J Clin Periodontol 2006; 33: 14–20 doi: 10.1111/j.1600-051X.2005.00859.x

Journal of Clinical Periodontology

A method to study sustained antimicrobial activity of rinse and dentifrice components on biofilm viability in vivo

H. C. van der Mei[1], D. J. White[2], J. Atema-Smit[1], E. van de Belt-Gritter[1] and H. J. Busscher[1]

[1]Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [2]The Procter & Gamble Company, Mason, OH, USA

van der Mei HC, White DJ, Atema-Smit J, van de Belt-Gritter E, Busscher HJ. A method to study sustained antimicrobial activity of rinse and dentifrice components on biofilm viability in vivo. *J Clin Periodontol 2006; 33: 14–20. doi: 10.1111/j.1600-051X.2005.000859.x.* © Blackwell Munksgaard, 2006.

Abstract

Aim: To develop an improved method for quantitative assessment of antimicrobial efficacy and substantivity of mouth rinses and dentifrices on in vivo treated plaque.

Material and Methods: Nine- and 72-h-old plaques were formed in volunteers carrying out standardized hygiene using NaF-containing dentifrice. Plaques were collected before (baseline) in vivo treatment with dentifrices or chlorhexidine mouth rinse, immediately post-treatment and after 1 or 6 h, dispersed in demineralized water and stained with live/dead stain after which bacteria were enumerated. Dispersed baseline plaques were treated with dentifrices or chlorhexidine to determine antimicrobial efficacy against planktonic bacteria.

Results: Baseline plaques revealed 56–41% viable organisms in 9- and 72-h-old plaques, respectively. Treatment of planktonic (dispersed baseline plaque) bacteria resulted in 1–4% viable organisms. Chlorhexidine mouth rinse and dentifrices produced strong immediate antimicrobial effects, but after 1 or 6 h, the proportion of viable organisms in 9-h-old plaques rebounded significantly with only chlorhexidine mouth rinse retaining significant efficacy. Seventy-two-hour-old plaques were less susceptible to antimicrobials, although dentifrices appeared more effective after 6 h than initially, whereas efficacy of chlorhexidine rinse continued to drop with time post-treatment.

Conclusions: The proposed method holds promise for assessment of both immediate and retained antimicrobial actions of oral treatments against dental plaque in vivo.

Key words: chlorhexidine mouth rinse; confocal scanning laser microscopy; dentifrices; plaque; substantivity

Accepted for publication 28 September 2005

Gingivitis and periodontitis are the direct result of microbial dental plaque infection at the gingival tooth interface modulated by the host response (Robinson 1995, Mombelli 1998). While specific microbial populations and pathogenicity have been correlated with disease processes, the more generalized non-specific correlation between dental plaque and soft tissue disease is supported by the universal development of experimental gingivitis in patients and rapid resolution of disease upon reinstitution of hygiene measures (Loe et al. 1965, Theilade et al. 1966). Although frequent and effective hygiene presents a proven and effective route to the maintenance of soft tissue health, most patients do not exhibit the motivation, skill or discipline for adequate plaque control (Schou 1998). As a result, gingivitis prevalence remains high, even among educated patients regularly visiting their dentist (Van der Ouderaa 1992, Baehni & Bourgeois 1998).

While most patients find it difficult to completely control plaque through routine oral hygiene measures, virtually all patients use commercial dentifrices and mouth rinses as part of their attempts towards a daily hygiene regimen. Because of their frequent application, dentifrices and mouth rinses therefore represent opportunistic vehicles providing chemotherapeutic benefits. The formulation of antimicrobial ingredients into dentifrice and rinse formulations has thus been the focus of significant academic and industrially sponsored research, although only few antimicrobial ingredients have found their way into consumer products. Ingredients used in commercial formulations

nowadays include chlorhexidine, triclosan, mixtures of essential oils, and various metal salts including zinc compounds and stannous fluoride (Cummins & Creeth 1992, Ciancio 1992, Van der Ouderaa 1992, White 1995, Jackson 2000, Davies et al. 2004). As a general rule, formulations containing these ingredients exhibit some modicum of efficacy in the control of dental plaque, plaque-induced gingival inflammation and bleeding. However, the effectiveness of these formulations is highly variable and appears influenced by a variety of factors, including the patient plaque level (McClanahan & Bartizek 2002). Of the ingredients commonly used in commercially available products, only chlorhexidine has a ubiquitously recognized efficacy. Chlorhexidines' efficacy is related to its aggressive and broad spectrum antimicrobial activities, rapid diffusivity into dental plaque and its adsorptive and release properties to oral tissues providing a long duration of action (Goodson 1989, Mandell 1994). Unfortunately, side effects such as staining and taste perception limit the broad scale use of chlorhexidine over the counter (Addy & Moran 1995, Nathoo & Gaffar 1995).

