Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine

J. Pratten a, A. W. Smith b and M. Wilson a

a Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences, University of London, 256 Grays Inn Road, London WC1X 8LD; b SmithKline Beecham Consumer Healthcare, Oral Health Category, St George’s A venue, Weybridge, Surrey KT13 O DE, UK

The aim of this study was to determine the effect of pulsing chlorhexidine gluconate, at concentrations commonly used in mouthwashes, on Streptococcus sanguis biofilms and microcosm dental plaques in vitro. Biofilms were grown on bovine enamel and nutrients were supplied in the form of artificial saliva. Pulsing experiments were carried out on steady-state biofilms using 0.05 or 0.2% chlorhexidine solutions delivered twice daily for 1 min. In a separate study, the enamel discs on which the biofilms were formed were pre-treated with chlorhexidine and pulsed directly after inoculation and then at regular intervals. With both concentrations of chlorhexidine used, a c.2 log10 reduction in the viable counts of S. sanguis was achieved with the initial pulse, but as pulsing continued, the bacterial population recovered, albeit not to the previous level. A c.1 log10 reduction in the total viable counts of the microcosm plaques was seen after the first pulse with 0.2% chlorhexidine. The total count then recovered rapidly and, after the fifth pulse, the total viable counts were not significantly different from those before pulsing. The total counts then remained at a similar level throughout the course of the experimental runs. Pre-treatment of the enamel discs with 0.2% chlorhexidine before inoculation produced viable counts of c.10^5 cfu/mm², a 1 log10 reduction compared with untreated discs. After pulsing with 0.2% chlorhexidine at 8 h, a 3 log10 reduction was seen in the total aerobic and anaerobic counts, but again the viable counts subsequently increased despite twice-daily chlorhexidine pulsing. Regardless of the nature of the biofilm, pulsing initially achieved substantial kills, but the viability of the biofilms subsequently increased despite continued pulsing. Chlorhexidine was effective at reducing the viability of microcosm plaques when it was applied to the substratum before exposure to bacteria and subsequently pulsed on to the biofilms.

Introduction

The microflora of the oral cavity is a complex community and is found as a film, known as plaque, on the tooth surface. Plaque comprises bacteria, extracellular products and polymers of salivary origin and is therefore a typical biofilm. Bacteria living in biofilms are generally less susceptible to antimicrobial agents than their corresponding planktonic forms which are normally used for in-vitro susceptibility testing. For example, it has been shown that various antibiotics used at concentrations of 500 x MIC affected the viability of Streptococcus sanguis biofilms only after 48 h exposure. In clinical trials, however, antimicrobial agents have been shown to be effective in the control and prevention of periodontal diseases and caries. The most effective agent to date has been chlorhexidine, which has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. Chlorhexidine has been shown to reduce plaque, caries and gingivitis in humans, but it is not ideal for prolonged periods of use as treated teeth become stained. At high concentrations, chlorhexidine is bactericidal and acts as a detergent by damaging the cell membrane. The benefits of chlorhexidine are not limited to this initial bactericidal effect, as other agents with a similar MIC do not have the same anti-plaque properties. Chlorhexidine is known to be a cationic antimicrobial, and hence is bound to enamel or the salivary pellicle at a
concentration sufficient to have a prolonged biostatic effect on adherent streptococci.\(^6\)

When developing new antibacterial agents for use in the complex environment of the oral cavity, it is important to have a model that mimics closely the nutrient source, substratum and bacterial species that are present in vivo. To compare the effectiveness of test agents for both antibacterial and anti-plaque purposes it is also important to deliver the compound in such a way that results are comparable with clinical studies. The purpose of this investigation was to evaluate the effects of pulsing a commonly used antimicrobial and anti-plaque agent on biofilm viability and formation using a constant-depth film fermentor (CDFF) as a means of generating biofilms under conditions similar to those which would exist in vivo.

