Recovery of Putative Periodontal Pathogens from Curette Sampling at Different Depths of Periodontal Lesions: An *In Vivo* Cross-Sectional Clinical Study

Erik F. Sahl[1], Jeffrey M. Henkin[1] and Nikola Angelov[2]

[1]School of Dentistry, Department of Periodontics, Loma Linda University, Loma Linda, CA; [2]University of Texas Health Science Center in Houston, School of Dentistry, Department of Periodontics and Dental Hygiene, Houston, TX

Abstract

Objective: The aim of this study was to evaluate the effect of the depth of curette sample collection from periodontal lesions on the recovery of putative periodontal pathogens using real-time polymerase chain reaction (PCR).

Methods: Twenty-two periodontal pockets 6 to 8 mm deep with bleeding on probing at a single-rooted tooth were sampled, yielding 66 separate samples. Curette samples were obtained at three different levels of the periodontal lesion (orifice, shallow - 2 mm into the pocket; or base of lesion), and processed using PCR to identify 10 periodontal pathogens. The chi-square procedure was used to determine whether probe depth affected the distribution of bacterial counts observed. A repeated measures analysis of variance tested the hypotheses related to level of probe and microorganism on mean rank of bacterial counts.

Results: The effect of probe level on mean bacterial counts depends on the type of microorganism. Likewise, the effect of microorganism type on mean bacterial counts significantly depends on probe level, where sampling from 2 mm into the periodontal pocket was found to yield significantly higher results than sampling from the orifice. Overall mean counts of pathogenic microorganisms were found to differ significantly across the three probe depths. The microorganisms differed in their observed levels over all three probe levels. Further analysis found several significant differences that characterize the nature of the interaction between probe level and microorganism type.

Conclusion: There is significant difference in the amount of putative periodontal pathogens at varying depths of the pocket when sampled with a periodontal curette.

Key words: Periodontal disease, microbiological sampling, curette, periodontal pathogens

Introduction

Periodontitis is a chronic, inflammatory disease of the tissues that support the teeth, resulting in a gradual loss of periodontal attachment, including the periodontal ligament and alveolar bone. Initiation and progression of the disease have been attributed to the presence of elevated levels of pathogenic microorganisms within the gingival crevice (Socransky and Haffajee, 2002; Zambon, 1996).

Patients with chronic periodontitis typically respond well to traditional mechanical therapies such as oral hygiene, scaling and root planing, and surgery (Cobb, 1996; Palcanis, 1996). According to Slots and Ting (2002), in patients with aggressive periodontitis it is often recommended that the mechanical treatment should be combined with the use of systemic antibiotics, based upon studies demonstrating a beneficial adjunctive effect of the use of appropriate antibiotic therapy in conjunction with mechanical therapies. In a recent meta-analysis, Sgolastra and his group (2012) concluded

Correspondence to: Dr. Erik F. Sahl, Department of Periodontics, School of Dentistry, Loma Linda University, 24860 Taylor St, Suite 212, Loma Linda, CA 92350, USA. Fax: 909-558-464. E-mail: esahl@llu.edu

that the combination of amoxicillin and metronidazole (AMX/MET) as an adjunctive treatment to scaling and root planing (SRP) seems to be effective in the treatment of chronic periodontal disease. Interestingly, some have questioned the use of microbiological testing for selecting the specific antimicrobial treatment. Even more, some authors have documented that standard combinations of antibiotics commonly used for treatment of periodontal disease, such as the combination of metronidazole and amoxicillin, yield better clinical and microbiological results, even in the absence of microbiological testing (Cionca *et al.*, 2009; 2010; Goodson *et al.*, 2012; Feres *et al.*, 2012). Nevertheless, the use of antimicrobials often warrants microbiological testing to determine which pathogens the diseased sites may harbor in a given patient. In addition, these bacteria demonstrate diverse sensitivities to antimicrobial drugs. Thus, selection of an appropriate antibiotic is also part of the therapeutic process (Slots and Ting, 2002). Even more, sampling material from the periodontal pocket may be useful for investigating several other aspects of periodontal diseases, such as initiation and progression of periodontal diseases, periodontal health, periodontal microbial ecology and results of different treatment modalities.