Consequently, the desire to develop improved antimicrobial formulations for patient use without side effects based upon improved delivery of antimicrobials to the oral cavity remains. Efforts to this end are hampered in part by limitations of evaluation techniques (Lang 1998). There is a particular problem in assessing the proportional efficacy of formulations in patients with variable levels of hygiene, specifically the efficacy of formulas under conditions of variable plaque coverage (McClanahan & Bartizek 2002). Methods used to study the strength and duration of antibacterial oral benefits include in vitro methods such as minimum inhibitory concentration, bactericidal time kill and various biofilm assays (Ramji et al. 2004) while in vivo techniques include plaque re-growth assessments (Addy et al. 1990). These methods and assessments of antimicrobial retention typically provide only a general prediction of formulation strength and duration of activity. White et al. (1995) described a plaque sampling technique, which permitted the time-dependent study of strength and duration of antimicrobial efficacy on in situ-treated dental plaques. Natural dental plaques were sampled prior to and at various periods following direct in vivo treatments with antimicrobial formulations. Normalized plaque dispersions were cultured and analysed for metabolic and re-growth activity ex vivo, permitting an assessment of strength and duration of activity following treatments. Results of this ''plaque glycolysis and regrowth method (PGRM)'' could be correlated to the antiplaque and antigingivitis efficacy of topical antimicrobial formulations containing both stannous fluoride and cetylpyridinium chloride and empirical use of these assays was recommended as part of the proposed guidelines for monograph antimicrobial formulations with the United States Food and Drug Administration (Federal Register 2003).

The empirical PGRM has limitations in that the technique relies on ex vivo culturing of sampled plaque. Traditional culturing of dispersed plaque samples only allows the evaluation of viable organisms. Complementary measures of antimicrobial effects on biofilm species viability without ex vivo culturing could provide a valuable corollary. Confocal scanning laser microscopy (CSLM) has recently developed as a powerful tool to study dead and live bacteria in dental plaque (Kuehn et al. 1998, Wood et al. 2000). However, as a drawback, the microscopic nature of the technique makes the selection of regions of the dental plaque for analysis highly observer dependent. Moreover, penetration of the required stains into the plaque is not always trivial, while staining and CSLM obviously cannot be applied on intra-oral plaque. This drawback has been circumvented in one protocol by using ex situ plaques, formed in 200 μm wide grooves cut in bovine dentin discs and worn by human volunteers up to 48 h (Zaura-Arite et al. 2001). CSLM on these plaques, after ex situ exposure to chlorhexidine, showed a reduction in viability only in the outer plaque layer because of the protection offered by the biofilm mode of growth of the plaque (Gilbert et al. 1997, Costerton & Stewart 2002). In order to study the substantivity of oral antimicrobials, application of the antimicrobials should not be done ex situ, but in vivo. Exploitation of the benefits of CSLM to study dead and live bacteria then requires dispersal and staining of intra-oral plaque samples. Therewith, it admittedly becomes impossible to study antimicrobial effects over the depth of the plaque layer, but it is just this point that bears the danger of introducing a strong observer dependence in the data as an observer has the unavoidable tendency to select for ''nice'' images.

The aims of this paper are (i) to propose a technique for quantitative assessment of antimicrobial efficacy and substantivity on in vivo-treated dental plaque based on dispersal, staining and CSLM examination of collected plaques, (ii) to compare the immediate antimicrobial efficacy of a number of oral antimicrobials applied intra-orally through four selected dentifrices or a chlorhexidine mouth rinse on in vivo treated 9- and 72-h-old plaques with the antimicrobial efficacy against planktonic bacteria, dispersed from untreated plaques and (iii) to compare the substantivity of these antimicrobials against 9- and 72-h-old plaques.

Material and Methods

Human volunteers, plaque formation and its treatment

Five healthy subjects volunteered to participate in this study according to the guidelines of the Medical Ethics Committee of the University Hospital of Groningen, the Netherlands, including the obtained informed consent of the volunteers and the tenets of the Declaration of Helsinki. Volunteers were requested to brush their teeth according to their habitual routine with a sodium fluoride-containing dentifrice (Crest® Regular, Procter & Gamble, Cincinnati, OH, USA) for the duration of the study, with the exception of treatment days. After having used this dentifrice for at least 7 days, the volunteers were first asked to come to the laboratory on treatment days 9 h after the last brushing, while in a second round of experiments the volunteers had refrained from brushing and all other oral hygiene 72 h after the last brushing (see also Fig. 1).

On treatment day, panellist's removed plaque from the buccal side of the tooth and molar surfaces down to the gingival margin from the left-upper quadrant of the dentition with sterile cotton swab sticks. A dental explorer was employed to remove the inter-proximal plaque from the buccal sides. The plaque collected was suspended in 1.5 ml demineralized water, dispersed by sonication and divided in two portions. One portion (baseline) was immediately analysed after *Bac*Light™ (Molecular Probes Europe BV, Leiden, the Netherlands) staining, while the second portion of dispersed plaque was 1 min. exposed in vitro to a