**Materials and methods**

**Bacterial strain and media**

The organism used in the single species studies was *S. sanguis* NCTC 10904. Saliva was used as an inoculum to provide a multi-species biofilm containing organisms found in the oral cavity. The saliva was collected from ten healthy individuals; equal amounts from each person were pooled and 1 mL aliquots were dispensed into cryovials and stored at -70°C for subsequent use. The nutrient source in all experiments was an artificial saliva, the composition of which was based on the work of Russell & Coulter\(^7\) and Shellis.\(^8\) The artificial saliva contained 1 g/L ‘Lab-lemco’ powder (Oxoid, Basingstoke, U K), 2 g/L yeast extract (Oxoid), 5 g/L proteose peptone (Oxoid), 2.5 g/L hog gastric mucin (Sigma Chemical Co., Poole, U K), 0.35 g/L sodium chloride (BDH Chemicals Ltd, Poole, U K), 0.2 g/L calcium chloride (BDH) and 0.2 g/L potassium chloride (BDH) in distilled water. After autoclaving, 1.25 mL of a 40% urea (Sigma) solution was added.

**Production of biofilms**

Biofilms were grown in a CDFF (University of Wales, Cardiff) as described by Wilson et al.\(^\text{9}\) The apparatus was maintained at 37°C in an incubator. The rotating turntable held 15 polytetrafluoroethylene (PTFE) pans located flush around its rim. Each pan contains six cylindrical holes containing PTFE plugs. The biofilms were grown on bovine enamel discs (4.7 mm in diameter) cut from bovine incisors which had been polished flat (Biomaterials Department, Eastman Dental Institute, University of London). These sit on PTFE plugs of the same diameter and recessed to a depth of 300 \(\mu\)m. The enamel discs were immersed in sterile artificial saliva for 1 min to form a conditioning film before the CDFF was assembled and autoclaved.

Inoculation of the CDFF

Ten millilitres of nutrient broth (Oxoid) was inoculated with a single colony of *S. sanguis* and incubated anaerobically at 37°C overnight. This was then used to inoculate 2 L of artificial saliva which was recirculated through the CDFF for 24 h. Recirculating in the case of the microcosm community was for 8 h using 500 mL of artificial saliva inoculated with 1 mL of pooled saliva. A portion of the pooled saliva sample was also serially diluted and plated on to selective media as described below. After the appropriate time, the inoculum flask was disconnected and the CDFF fed from a medium reservoir of sterile artificial saliva, the waste being collected in an effluent bottle. The artificial saliva was delivered at a rate of 0.72 L/day, corresponding to the resting flow rate of saliva in humans\(^10\)-\(^12\) using a peristaltic pump (Watson-Marlow, Falmouth, U K).

**Chlorhexidine pulsing of biofilms**

Pulsing was carried out twice daily (at 09.00 and 17.00 h) for 1 min with 10 mL of either 0.05 or 0.2% (w/v) chlorhexidine gluconate (Sigma) delivered via a peristaltic pump. The pulsing was started once the biofilms had achieved a steady state. However, when the discs were pre-treated with chlorhexidine before inoculation, pulsing took place after inoculation at 8 h.

**Culture methods**

Pans were removed from the CDFF at 24 h intervals, and the bovine enamel discs were aseptically removed and placed into neutralizing broth (Difco Laboratories, Detroit, MI, USA), to prevent any further action by chlorhexidine, before being vortexed for 1 min to disrupt the biofilm. *S. sanguis* was serially diluted in nutrient broth and duplicate 20 \(\mu\)L volumes of each dilution were plated on to Tryptone Soy A agar (Oxoid). The plates were incubated anaerobically overnight at 37°C and the resulting colonies were counted.

The microcosm biofilms were treated in a similar manner but were plated on to various selective media. *Actinomyces* spp. were isolated on cadmium fluoride/acriflavin/tellurite (CFA T) agar plates,\(^13\) Veillonella spp. on veillonella agar (Difco) containing vancomycin (Sigma) 7.5 mg/L and streptococci on Mitis Salivarius A agar (Difco). The total anaerobic count was obtained using 8% blood agar (Oxoid). Plates were incubated anaerobically for 4 days at 37°C. Total aerobic viable counts were performed on blood agar plates and incubated at 37°C aerobically.