Periodontal microbial testing requires acquisition of subgingival plaque samples from periodontal lesions. These have been obtained using gingival washings, custom-designed devices, curettes and paper points, with the latter two being most commonly used (Hartroth *et al.*, 1999; Tanner and Goodson, 1986). Paper point samples are obtained by introducing endodontic, absorbent paper points to the depth of the periodontal lesion (until resistance is met), but some authors (Baker *et al.*, 1991; Smola *et al.*, 2003) claim that a paper point sample might primarily reflect the microbiota of the orifice or the upper part of the lesion. Curette sampling typically involves obtaining samples supragingivally as well as from the depth of the periodontal lesion. A common sampling method is to place the curette at the base of the pocket and pull coronally to remove bacterial deposits from the length of the root surface.

The optimal curette sampling technique depends on the area to be studied, the subgingival depth of the lesion, and whether or not attempts are made to include the root surface. Regardless of the sampling technique, the method of identification of the putative periodontal pathogens is important for proper outcomes (Sanz *et al.*, 2004). In this study on different approaches to microbiological testing, bacterial culturing and molecular techniques were identified as the only two possessing the validity of well-controlled clinical studies.

The aim of this study was to evaluate the effect of the depth of curette sample collection of periodontal lesions (orifice, shallow or base of lesion) on the recovery of putative periodontal pathogens using real-time polymerase chain reaction (PCR) as a method of bacterial identification.

Materials and methods

Sample collection

Twenty-two periodontitis patients, each having at least one periodontal lesion 6 to 8 mm deep (clinical attachment loss of 5 mm or more) with bleeding on probing at a single-rooted tooth (maxillary or mandibular) were recruited at Loma Linda University School of Dentistry Advanced Periodontics clinic. All patients were previously diagnosed with severe chronic periodontitis per clinic protocols. One tooth per subject was sampled at the same pocket measuring 6 to 8 mm in depth at three different levels of the pocket consecutively (supragingivally, 2 mm into the lesion, and at the depth of the lesion (resulting in 66 samples total).

This study was reviewed and approved by the Loma Linda University (LLU) Institutional Review Board. Exclusion criteria were: 1) any systemic disease known to affect periodontal conditions; 2) any condition for which antibiotic premedication was required; 3) antimicrobial treatment within the last 3 months; 4) periodontal treatment within the last 6 months; 5) under 18 years of age; 5) pregnant and lactating females; 6) current smokers.

Clinical and laboratory procedures

One examiner (ES) performed all clinical procedures. At the baseline visit, qualifying and volunteering patients had a complete periodontal probing evaluation. Patients were asked to abstain from flossing this area for one week. The one-week interval between probing and sample collection was designed to ensure that the probing procedure did not distort the bacterial sample. After one week, the patients returned for bacterial sample collection. The samples were analyzed with PCR testing for the following periodontopathogenic bacteria: *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Tannerella forsythia (Tf), Prevotella intermedia (Pi), Treponema denticola (Td), Parvimonas micra (Pm), Fusobacterium nucleatum (Fn,), Campylobacter rectus (Cr), Eikenella corrodens (Ec),* and *Eubacterium nodatum (En).*

The tooth sampled was isolated with cotton rolls and dried. Curette samples were obtained at three different depths of the periodontal lesions consecutively:

1. At the orifice (Level 1) by utilizing three subsequent horizontal pulls of a curette following the coronal border of the gingival sulcus.
2. At a 2 mm depth (Level 2) of the periodontal lesion. The curette was placed 2 mm into the gingival pocket (confirmed by the placement of an endodontic stopper placed 2 mm from the apical extent of the curette) and then pulled coronally three subsequent times.
3. At the depth of the lesion (Level 3), (confirmed by the feeling of resistance upon placing the curette into

the lesion). The curette was inserted to the depth of the pocket and pulled coronally three times. This was done during the collection of each sample.

To obtain samples, new, sterilized Hu-Friedy Gracey 5/6 periodontal curettes were utilized. Three curettes were used per subject, resulting in three consecutive samples. The Gracey 5/6 curette was chosen to standardize the angulations of all three samples as well as to permit the most effective method of placing the curette inside the transport tube.