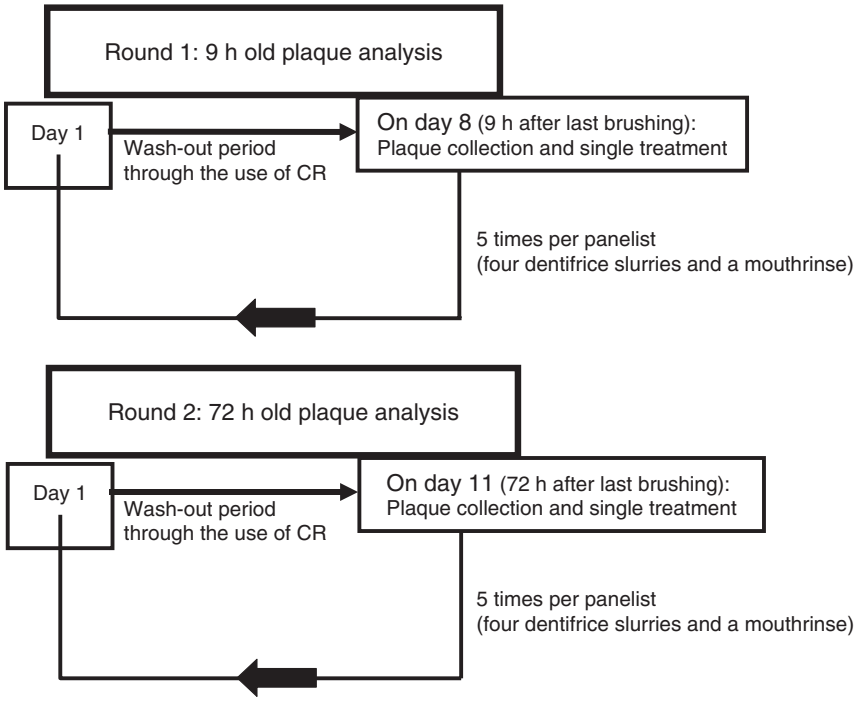

Fig. 1. Flowchart of the study protocol, outlining round one (9-h-old plaques) and round two (72-h-old plaques) of the study.

slurry of one of the four dentifrices or a chlorhexidine rinse by mixing 0.5 ml dispersed plaque with 0.5 ml dentifrice slurry or chlorhexidine mouth rinse (effects on planktonic organisms) prior to CSLM analysis. Subsequently, volunteers were requested to flush their mouth for 1 min. with 10 ml of a 0.2 wt% chlorhexidine containing mouth rinse (Corsodyl®, SmithKline Beecham Consumer Brands B.V., Rijswijk, the Netherlands) or 10 ml of a slurry of one of the four dentifrices (25% by weight in water used after centrifugation, 5 min. at 10,000 *g* to remove particulate matter) to study effects on biofilm organisms in vivo. Immediately after flushing, plaque was collected from the left-lower quadrant as well and subsequently from the right-upper and right-lower quadrants 1 and 6 h after flushing, respectively, and suspended as described above. As a control, possible differences in viability of the plaques collected from the different quadrants prior to application of the antimicrobials were determined in separate experiments.

Four dentifrices were selected in this study owing to their different chemical compositions and expected antimicrobial activity:

- a conventional sodium fluoride dentifrice containing no antimicrobial ingredients with the exceptions of foaming surfactants (Crest® Regular, Procter & Gamble), denoted CR;
- a conventional sodium fluoride dentifrice containing no antimicrobial ingredients with the exceptions of foaming surfactants and containing pyrophosphate antitartar ingredient (Crest® Tartar Control, Procter & Gamble), denoted CTC;
- a known antimicrobial (Davies et al. 2004) containing dentifrice, Colgate Total (Colgate-Palmolive Company, New York, NY, USA), containing sodium fluoride and a copolymer of maleic anhydride and methyl vinyl ether Gantrez™ polymer as well as triclosan broad spectrum antimicrobial, denoted Total;
- a high cleaning performance sodium fluoride dentifrice containing no added antimicrobial ingredients with the exceptions of foaming surfactants and containing sodium hexametaphosphate antistain/antitartar ingredient (Crest® Dual Action Whitening, Procter & Gamble), denoted CDAW.

The surfactant systems in the dentifrices were generally similar and based on sodium lauryl sulphate (SLS) and though three of the four dentifrices did not have added antimicrobial components, the addition of SLS might impart antimicrobial effects (Pader 1985).

In order to eliminate the effects of an evaluation, 7 days of habitual oral hygiene were allowed after each evaluation during which the volunteers were requested to brush with a sodium fluoride containing dentifrice (Crest® Regular).

CSLM

Thirty microlitres of each dispersed plaque was put on a microscope glass slide and stained for 30 min. in the dark with a live and dead stain (*Bac*Light™, Molecular Probes Europe BV). Confocal images were collected using a Leica TCS-SP2 CSLM (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with beam path settings for fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate-like labels. Three images ($375 \times 375\,\mu$m) were randomly taken from each plaque sample immediately after focussing, the number of dead and live bacteria in each images was determined and averaged for the assessed effect per sample.

