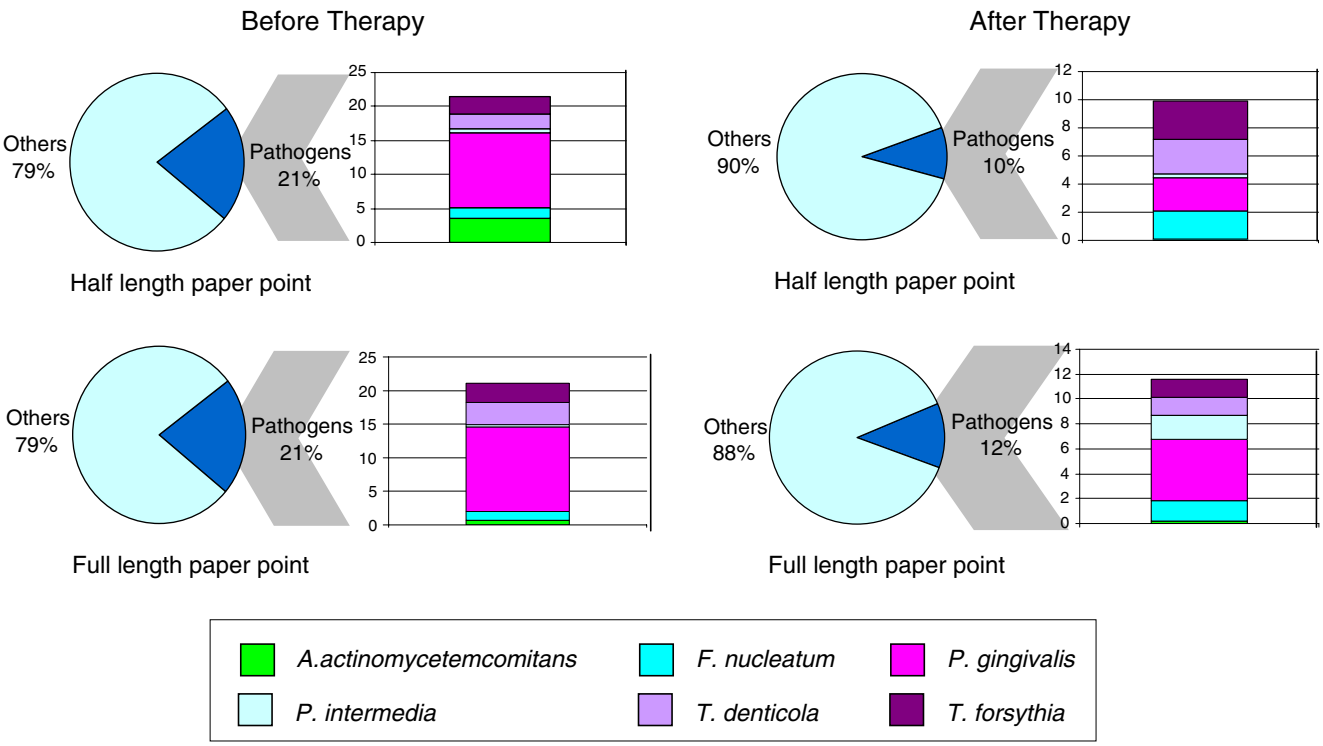

Fig. 2 Means of proportions of half length paper point samples and full length paper point samples in group A