**Vital staining of bacteria**

Vital staining was carried out using a BacLight viability testing kit (Molecular Probes Inc., Eugene, OR, USA).
The discs on which the biofilms formed were vortexed for 1 min and transferred into vials containing 1 mL of phosphate buffered saline (PBS; Oxoid) before adding the reagents, according to the manufacturer’s specifications. After 15 min, 5 μL of the suspension was added to a counting chamber. Green fluorescence indicated live bacteria whereas dead bacteria appeared red when viewed with light at a wavelength of 300 nm.

Results

The effects of chlorhexidine pulses on the viability of S. sanguis biofilms are shown in Figure 1. Figure 1a shows the effect of pulsing 0.05% and 0.2% chlorhexidine on S. sanguis biofilms at 120 h. When sampled at 144 h the biofilms pulsed with 0.05% chlorhexidine had reductions in total viable counts of 98.7%. By 168 h there was an increase in the total viable count, although this was not significant; after subsequent pulsing, growth of the biofilms continued to occur, but the number of viable bacteria did not reach the levels seen before pulsing and there was still a 90.3% reduction (compared with pre-pulsing levels) in the number of viable bacteria after 216 h. The initial pulse with 10 mL of 0.2% chlorhexidine produced a reduction (99.7%) in the number of viable bacteria similar to that achieved using 0.05% chlorhexidine. This was followed by a significant rise (P < 0.05) in numbers 24 h after the initial pulse. However, the viable count after 216 h was 94% lower than that before pulsing.

Figure 1b shows the proportion of live/dead bacteria within the S. sanguis biofilms as revealed by vital staining. The total number of live bacteria before pulsing was 4 × 10⁶/mm². With direct visualization, a c.1 log₁₀ reduction in live cells was observed as a direct response to the first pulse of 0.05% chlorhexidine, a kill of 85.4%. There was a corresponding increase of 52.2% in the number of dead cells counted, which comprised 90.0% of the total bacteria in the biofilm.

The microcosm plaques produced total viable counts in

![Figure 1](image-url)
the region of $10^8$ cfu/mm$^2$. When these had reached a steady state they consisted of 19% Actinomyces spp., 25% streptococci and 7% Veillonella spp. (Table). The proportions of these species, expressed as a percentage of the total anaerobic count, are also given for the pooled saliva used as an inoculum. The streptococci accounted for 86% of the enumerated species, which was more than three times greater than that observed in the steady-state microcosm plaques.

When pulsing was carried out on these biofilms, the trends observed (Figure 2) were similar to those obtained using the single species biofilms. A reduction of c.1 log$_{10}$ in the total viable counts (aerobic species, $P < 0.05$; anaerobic species, $P < 0.001$) was seen after the first treatment with 0.2% chlorhexidine. After this first pulse, the total aerobic count rapidly recovered and by 240 h the total viable count was not significantly different ($P < 0.05$) from that before pulsing. The counts then remained at similar levels throughout the course of the experiment. With respect to the total anaerobic count, the number of viable bacteria did reach levels seen before pulsing after 336 h. The Actinomyces spp. and Veillonella spp. viable counts decreased significantly ($P < 0.001$) after the initial pulse. Between 240 and 288 h the numbers did not significantly change but after this period there was an increase until at 336 h they had recovered to pre-pulsing levels. The proportions of these species, compared with the total anaerobic count, at 336 h had increased considerably, collectively making up >90% of the community. The streptococci seemed the most susceptible to chlorhexidine in the microcosm community and, although the number of viable bacteria recovered to some extent following initial pulsing, this growth was not sustained. At the final sampling point the proportion of streptococci (as a percentage of the total anaerobic count) was 30% less than that seen before pulsing.