The strokes had sufficient force to remove plaque while no attempt was made to remove cementum from the root surface. Immediately upon collection of the samples, the curettes were shaken into a transport tube with 200 µl of sodine solution (sterile sodium chloride) transport solution. The samples were then sealed and sent immediately to the testing facility. Results were analyzed using the statistical methods described below. The micro-IDent test, which was performed by Hain Lifescience (Germany), employs PCR to amplify the sample DNA, and specific DNA probes on a DNA-STRIP® matrix to hybridize with the amplified DNA. This reaction produces a color reaction on the DNA-STRIP®, the intensity of which corresponds to the initial level of DNA in the sample. This was semi-quantification because not every sample had an identical amount of plaque. The quantification method is described in Table 1.

Statistical analysis

The chi-square procedure was used to determine whether probe depth affected the distribution of bacterial counts observed. A repeated measures analysis of variance (ANOVA) was conducted to test influence level of probe and type of microorganism on mean rank of bacterial counts. Student's *t*-test was used to detect significant differences between the overall probe level counts at $p < 0.05$. All statistical tests were two sided and conducted at an alpha level of 0.05. Analysis was performed with SAS v 9.2 (SAS Institute, Cary, NC).

Results

Figure 1 presents the mean overall counts for each microorganism type across all three probe levels. The mean counts by probe level for each microorganism type are presented graphically in *Figure 2*. As previously described, nominal values were assigned to different quantity distribution of bacteria from *Table 1*. Several major periodontal pathogens appear to have different distribution at different levels of the periodontal pocket, when sampled with a curette. *Pg* appears to be more frequently sampled more coronally (Levels 1 and 2), while *Aa* was only detected at the bases of the periodontal lesions sampled (Level 3). Similarly, *Pi* was detected in all three levels, but was detected in twice the quantity at Level 3, at the base of the periodontal lesion. Other major periodontal pathogens that demonstrated tendency for increased presence, possibly related to the depth of the lesion, were *Cr, En,* and *Td.*

Table 1. Quantification of bacteria in curette samples.

Score (number) assigned	Quantity of bacteria (Qb)
(0)	$Qb < 10^4$
(+) (0.5)	$Qb = 10^4$
+ (1)	$10^4 < Qb < 10^5$
++ (2)	$10^5 < Qb < 10^6$
+++ (3)	$Qb > 10^7$

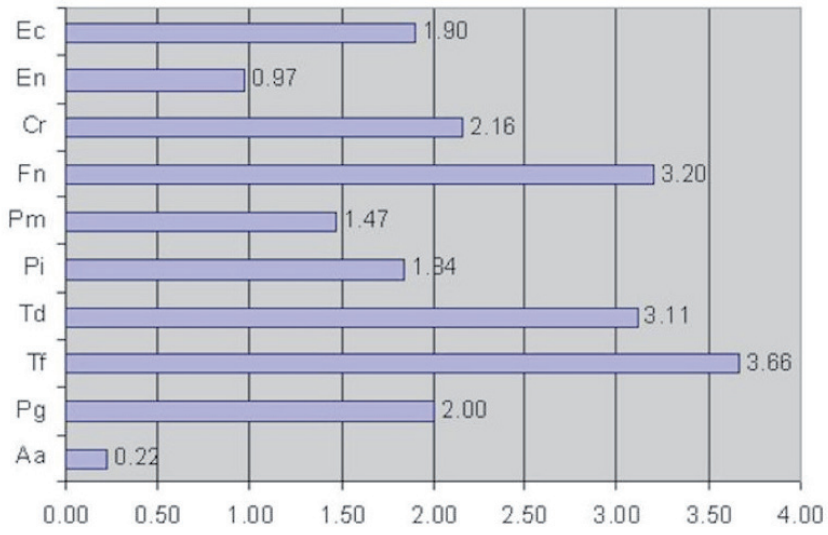

Figure 1. Sum of the mean overall counts for each microorganism type across all three probe levels