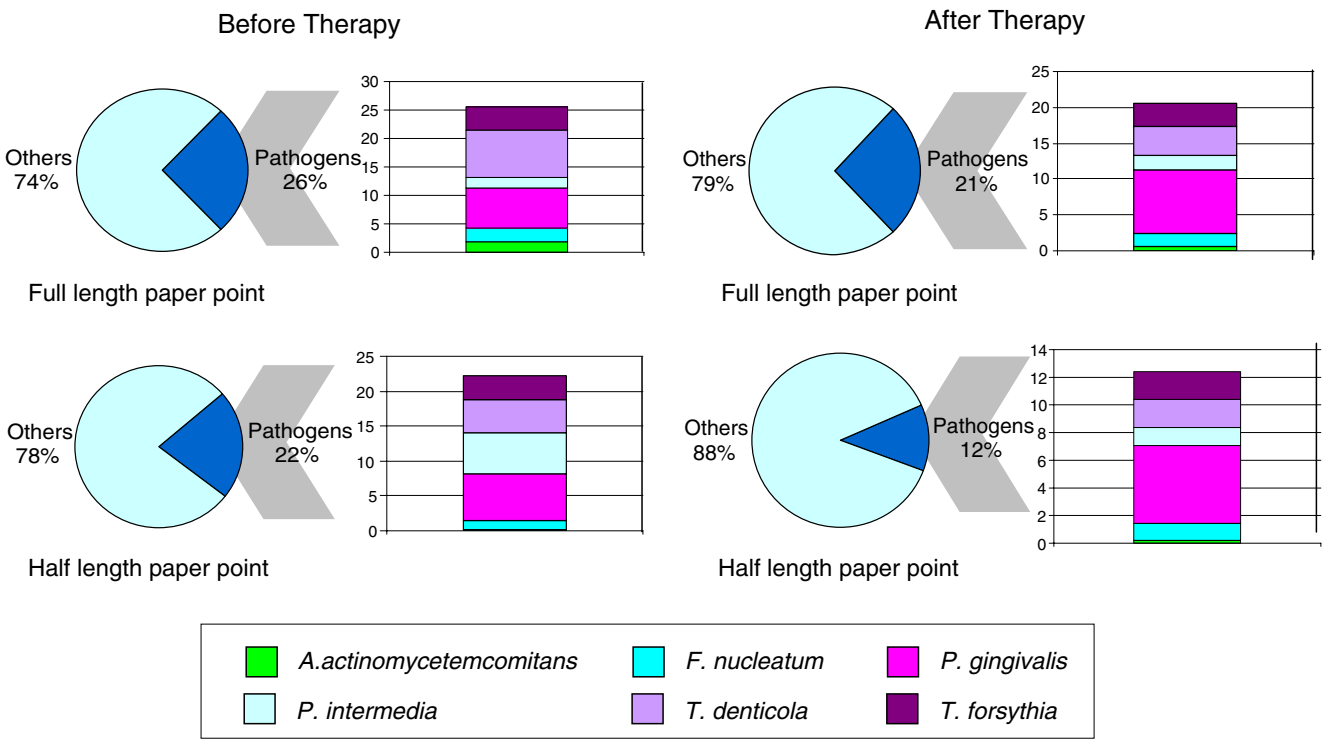

Fig. 3 Means of proportions of full length paper point samples and half length paper point samples in group B

study. As the reduction of probing depths minimizes the physical difference between full and half length paper points, also the amount of bacterial material collected by the two lengths of paper points would be less different.

No significant differences in bacterial composition between both paper point samples were neither for total target pathogen proportions nor for selected bacteria observed. In fact, there was a relatively good agreement between apical and coronal paper point samples at this detection level.

It was earlier shown, that periodontopathogenic bacteria are localized in definite areas in the human periodontal pocket, using immunohistochemical investigation methods on extracted teeth and their surrounding tissues [6]. Thus, quantitative and qualitative differences in the distribution of subgingival plaque from the apical and coronal aspect may exist; they may not be easily detected by paper point samples. Earlier in vitro evaluations of the paper point sampling technique using cultivation may support this assumption [16]. Here paper points were unable to sample deeper regions of multiple layers of liquid cultures of periodontopathogenic species, suggesting that paper points inserted to the depth of a periodontal pocket already may be saturated by plaque from immediately inside the gingival margin before reaching their ultimate sampling destination. This was also found in a clinical study comparing paper point samples with a "Pocket-out" sampling method [13]. Recovery of periodontal pathogens with both methods gave similar results, implying a possibility of cross-contamination of the paper point by supragingival biologic material, during insertion and removal from the sulcus.