Results

Figures 2 and 3 show examples of CSLM images of dispersed, 9-h-old plaques, prior to and after exposure to antimicrobials in the planktonic and biofilm state. In Fig. 2 it can be seen that the baseline plaque contains a large number of viable organisms (green), while exposure to the chlorhexidine mouth rinse yields a large decrease in viability both for planktonic as well as for biofilm organisms, i.e. nearly all organisms are coloured red. In contrast, exposure of a plaque to CDAW, yields a comparable decrease in viability for planktonic organisms but only a small decrease in viability of the plaque in biofilm state, i.e. a sizeable number of green-coloured organisms remains after exposure to the dentifrice slurries (Fig. 3). The enumeration of viable and non-viable bacteria in such images represents the quantitative evaluation of treatment effects.

Table 1 shows plaque viability as assessed in different quadrants sampled simultaneously 9 h post-brushing. Note, that the total number of bacteria sampled is higher (no significant difference, $p > 0.05$ paired Student's t test) in maxillary quadrants than in mandibular quadrants. Furthermore, the percentage of viable organisms is similar across quadrants (no significant difference).

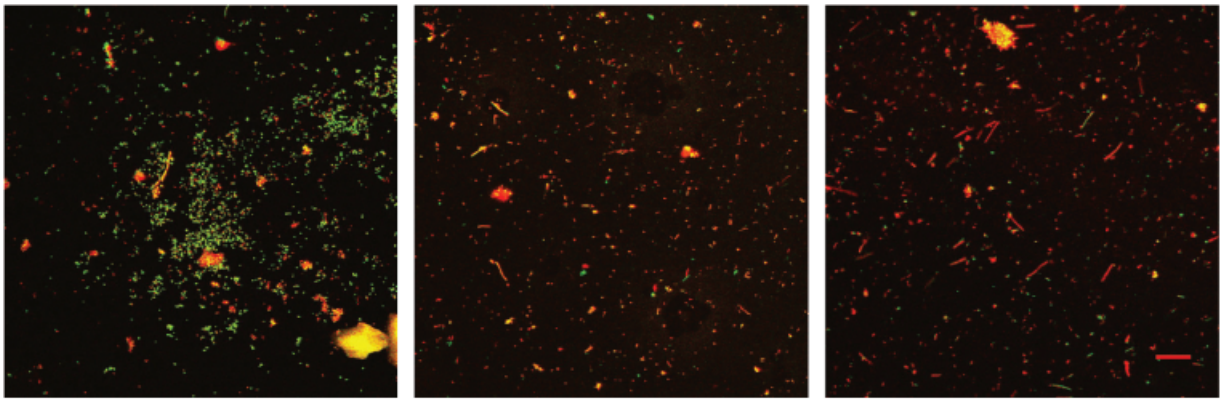

Fig. 2. Examples of 9-h-old plaques collected from human volunteers after dispersal and dead (green)–live (red) staining for confocal scanning laser microscopy. The bar denotes 40 μm. (a) Plaque collected prior (baseline) to exposure to the chlorhexidine mouth rinse (left), (b) plaque exposed (in vitro) to the chlorhexidine mouth rinse in a planktonic state (middle) and (c) plaque collected immediately after exposure (in vivo) to the chlorhexidine mouth rinse in a biofilm state (right).

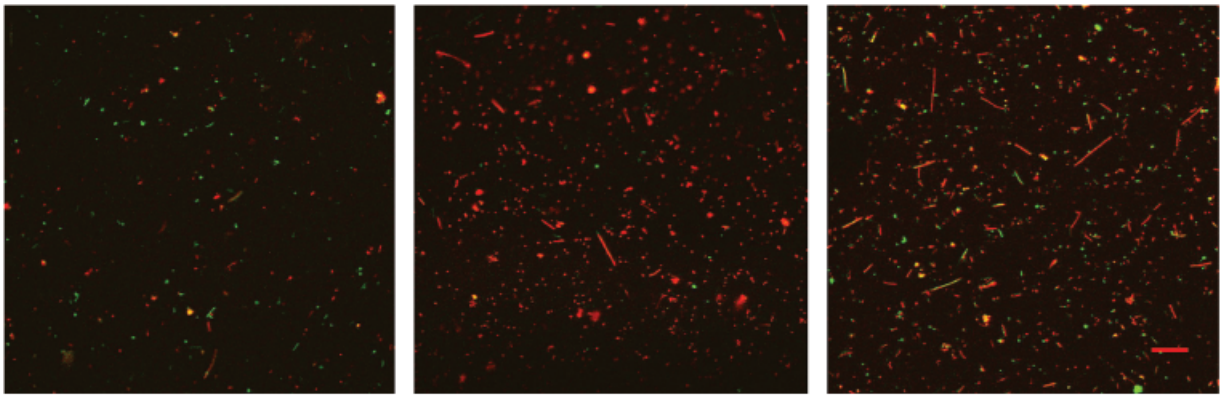

Fig. 3. Examples of 9-h-old plaques collected from human volunteers after dispersal and dead (green)–live (red) staining for confocal scanning laser microscopy. The bar denotes 40 μm. (d) Plaque collected prior (baseline) to exposure to CDAW (left), (e) plaque exposed (in vitro) to CDAW in a planktonic state (middle) and (f) plaque collected immediately after exposure (in vivo) to CDAW in a biofilm state (right).