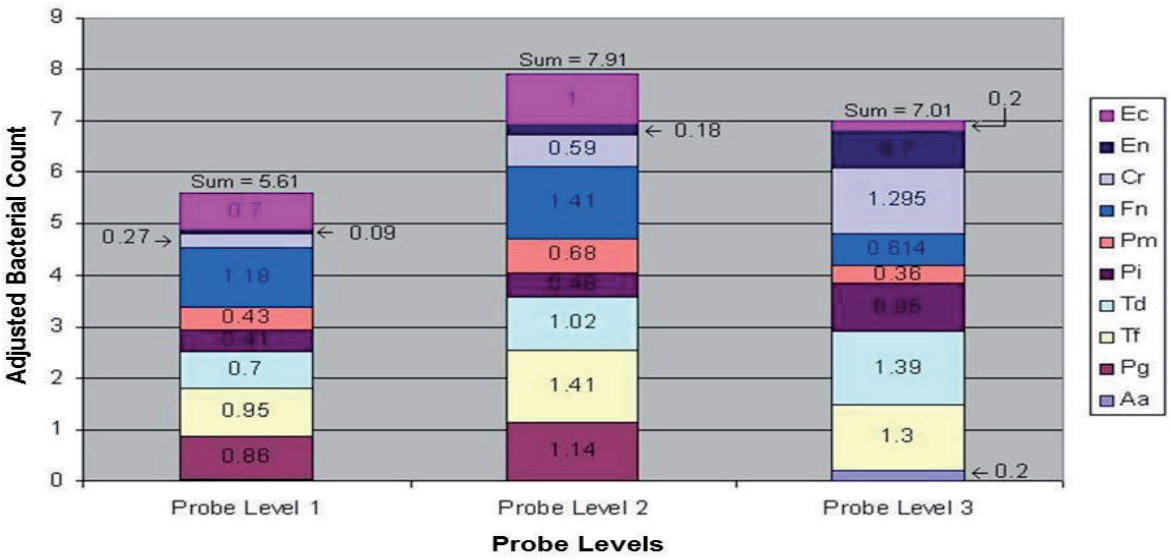

Figure 2. Mean counts of each microorganism type within each probe level

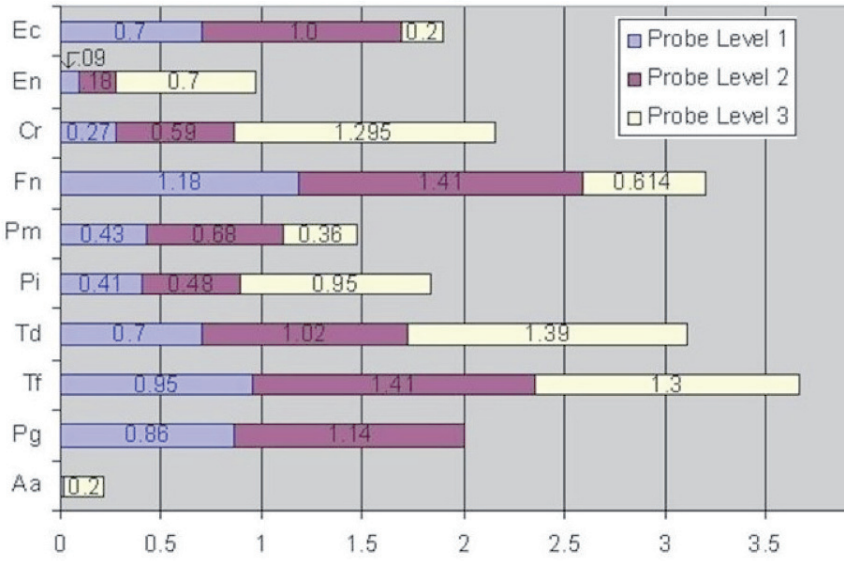

Figure 3. Mean counts of each microorganism type by probe level

Table 2. Results of repeated measures ANOVA of microorganism counts for probe level and microorganism type. [a]Greenhouse-Geisser correction applied to df; [b]Values enclosed in parentheses represent mean square error; [c]Mauchly test for sphericity

Source	Sphericity[c] assumption met?	df	F[b]	*p*	Eta Squared
Probe level	Yes	2	5.190	0.010	0.015
Error (probe level)		42	(0.556)		
Bacteria type	No[a]	5.049	14.756	<0.001	0.188
Error (bacteria type)		106.038	(0.978)		
Probe level * bacteria type	No[a]	6.177	8.938	<0.001	0.140
Error (probe level * bacteria type)		129.716	(0.984)		

As shown in *Table 2*, the F-test for the significance of main effects for both probe level and microorganism type were significant, as was the interaction. The significance of the interaction effect indicates that the effect of probe level on mean rank of bacterial counts depends on the type of microorganism (*Figure 2*). Likewise, the effect of microorganism type on mean bacterial counts significantly depends on probe level (*Figure 3*).