Early studies on anaerobic sampling from the deeper parts of the periodontal pocket tried to address these problems of possible cross-contamination by means of a barbed broach device [17, 18]. The idea behind this sampling design was to protect the sampling device during insertion in the pocket, so that mainly bacteria from the bottom of the periodontal pocket were harvested. The data indicated a difference in the microbiota of diseased and healthy pockets in five patients [17]. Unfortunately, no additional samples of superficial parts of the diseased sites were taken in order to evaluate if plaque samples really originated from the apical parts of the pocket.

In a recent clinical study, employing real-time PCR, comparing paper point samples and curette samples, both from the full depth of the pocket, a relatively good agreement for the results was found [19]. Assuming that curettes enable a targeted plaque collection from the apical region [11], these results indicate that fully inserted paper points may accomplish the same task. Looking at the frequency of detection and the relative proportions of the target bacteria, the results of the present study confirm the findings of our previous study [19]. Comparing the data for full length paper point samples in our previous study with the outcomes of full

Table 2 *t* test results of total bacterial counts (TBC) as well as single target bacteria comparing sampling with half and full length paper points (half length minus full length)

Bacteria	Mean	SD	95% CI of the difference		*t* value	*p* (2-tailed)
			Lower	Upper		
Before therapy						
TBC	−0.791	0.770	−0.515	−0.068	−2.300	0.034*
Aa	−0.666	1.391	−1.550	0.218	−1.580	0.131
Fn	−0.995	0.961	−1.899	−0.092	−2.320	0.033*
Pg	−0.295	0.890	−1.870	−0.298	−2.900	0.010*
Pi	−0.436	1.000	−1.372	0.500	−0.980	0.341
Td	−1.088	0.940	−1.972	−0.205	−2.590	0.019*
Tf	−0.991	1.366	−2.274	0.293	−1.620	0.122
After therapy						
TBC	−0.563	0.625	−1.151	0.024	−2.010	0.059
Aa	−0.038	0.825	−0.814	0.737	−0.100	0.919
Fn	−1.034	1.043	−2.013	−0.054	−2.220	0.040*
Pg	−1.023	1.078	−2.036	−0.009	−2.120	0.048*
Pi	−0.730	1.030	−1.697	0.238	−1.580	0.130
Td	−0.813	0.941	−1.698	0.071	−1.930	0.069
Tf	−1.041	0.947	−1.930	0.202	−2.460	0.024*

Aa Aggregatibacter actinomycetemcomitans, *Fn Fusobacterium nucleatum*, *Pg Porphyromonas gingivalis*, *Pi Prevotella intermedia*, *Td Treponema denticola*, *Tf Tannerella forsythia*

*$p<0.05$, significant

length paper point samples in the present study, interesting results are found. In the groups of both studies, where full length paper points were used first (before curette or half length paper point) a similar reduction after subgingival scaling and root planing of total target pathogen proportions was noticed ([19]: from 27% to 24%; present study: from 26% to 21%); this might perhaps be expected. But in the groups of the former respectively the present study, where full length paper points were used immediately after one of two completely different sampling methods, either curette (highly invasive) or half length paper point (minimal invasive), similar amounts of reduction were found in both studies ([19]: from 24% to 16%; present study: from 21% to 12%). As the two preceding sampling methods differed in their invasiveness, second sampling with full length paper points should be expected to result in different degrees of reduction of bacteria in the two studies. Presumably curettes would leave fewer bacteria than half length paper points left to sample. This however, could not be found.

The interesting point by comparing the results for full length paper point sampling in the two studies however, is the reduction of TTPP. The reduction of TTPP with different sampling methods depicts either the true reduction of bacteria or the ability of the used methods to sample bacteria from different parts of the pocket. If, in both studies, full length paper points were used first, still high percentages of TTPP were found after anti-infective therapy with reductions of only 3–5%. Curette or half length paper point sampled always less amounts of TTPP than full length paper points after therapy, independent of sampling

Table 3 Spearman correlation and Kappa coefficients between half and full length paper point samples in groups A and B at all time points