Table 1. Number of bacteria in 9-h-old plaques collected from the different quadrants in a group of five volunteers, using Crest® Regular as a control, as well as the viability of the plaques

Quadrant	Number of bacteria (10^8/ml)*	Viability (%)*
Left upper	7.3 ± 3.1	42 ± 22
Left under	5.6 ± 3.4	42 ± 22
Right upper	8.5 ± 4.5	37 ± 12
Right under	5.6 ± 3.0	37 ± 12

*No statistically significant difference among groups ($p > 0.05$, paired Student's *t*-test).

Table 2. Percentage viability of organisms in 9 and 72 h old dental plaques formed in vivo (baseline) and immediately after exposure to antimicrobials in a planktonic (in vitro) and biofilm (in vivo) state with ± indicates the SD over the group of volunteers

Treatment	9-h-old biofilm			72-h-old biofilm		
	baseline	planktonic organisms	biofilm organisms	baseline	planktonic organisms	biofilm organisms
CR	47 ± 17	2 ± 1*	14 ± 6	49 ± 18	1 ± 0*	28 ± 15
CTC	48 ± 14	4 ± 5*	14 ± 11	29 ± 17	1 ± 0*	14 ± 4
Total	66 ± 17	1 ± 1*	30 ± 19	48 ± 8	1 ± 0*	30 ± 15
CDAW	66 ± 10	2 ± 2*	30 ± 17	37 ± 10	1 ± 1*	42 ± 12
CHX	55 ± 10	4 ± 3*	18 ± 23	44 ± 12	1 ± 0*	8 ± 2

*Statistically different from baseline and biofilm viability at $p < 0.01$, Student's *t*-test.

This observation is important, as it provides the key validation for the methodology – use of baseline quadrant viabilities to compare the sustained antimicrobial effects following treatment based on sampling of different quadrants.

Table 2 summarizes the percentage viability of the 9- and 72-h-old plaques prior to and immediately after exposure to the antimicrobials in the planktonic and biofilm state. The average viability of the plaques prior to exposure to antimicrobials clearly decreases from a 9-h-old plaque (average viability 56 ± 8%) to a 72-h-old plaque (average viability 41 ± 8%). The viability of the planktonic organisms after in vitro exposure to both the chlorhexidine mouth rinse as well as after exposure to dentifrice slurries decreases significantly. Also in the biofilm state (9-h-old), organisms appeared surprisingly

sensitive to the antimicrobials and the viability of the plaques decreased significantly to between 14% and 30% (no statistically significant difference between products). A 72-h-old plaque left on average 13% more organisms in a viable state after exposure to CR and CDAW slurries than did a 9-h-old plaque. Similar viabilities resulted immediately after exposure to Total, CTC, and the chlorhexidine mouth rinse after exposure of a 9- and 72-h-old plaque.

Figure 4 provides a summary of the data for treatment groups compared in the study. For each treatment period, subjects used Crest® Regular prior to quadrant plaque sampling. The percentage of dead bacteria increases dramatically for all treatments immediately following application, but generally increases towards baseline levels after some time, except for the chlorhexidine mouth rinse. A statistical evaluation of baseline plaque viability, however, showed that the percentage of viable bacteria was slightly different for baseline quadrants from day to day per person and within different persons. The variation from day to day per person and within different persons was similar, however. To standardize comparisons, it was determined to assess the percentage change in viable bacteria, i.e. the percentage bacteria killed, relative to the internal control of each panellist in a group according to

$$\%\text{killed at time } t = \left(1 - \frac{\%\text{viable at time } t}{\%\text{viable at baseline}}\right) \times 100$$

A quantitative statistical analysis of the percentage of plaque bacteria actually ''killed'' by each treatment is shown in Table 3 for 9- and 72-h-old plaques. Immediately post-treatment, all formulations produced over 50% kill of viable organisms in 9-h-old plaques with no significant differences between treatments and the chlorhexidine mouth rinse exhibiting antimicrobial activity in the high range without being significantly more effective. However, all formulations except the chlorhexidine mouth rinse were less effective against a 72-h-old plaque than against a 9-h-old plaque. One hour post-treatment, the percentage of bacteria killed was reduced for all treatments, with no significant difference between dentifrices. The efficacy of the chlorhexidine rinse decreased only against 72-h-old plaques, but not against 9-h-old plaque. Six hours post-treatment, the efficacy of the chlorhexidine mouth rinse against 9-h-old plaques remains high and the distinction between the chlorhexidine rinse and the dentifrices becomes more pronounced (statistically significant at $p < 0.05$), although the efficacy of the chlorhexidine rinse drops off rapidly after 6-h against a 72-h-old plaque. Interestingly, the efficacy of the dentifrice components against 72-h-old plaque increases 6 h post-treatment as compared with 1 h post-treatment.