The results in *Table 3* indicate that only one of the three pairwise comparisons between probe levels demonstrated a significant difference, that being the comparison between probe Levels 1 and 2, in which the microorganism count for probe Level 2 was found to be significantly higher than that for probe Level 1.

The overall levels of *Aa* were lower than those of all nine other microorganisms, a difference that reached significance in all cases except for *Pm* and *En*. The incidence of *Pg* was significantly lower than that for *Tf*, whereas the incidence of *Tf* was significantly higher than that for *Pi*, *Pm, En,* and *Ec*. The incidence of *Td* was significantly higher than that for *En*. The incidence of *Pi* was significantly lower than that for *Fn*, and the incidence of *Fn* was significantly higher than that for *En*. And finally, the incidence of *C*r was significantly higher than that for *En*.

The probe levels differed in their counts of the various microorganism types. These differences are depicted graphically in *Figure 2*.

The differences between probe levels within each microorganism type were addressed in two different ways. First, the actual counts of microorganisms were used as the measure of occurrence rate, which was also the measure used as the dependent variable in the two within-subject ANOVAs reported above. The differences between probe levels on this measure within each microorganism type are displayed graphically in *Figure 3*.

The differences within each probe level reflected in *Figure 3* were tested for significance through the evaluation of pairwise contrasts between the three probe levels for all microorganism types where the overall F for the type was statistically significant.

A final analysis was conducted that operationalized the occurrence of a microorganism as a binary event rather than a scored (*i.e.*, counted) event. In this analysis, all non-zero probe results were scored as 1.0 and all zero probe results were scored as 0. Thus, the dependent measure for each microorganism type was the number of occasions it had been detected at all in a probe at each depth level. The results of this analysis are presented in *Figure 4*.

In order to evaluate these detection rates statistically, the observed number of cases in which a given microorganism was detected at each probe level was used as the "observed frequencies" in a chi-square goodness-of-fit test. The expected frequencies for each of the three probe levels was specified as 1/3 the sum of the observed frequencies for the given microorganism. The resulting chi-square test assessed whether the observed frequencies at the 3 probe levels departed from what would be expected to occur by chance. Significant results were reported in the analyses for five of the ten types of microorganisms, as reported in *Table 4*. These indicate that significant departures from chance were obtained in two cases (*A.a* and *P.i*), which did not emerge as significant in the analysis of the clinical counts of microorganisms. Furthermore, in the cases of three microorganism types, which were found to have significantly different counts over the three probe levels (*viz.*, *Td, Fn*, and *Ec*) and their binary detection rates did not differ significantly from chance. Thus, it seems reasonable to infer that strength of occurrence and mere presence vs. absence measure quite different aspects of the behavior of the microorganisms targeted by this study.

Discussion

This study investigated the effectiveness of a curette sampling to detect periodontal pathogens at different depths of periodontal lesions using the same protocol used by Angelov *et al.* (2009) in their study of paper point sampling. In addition to evaluating a different method of sampling, the current study used the sampling procedure to detect 10 types of pathogenic microorganisms, as compared with the three microbial types analyzed by Angelov *et al.* (2009). In this study, a micro-IDent kit was used for identification of periodontal pathogens. A recent controlled study compared the micro-IDent kit with culture for the detection of *Aa, Pg, Pi, Tf, and Td* in 122 plaque samples from sites with different pocket depths. Both techniques showed a positive correlation between pocket depth and the quantity of the test species (Eick and Pfister, 2002). Statistical methods also differ in the two studies. The current study employed repeated measures analysis of variance to analyze the correlated data obtained through multiple measurements on a single periodontal lesion, while Angelov *et al.* (2009) used the nonparametric Friedman test for dependent samples. The two-factor repeated measures analysis of variance examined the effect on mean pathogen counts of two factors: within-probe level (3 levels) and microorganism type (10 types).

The overall mean counts of pathogenic microorganisms were found to differ significantly across the three probe depths. Our results of significant difference between bacterial counts at probe Levels 1 and 2, with the mean count at Level 2 exceeding that for Level 1 results contrast with those of Angelov *et al.* (2009) who reported similar microbial counts at the same three probe depths using paper point sampling.