	Spearman Coefficient	*p* value	Kappa	95% CI		Evaluation
				Lower	Upper	
Total bacteria counts	0.588	0.000				
A. actinomycetemcomitans	0.778	0.000	0.807	0.597	1.000	Excellent agreement
F. nucleatum	0.772	0.000	0.573	0.292	0.854	Fair agreement
P. gingivalis	0.824	0.000	0.733	0.488	0.979	Good agreement
P. intermedia	0.756	0.000	0.480	0.205	0.756	Fair agreement
T. denticola	0.814	0.000	0.807	0.597	1.000	Excellent agreement
T. forsythia	0.695	0.000	0.692	0.403	0.972	Good agreement

$n=40$

sequence (reduction, 9–13%). Full length paper points used after a preceding sampling demonstrated reductions of 8–9%. So, full length paper points might have a better ability to sample representative amounts of periodontopathogenic bacteria from the pocket after periodontal therapy. Curettes are known to sample biofilm-associated bacteria; half length paper points would be expected to sample more coronal planktonic contents of the pocket. Perhaps this is only important after therapy, where the amount of microorganisms in the periodontal pocket is reduced. These findings could point out the importance of using a full length paper point for the evaluation of the impact of periodontal therapy on periodontopathogenic bacteria.

Any comparison of two different subgingival plaque sampling techniques has problems associated with the fact that once one sampling technique has been performed in a site, the content of the pocket has been changed. The present study was designed to compensate for these problems. In the present study as in the former study [19] with the applied cross-over design there may be a problem with the so called carry-over effect; the second sample will be differentially affected by the kind of the first sampling. This may induce a bias in the estimation of the difference between the sampling methods and also lead to a reduction of the power to detect differences between the sampling techniques. Although it was not the scope of our previous study, the question if repeated sampling introduces a bias on the results of the second sampling was refuted [19], supporting the findings of other studies [20–22]. Therefore the present study was designed according to the design of the former study. Very recently the outcome of repeated sampling of subgingival bacteria with curettes within a very short period was described [23]. Each of seven strokes from the same site provided samples that were very similar in terms of proportions of the test species, although the bacterial counts decreased. The reproducibility of sampling with curettes led to the finding that one curette stroke was not enough to remove all of the plaque present in one site. Visually detectable plaque samples were collected up to the fourth stroke in most of periodontal diseased pockets. This study clearly demonstrated that it is not possible to empty the periodontal pocket even with a curette because removal of plaque might create an influx of plaque from adjacent areas. These findings support the presumptions in the present study that two consecutive inserted paper points do not interact with each other in terms of relative proportions of bacteria. The use of different analysis methods complicates the comparison between the present and other studies, as the outcome of microbiological investigations depends on the identification method used for microbiological analysis [11]. Omar et al. [5], Strand et al. [21], and Mombelli et al. [22] used dark field microscopy; Noiri et al. [6–8], an immunohistochemically based method; Baker et al. [16] and Mombelli et al. [22], cultivation; Renvert et al. [20], phase-contrast microscopy; and Teles et al. [23] used DNA–DNA hybridization for microbiological identification. The present study employed a real-time PCR-based method which enables quantification of subgingival bacteria [9, 10].

The suitability of this particular method to quantify the respective bacteria was also shown previously in a comparative study in which the results were compared with quantification results obtained by cultivation methods [10].

In summary, the results of the present study provide new information on the currently widely accepted paper point sampling technique in microbiological diagnostic assays of periodontal lesions. Even though paper points inserted to full length of the probing depth aiming at the apical region collected higher amounts of total bacteria than paper points inserted to the coronal half only, probably due to their greater sampling volume, there was a relatively good agreement between both samples for the recovery of selected target pathogens. However, after periodontal therapy there might be an indication of paper points inserted to the full depth of the periodontal pocket would be more suitable to collect periodontopathogenic bacteria. Probably due to the reduced amounts of microorganisms as a result of instrumentation, demanding as much absorbing material in the pocket as possible.

These findings seem to indicate that the anaerobic periodontal pathogens do not primarily inhabit deeper regions, but may be present in similar proportions throughout the various depths of the periodontal pocket.

Acknowledgment The authors would like to thank GABA International, Münchenstein, Switzerland for their financial support. Max Koltzscher is head of research and development at Carpegen.