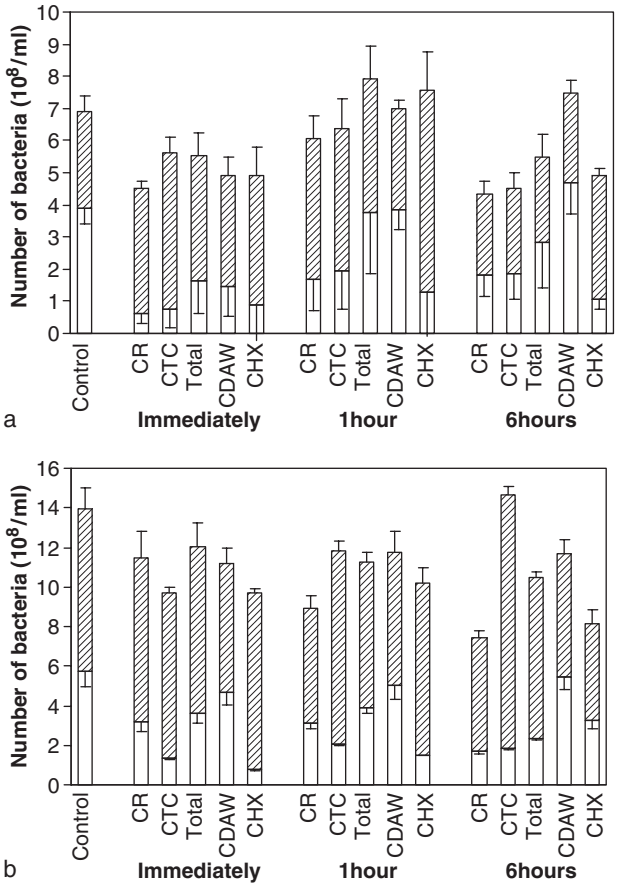

Fig. 4. Viability of dental plaques formed in vivo as determined after collection, dispersal and staining for confocal scanning laser microscopy, together with immediate effects of in vivo exposure to dentifrice slurries and a chlorhexidine mouth rinse as well as effects determined 1 and 6 h after exposure. The white areas indicate live bacteria while the dashed areas represent dead bacteria. (a) Nine-hour-old plaques and, (b) 72-h-old plaques. Bars denote SD over experiments in five human volunteers.

Discussion

The purpose of this study was to propose a novel method for the examination of antimicrobial effectiveness and substantivity of oral antimicrobials on in vivo dental plaques and to compare a variety of commercial oral products. The proposed method is considered preferable to ex vivo development of plaque or dye reactivity with thick films, because the plaques examined have been formed naturally and dye reactivity is standardized. Furthermore, application of the antimicrobials is in vivo and antimicrobial effects normalized for different participants and treatment days. This latter benefit of the model is crucial in order to account for diurnal, subject and day-to-day variations in plaque quality.

The antimicrobial efficacies of all dentifrices and the chlorhexidine rinse are similarly high against planktonic bacteria owing to the antimicrobial effects imparted by SLS (Pader 1985), regardless of whether other antimicrobials were added to the products. When in the biofilm mode of growth, however, organisms are more resistant against SLS and effects of the antimicrobials added become more evident in older plaques, most notably 6 h post-treatment. This indicates that dead bacteria and exopolymeric substances produced

Table 3. Plaque viability changes post-treatment with various dentifrices and the chlorhexidine mouth rinse, expressed as percentage killed *versus* the baseline plaque sampled in a group of five volunteers, using Crest® Regular as a control and ± denoted the SD over the group of volunteers after correction for day-to-day and person-to-person variations

Treatment	Immediate post-treatment % killed	1 h post-treatment % killed	6 h post-treatment % killed
9-h-old plaques			
CR	70.0 ± 19.4^{a}	$38.0 \pm 27.3^{a,b}$	7.8 ± 26.9^{b}
CTC	73.4 ± 14.8^{a}	$39.5 \pm 36.0^{a,b}$	15.1 ± 23.0^{b}
Total	57.1 ± 19.4^{a}	32.4 ± 23.9^{b}	26.4 ± 25.7^{b}
CDAW	51.5 ± 34.7^{a}	13.3 ± 25.8^{b}	4.8 ± 10.8^{b}
CHX	70.7 ± 33.8^{a}	70.1 ± 28.0^{a}	60.6 ± 12.8^{a}
72-h-old plaque			
CR	39.4 ± 40.7^{b}	22.7 ± 32.9^{bc}	46.2 ± 31.7^{ab}
CTC	29.7 ± 48.8^{bc}	21.1 ± 38.5^{bc}	41.2 ± 34.2^{ab}
Total	38.7 ± 22.2^{b}	26.9 ± 12.8^{ab}	52.6 ± 7.6^{a}
CDAW	12.4 ± 8.9^{c}	14.8 ± 23.7^{c}	31.4 ± 31.5^{c}
CHX	80.8 ± 5.6^{a}	62.5 ± 25.6^{a}	7.5 ± 33.7^{bc}

$a \neq b \neq c$ $p < 0.05$ (paired Student's *t*-test).

impede fast penetration of the antimicrobials through the biofilm, while on the other hand they may also contribute to a reservoir function.