Table 3. Post hoc tests of differences between probe levels in microorganism counts

Probe Level (I)	Probe Level (J)	Mean Difference (I – J)	Std. Error	*p*	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-0.227*	0.070	0.011	-0.409	-0.046
1	3	-0.139	0.085	0.349	-0.359	0.082
2	3	0.089	0.056	0.387	-0.057	0.235

*The mean difference was significant at the 0.05 level; the Bonferroni adjustment for multiple comparisons was used

Table 4. Results of chi-square goodness-of-fit tests of observed detection frequencies of the target microorganisms.

Microorganism	Chi-square	DF	*p*
Aggregatibacter actinomycetemcomitans	10.06	2	0.007
Porphyromonas gingivalis	13.91	2	0.001
Tannerella forsythia	0.16	2	0.923
Treponema denticola	0.81	2	0.667
Prevotella intermedia	7.13	2	0.028
Parvimonas micra	4.78	2	0.092
Fusobacterium nucleatum	1.96	2	0.375
Campylobacter rectus	8.45	2	0.015
Eubacterium nodatum	7.66	2	0.022
Eikenella corrodens	4.12	2	0.128

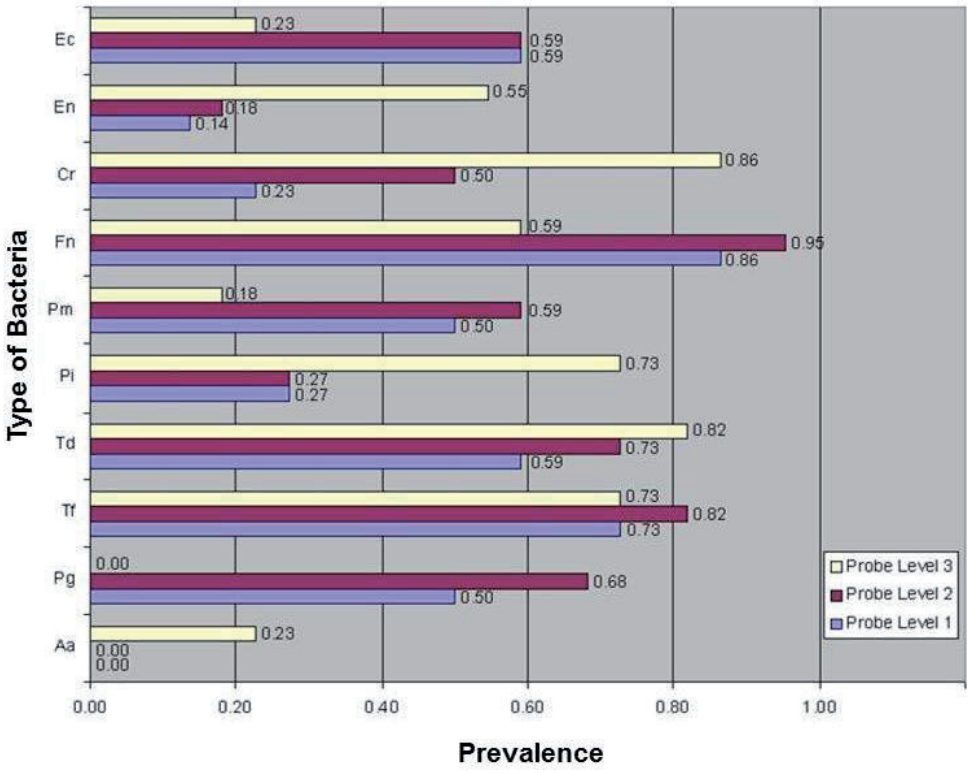

Figure 4. Detection rates of each microorganism type by probe level

This similarity of the putative periodontal pathogens might be explained by 2 circumstances. Based upon the similarity in recovery by the 2 sampling methods, the authors suggested that both paper point and 'pocket-out' samples may primarily represent the microbiota present along the gingival margin. Second, it is also conceivable that the pathogens may be present in similar proportions throughout the various depths of the periodontal lesions. If so, this seems to be contrary to the notion that the anaerobic putative periodontal pathogens primarily are harbored in the deeper parts of the lesions (Socransky and Haffajee, 2002).

Additionally, the types of microorganisms differed in their observed levels over all three probe levels. Interpretation of this significant main effect for microorganism type, as well as that for probe level discussed above, however, must take into consideration the significant interaction found between the two factors. The significance of the interaction indicates that the effect of probe-level on mean bacterial counts depends on the type of microorganism. Likewise, the effect of microorganism type on mean bacterial counts significantly depends on probe level.