Conflicts of interest The authors declare that they have no conflict of interest.

References

1. Marsh PD (2005) Dental plaque: biological significance of a biofilm and community lifestyle. J Clin Periodontol 32(Suppl. 6):7–15
2. Socransky SS, Haffajee AD (2002) Dental biofilms: difficult therapeutic targets. Periodontol 2000 28:12–55
3. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1998) Microbial complexes in subgingival plaque. J Clin Periodontol 25:134–144
4. Socransky SS, Haffajee AD (2005) Periodontal microbial ecology. Periodontol 2000 38:135–187
5. Omar AA, Newman HN, Bulman J, Osborn J (1990) Darkground microscopy of subgingival plaque from the top to the bottom of the periodontal pocket. J Clin Periodontol 17:364–370
6. Noiri Y, Ozaki K, Nakae H, Matsuo T, Ebisou S (1997) An immunohistochemical study on the localization of *Porphyromo-*

nas gingivalis, Campylobacter rectus and *Actinomyces viscosus* in human periodontal pockets. J Periodontal Res 32:598–607
7. Noiri Y, Ebisou S (2000) Identification of periodontal disease-associated bacteria in the "Plaque-Free-Zone". J Periodontol 71:1319–1326
8. Noiri Y, Li L, Ebisou S (2001) The localization of periodontal-disease-associated bacteria in human periodontal pockets. J Dent Res 80:1930–1934
9. Boutaga K, van Winkelhoff AJ, Vandenbrouke-Grauls CMJE, Savelkoul PHM (2006) The additional value of real-time PCR in the quantitative detection of periodontal pathogens. J Clin Periodontol 33:427–433
10. Jervøe-Storm PM, Koltzscher M, Falk W, Dörfler A, Jepsen S (2005) Comparison between culture and real-time PCR for detection and quantification of 5 putative periodontopathogenic bacteria in subgingival plaque samples. J Clin Periodontol 32:778–783
11. Loomer PM (2004) Microbiological diagnostic testing in the treatment of periodontal diseases. Periodontol 2000 34:49–65
12. Tanner AC, Goodson JM (1986) Sampling of microorganisms associated with periodontal disease. Oral Microbiol Immunol 1:21–22
13. Smola SF, Rettenberger G, Simmet T, Burysek L (2003) Comparison of sample collection methods for the PCR detection of oral anaerobic pathogens. Lett Appl Microbiol 36:101–105
14. O'Leary TJ, Drake RB, Naylor JE (1972) The plaque control record. J Periodontol 43:38–40
15. Senn SJ (1993) Cross-over trials in clinical research. Wiley, Chichester, pp 62–87
16. Baker PJ, Butler R, Wikesjö UM (1991) Bacterial sampling by absorbent paper points, an in vitro study. J Periodontol 62:142–146
17. Newman MG, Socransky SS (1977) Predominant cultivable microbiota in periodontosis. J Periodontol Res 12:120–128
18. Olsen I, Socransky SS (1981) Comparison of three anaerobic culture techniques and media for viable recovery of subgingival plaque bacteria. Scand J Dent Res 89:165–174
19. Jervøe-Storm P-M, AlAhdab H, Koltzscher M, Fimmers R, Jepsen S (2007) Comparison of curette and paper point sampling of subgingival bacteria as analysed by real-time PCR. J Periodontol 78:909–917
20. Renvert S, Wikström M, Helmersson M, Dahlen G, Claffey N (1992) Comparative study of subgingival microbiological sampling techniques. J Periodontol 63:797–801
21. Strand P, Palmer RM, Wilson RF (1987) Sampling of subgingival plaque: a comparison of two methods using darkfield microscopy. Oral Microbiol Immunol 2:142–144
22. Mombelli A, Minder CE, Gusberti FA, Lang NP (1989) Reproducibility of microscopic and cultural data in repeated subgingival plaque samples. J Clin Periodontol 16:434–442
23. Teles FR, Haffajee AD, Socransky SS (2008) The reproducibility of curet sampling of subgingival biofilms. J Periodontol 79:705–713