All dentifrices and the chlorhexidine mouth rinse have sizeable immediate antimicrobial effects against 9-h-old plaque, which disappear over time for most antimicrobials evaluated. Nevertheless, some of the oral antimicrobials added, like chlorhexidine and triclosan, are able to maintain antimicrobial activity over several hours as measured in the proposed method, i.e. measured as substantivity of the killing action, which is meaningful to actual in vivo therapeutic actions. However, only the chlorhexidine mouth rinse demonstrated sustained and clear statistically significant efficacy in rendering dental plaques with reduced viability. The killing efficacy of the dentifrices, most notably of Total and CDAW, increases 1 h post-treatment of 72-h-old plaques, as opposed to 9-h-old plaques, which likely attest to the slow penetration of the antimicrobials through the 72-h-old plaques mentioned before, showing antimicrobial benefits only after extended time intervals.

The major advantage of the proposed method over many others, demonstrating in vitro or in vivo effects of the chlorhexidine mouth rinse and other antimicrobials, is that it involves a fully natural plaque in addition to a natural route of antimicrobial administration. In vitro studies with, for instance a constant depth film fermenter (Pratten et al. 1998), or in vivo studies with a dentin groove model (Zaura-Arite et al. 2001), yield plaques with an elevated presence of lactobacilli (Van Houte et al. 1972, Van Houte 1994), thriving in retention sites in the oral cavity. Also, because of the confined area in which these plaques develop, the biofilm compresses with potential effects the penetration of antimicrobials. A minor disadvantage of this study design is that it is not possible to study the localization of antimicrobial effects on sample plaques as they are dispersed for analysis. This however, does not limit studies on the effects of biofilm thickness on antimicrobial activity, which is of primary interest in formulation efficiency and delivery.

The immediate effects of the chlorhexidine mouth rinse and the dentifrice components on the viability of 9-h-old plaques are unexpectedly high and raise the question of how much protection (Stewart et al. 2000) young oral biofilms actually offers. Apart from the fully natural way our plaques have developed in the present study, the minor protection offered by these young biofilms may be because of the use of only overnight plaques. These plaques can be expected to form a relatively small, but thick biofilm near the gingival margins, with scattered micro-colonies over other parts of the dentition. Evidently, these micro-colonies do not experience the full benefits of the biofilm mode of growth, as seen in his study for 72-h-old plaques.

Acknowledgements

This study was supported by the University of Groningen, Groningen, the Netherlands and The Procter & Gamble Company, Cincinnati, OH, USA.

References

Addy, M., Jenkins, S. & Newcombe, R. G. (1990) The effect of tricosan, stannous fluoride and chlorhexidine products on: (I). Plaque regrowth over a 4-day period. *Journal of Clinical Periodontology* **17**, 693–697.

Addy, M. & Moran, J. (1995) Mechanisms of stain formation on teeth in particular associated with metal ions and antiseptics. *Advances in Dental Research* **9**, 450–456.

Baehni, P. C. & Bourgeois, D. M. (1998) Epidemiology of periodontal health and disease. In: Lang, N. P., Atstrom, R. & Loe, H. (eds). *Proceedings of the European Workshop on Mechanical Plaque Control*, pp. 19–34. Berlin, Germany: Quintessence Publishing Company Inc.

Ciancio, S. G. (1992) Agents for the management of plaque and gingivitis. *Journal of Dental Research* **71**, 1450–1454.

Costerton, J. W. & Stewart, P. S. (2002) Battling biofilms. *Scientific American* **285**, 74–82.

Cummins, D. & Creeth, J. E. (1992) Delivery of antiplaque agents from dentifrices, gels and mouthwashes. *Journal of Dental Research* **68** (Special Issue), 1609–1616.

Davies, R. M., Ellwood, R. P. & Davies, G. M. (2004) The effectiveness of a toothpaste containing triclosan and polyvinyl–methyl ether maleic acid copolymer in improving plaque control and gingival health. *Journal of Clinical Periodontology* **31**, 1029–1033.

Federal Register. (2003) Oral health care drug products for over-the-counter human use. Antigingivitis/antiplaque drug products; establishment of a monograph proposed rules. May 29, pp. 32,232–32,287.

Gilbert, P., Das, J. & Foley, I. (1997) Biofilm susceptibility to antimicrobials. *Advances in Dental Research* **11**, 160–167.

Goodson, J. M. (1989) Pharmacokinetic principles controlling efficacy of oral therapy. *Journal of Dental Research* **68** (Special Issue), 1625–1632.

Jackson, R. J. (2000) Metal salts, essential oils and phenols – old or new? *Periodontology* **15**, 63–73.

Kuehn, M., Hausner, M., Bungartz, H. J., Wagner, M., Wilderer, P. A. & Wuertz, S. (1998) Automated confocal laser scanning microscope and semi-automated image processing for analysis of biofilms. *Applied and Environmental Microbiology* **64**, 4115–4127.

Lang, N. P. (1998) Commonly used indices to assess oral hygiene and gingival and periodontal health and diseases. In: Lang, N. P., Atstrom, R. & Loe, H. (eds). *Proceedings of the European Workshop on Mechanical Plaque Control*, pp. 50–71. Berlin, Germany: Quintessence Publishing Company Inc.