Interestingly, our findings about the low levels of *Pg* in the deep areas of the periodontal lesions sampled is in direct contrast with some previous reports where *P.g.* was found in higher levels in the deeper portions of the periodontal pocket (Kigure *et al.*, 1995; Noiri *et al.*, 1997; 2001; 2004). A potential reason for this discrepancy is that all these studies used immunohistochemical methods for detection of bacteria on site compared to the mechanical curette sampling, which may lead to more errors. Therefore, the outcomes of our study should not be used to draw conclusions about the detailed microbiological composition of the dental plaque at different areas of the periodontal pocket.

The clinical significance of the current findings is in contrast to most recently published studies regarding isolation of different bacteria at different probing depths (Persson *et al.*, 2009; Jervøe-Storm *et al.*, 2009). The majority of these studies addressed the current standard of paper point sample retrieval as opposed to the use of a periodontal curette. In a comparison study, Jervøe-Storm *et al.* (2007) found no significant differences in the plaque composition between the curette and paper point sampling methods, although the total bacterial count was much higher in the curette sampling group. It is worth mentioning that paper point method helps collect mainly planktonic bacteria in periodontal pocket, whereas curettage method can collect mainly biofilm-forming bacteria on root surface exposed in periodontal pocket. The different capabilities of these sampling methods raise the concern if we can in fact detect different spices of bacteria or micro-flora.

Still, the distribution of the microorganisms within the periodontal pocket may be of vital significance to obtain accurate data when sampling periodontal pockets. Crespi *et al.* in 1996 determined that there is significant difference in the presence of filamentous forms, fusiform rods, coccoid forms and loosely aggregated spirochetes between the coronal, middle and apical portions of the root surface in the periodontal pocket. Therefore, it is of paramount importance to use an appropriate sampling method when collecting dental plaque from the periodontal lesion. To our knowledge, this is the first publication to evaluate the distribution of the microflora within the periodontal lesion and its correlation with different pocket depths when using a curette for microbiological sampling.

These data have a multitude of clinical significance. Most notable would be the paradigm shift of routine microbial sampling with a paper point to the use of a curette. Teles *et al.* (2008) found that curette sampling provides a reproducible and reliable method to obtain samples of microbial pathogens from periodontal lesions. The current study found that, in contrast to paper point sampling of periodontal lesions (Angelov, 2009), curette sampling is capable of detecting differences in microbial counts at different probe levels within the pocket. Although our study did not compare directly the curette sampling effectiveness to the one using paper points, we feel that using a curette for sampling can provide more realistic baseline and follow-up bacteriological data. Obtaining periodontal pocket bacterial samples with paper points may only reveal which microorganisms are present at the gingival margin, while the curette sampling method was shown to reflect significantly different presence and quantity of bacteria at differing probing depths. This should result in a more accurate analysis and result in more precise local and/or systemic antimicrobial therapy. A follow-up study that will directly compare the effectiveness of paper point sampling to curette sampling is recommended and currently being considered.

One of the limitations of this study was that the data were only semi-quantifiable because not every sample had an identical amount of plaque. Because of this, and because of the relatively small sample size, these pilot study results should encourage larger, prospective studies to not only confirm the current findings, but possibly lead to the development of more precise diagnostic methods, and it turn lead to application of specific periodontal and/or antibiotic therapies.

Conclusion

There is a significant difference in the amount of putative periodontal pathogens at varying depths of periodontal pockets when sampled with a curette. The curette sampling method is an efficient method that can be used for recovery of periodontal pathogens at different depths of the periodontal lesion.

Conflict of interest and source of funding statement:

The author declares there is no conflict of interest. The study was self-supported, with the exception of the laboratory tests, which were generously provided by Hain Lifescience (Germany).

References

Angelov N, Aprecio RM, Kettering J, Lundgren T, Riggs M and Egelberg J. Recovery of putative pathogens from paper point sampling at different depths of periodontal lesions. *Clinical, Cosmetic, and Investigational Dentistry* 2009; **1**:1-5.

Baker PJ, Butler R and Wikesjo UM. Bacterial sampling by absorbent paper points. An *in vitro* study. *Journal of Periodontology* 1991; **62**:142-146.

Cionca N, Giannopoulou C, Ugolotti G, and Mombelli A. Amoxicillin and metronidazole as an adjunct to full-mouth scaling and root planing of chronic periodontitis. *Journal of Periodontology* 2009; **80**:364-371.

Cionca N, Giannopoulou C, Ugolotti G, and Mombelli A. Microbiologic testing and outcomes of full-mouth scaling and root planing with or without amoxicillin/metronidazole in chronic periodontitis. *Journal of Periodontology* 2010; **81**:15-23.

Cobb CM. Non-surgical pocket therapy: mechanical. *Annals of Periodontology* 1996; **1**:443-490.

Crespi R, Covani U, Margarone JE and Andreana S. Topographic distribution of subgingival plaque along root surfaces of human periodontally diseased teeth. A descriptive study. *Journal of Clinical Periodontology* 1996; **23**:698-703.

Eick S and Pfister W. Comparison of microbial cultivation and a commercial PCR based method for detection of periodontopathogenic species in subgingival plaque samples. *Journal of Clinical Periodontology* 2002; **29**:638-644.

Feres M, Soares GM, Mendes JA, *et al.* Metronidazole alone or with amoxicillin as adjuncts to non-surgical treatment of chronic periodontitis: a 1-year double-blinded, placebo-controlled, randomized clinical trial. *Journal of Clinical Periodontology* 2012; **39:**1149-1158.

Goodson JM, Haffajee AD, Socransky SS, *et al.* J. Control of periodontal infections: a randomized controlled trial I. The primary outcome attachment gain and pocket depth reduction at treated sites. *Journal of Clinical Periodontology* 2012; **39**:526-536.

Hartroth B, Seyfahrt I and Conrads G. Sampling of periodontal pathogens by paper points: evaluation of basic parameters. *Oral Microbiology and Immunology* 1999; **14**:326-330.

Jervøe-Storm PM, Alahdab H, Koltzscher M, Fimmers R and Jepsen S. Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. *Journal of Periodontology* 2007; **78**:909-917.

Jervøe-Storm PM, Al-Ahdab H, Koltzscher M, Fimmers R and Jepsen S. Quantification of periodontal pathogens by paper point sampling from the coronal and apical aspect of periodontal lesions by real-time PCR. *Clinical Oral Investigations* 2010; **14**:533-541.

Kigure T, Saito A, Seida K, Yamada S, Ishihara K and Okuda KJ. Distribution of *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. *Journal of Periodontal Research* 1995; **30:**332-41.

Mousques T, Listgarten MA and Stoller NH. Effect of sampling on the composition of the human subgingival microbial flora. *Journal of Periodontal Research* 1980; **15**:137-143.

Palcanis KG. Surgical pocket therapy. *Annals of Periodontology* 1996; **1**:589-617.

Persson GR, Weibel M, Hirschi R and Katsoulis J. Similarities in the subgingival microbiota assessed by a curet sampling method at sites with chronic periodontitis. *Journal of Periodontology* 2008; **79**:2290-2296.

Sanz M, Lau L, Herrera D, Morillo JM and Silva A. Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *Journal of Clinical Periodontology* 2004; **31**:1034-1047.

Sgolastra F, Gatto R, Petrucci A and Monaco A. Effectiveness of systemic amoxicillin/metronidazole as adjunctive therapy to scaling and root planing in the treatment of chronic periodontitis: a systematic review and meta-analysis. *Journal of Periodontology* 2012; **83**:1257-1269.

Slots J and Ting M. Systemic antibiotics in the treatment of periodontal disease. *Periodontology 2000* 2002; **28**:106-176.

Smola SF, Rettenberger G, Simmet T and Burysek L. Comparison of sample collection methods for the PCR detection of oral anaerobic pathogens. *Letters in Applied Microbiology* 2003; **36**:101-105.

Socransky SS and Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontology 2000* 2002; **28**:12-55.

Tanner AC and Goodson, JM. Sampling of microorganisms associated with periodontal disease. *Oral Microbiology and Immunology* 1986; **1**:15-22.

Teles R, Haffajee AD and Socransky SS. The reproducibility of curet sampling of subgingival biofilms. *Journal of Periodontology* 2008; **79**:705-713.

Zambon JJ. Periodontal diseases: microbial factors. *Annals of Periodontology* 1996*;* **1**:879-925.