Loe, H., Theilade, E. & Jensen, S. B. (1965) Experimental gingivitis in man. *Journal of Periodontology* **36**, 177–187.

Mandell, I. D. (1994) Antimicrobial mouthrinses: overview and update. *Journal of American Dental Association* **25** (Suppl. 2), 2S–10S.

McClanahan, S. F. & Bartizek, R. D. (2002) Effects of triclosan/copolymer dentifrice on dental plaque and gingivitis in a 3-month randomized controlled clinical trial: influence of baseline gingivitis on observed efficacy. *Journal of Clinical Dentistry* **13**, 167–178.

Mombelli, A. W. (1998) The role of dental plaque in the initiation and progression of periodontal diseases. In: Lang, N. P., Atstrom, R. & Loe, H. (eds). *Proceedings of the European Workshop on Mechanical Plaque Control*, pp. 85–97. Berlin, Germany: Quintessence Publishing Company Inc.

Nathoo, S. A. & Gaffar, A. (1995) Studies on dental stains induced by antibacterial agents and rational approaches for bleaching dental stains. *Advances in Dental Research* **9**, 462–470.

Pader, M. (1985) Surfactants in oral hygiene products. In: Rieger, M. M. (ed). *Surfactants in Cosmetics*, pp. 293–347. New York: Marcel Dekker Inc.

Pratten, J., Barnett, P. & Wilson, M. (1998) Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. *Applied and Environmental Microbiology* **64**, 3515–3519.

Ramji, N., Baig, A., He, T., Lawless, M. A., Saletta, L. & Suszcynsky-Meister, E. (2004) Sustained antibacterial actions of a new stabilized stannous fluoride dentifrice containing sodium hexametaphosphate. *Compendium Continuing Education in Dentistry* **25** (Suppl. 1), 19–28.

Robinson, P. J. (1995) Gingivitis: a prelude to periodontitis? *Journal of Clinical Dentistry* **6**, 41–45.

Schou, L. (1998) Behavioral aspects of dental plaque control measures: an oral health promotion perspective. In: Lang, N. P., Atstrom, R. & Loe, H. (eds). *Proceedings of the European Workshop on Mechanical Plaque Control*, pp. 287–299. Berlin, Germany: Quintessence Publishing Company Inc.

Stewart, P. S., McFeters, G. A. & Huang, C. T. (2000) Biofilm control by antimicrobial agents. In: Bryers, J. D. (ed). *Biofilms II: Process Analysis and Applications*, pp. 373–405. New York: Wiley-Liss Inc.

Theilade, E., Wright, W. H., Jensen, B. S. & Loe, H. (1966) Experimental gingivitis in man II. A longitudinal clinical and bacteriological investigation. *Journal of Periodontal Research* **1**, 1–13.

Van der Ouderaa, F. J. G. (1992) Human clinical studies of antiplaque and antigingivitis agents dosed from a dentifrice. In: Rolla, G. & Embery, G. (eds). *Clinical and Biological Aspects of Dentifrices*, pp. 181–204. Oxford: Oxford University Press.

Van Houte, J. (1994) Role of microorganisms in caries etiology. *Journal of Dental Research* **73**, 672–681.

Van Houte, J., Gibbons, R. J. & Pulkkinen, A. (1972) Ecology of human oral lactobacilli. *Infection and Immunity* **6**, 723–729.

White, D. J. (1995) A ''Return'' to stannous fluoride dentifrices. *Journal of Clinical Dentistry* **6**, 29–36.

White, D. J., Cox, E. R., Liang, N., Macksood, D. & Bacca, L. (1995) A new plaque glycolysis and regrowth method (PGRM) for the in vivo determination of antimicrobial dentifrice/rinse efficacy toward the inhibition of plaque growth and metabolism – method development, validation and initial activity screens. *Journal of Clinical Dentistry* **6**, 59–70.

Wood, S. R., Kirkham, J., Marsh, P. D., Shore, R. C., Natress, B. & Robinson, C. (2000) Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *Journal of Dental Research* **79**, 21–27.

Zaura-Arite, E., Van Marle, J. & Ten Cate, J. M. (2001) Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *Journal of Dental Research* **80**, 1436–1440.

Address:
H.C. van der Mei
Department of Biomedical Engineering
University Medical Center Groningen
University of Groningen
Antonius Deusinglaan 1,9713 AV Groningen
The Netherlands
E-mail: h.c.van.der.mei@med.umcg.nl

Clinical Relevance

This paper presents a new quantitative method to determine the clinical efficacy of antimicrobials based on CLSM examination of dispersed plaque, formed and treated in vivo. Dentifrice slurries and a chlorhexidine mouth rinse were all effective in killing dispersed, planktonic bacteria. Sodiumlaurylsulphate contributed to the immediate antimicrobial effects on 9- and 72-h-old plaques, but sustained effects were only seen for chlorhexidine on 9-h-old plaques. Dentifrice slurries on the other hand, became slightly more effective overtime against 72-h-old plaques.