In the case of the discs treated before inoculation and then subsequently pulsed (Figure 3), after 8 h the biofilms had total viable counts of $2.6 \times 10^5$ cfu/mm$^2$. This constituted approximately a 1 log$_{10}$ reduction in the

Table. Comparison of the bacterial composition of approximal dental plaque, microcosm plaques and the pooled human saliva used as an inoculum. Data represent the proportion of each type of organism expressed as a percentage of the total anaerobic viable count. Means and ranges are from four runs.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Approximal dental plaque</th>
<th>Microcosm plaques</th>
<th>Pooled human saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean  range</td>
<td>mean  range</td>
<td>mean  range</td>
</tr>
<tr>
<td>Actinomyces spp.</td>
<td>28  4–47</td>
<td>19  17–21</td>
<td>7  1–16</td>
</tr>
<tr>
<td>Veillonella spp.</td>
<td>11  &lt;1–30</td>
<td>7  3–12</td>
<td>4  1–14</td>
</tr>
</tbody>
</table>

Figure 2. Effect of pulsing 0.2% chlorhexidine on the viability of microcosm plaques ($n = 4$). Arrows represent 10 mL pulses. Error bars represent standard deviations. ◆, Total aerobic; ▲, total anaerobic; △, Actinomyces spp.; ○, Streptococcus spp.; ■, Veillonella spp.
Effect of chlorhexidine on oral biofilms

Figure 3. Growth of a microcosm plaque community on bovine enamel discs treated with 0.2% chlorhexidine and then pulsed with chlorhexidine after 8 h (n = 4). Arrows represent 10 mL pulses. Error bars represent standard deviations. ●, Total aerobic; ▲, total anaerobic; △, Actinomyces spp.; ○, Streptococcus spp.; ■, Veillonella spp.

The number of colonizing bacteria compared with untreated discs. After the initial pulse, a 3 log₁₀ reduction (99.8% kill) was seen in the viable count of aerobes, anaerobes and streptococci (P < 0.001). The Veillonella spp. and Actinomyces spp. were undetectable but numbers did recover later in the run. However, the Veillonella spp. were again undetectable after 80 h. After sampling at 56 h, the total aerobic, total anaerobic and streptococci counts had all significantly (P < 0.001) recovered, the total aerobic count continued to increase reaching levels similar to those before pulsing but still significantly lower than those found on discs which had not been treated with chlorhexidine.

Discussion

The aims of this study were to determine the effects of chlorhexidine pulsing on the viability of S. sanguis biofilms and microcosm plaques in vitro. There was no significant difference in the viability of the S. sanguis biofilms when pulsed with different concentrations of chlorhexidine. These results were corroborated with live/dead staining, where we were able to assess the viability of the biofilms in terms of the numbers of both live and dead bacteria. This surprising finding may be attributable to the dynamic nature of the experimental system used. Certainly, in a static system, we were able to show that the effect of chlorhexidine on S. sanguis biofilms was dose-related.14 It is important to note that the 1 min chlorhexidine pulses were followed by a continuous flow of artificial saliva (mimicking the in-vivo situation) which would exert a flushing action so reducing the contact time of the chlorhexidine with the biofilm as well as the time available for biofilm penetration. Therefore, it may be that the similar kills attained with both chlorhexidine concentrations were because in each case only the superficial layers of the biofilm were affected. Interestingly, the absence of a concentration-dependent effect has also been observed in a clinical study with chlorhexidine, there being no significant differences in mean plaque scores following the use of 0.1% and 0.2% chlorhexidine.15

The initial pulse of chlorhexidine produced reductions of approximately 1 and 2 log₁₀ in the total viable counts of the microcosm plaques and single species biofilms, respectively. However, as a result of the higher total viable counts of bacteria comprising the microcosm plaques, there were substantially more bacteria killed in these biofilms than in the S. sanguis biofilms. Following the initial pulses, there was a recovery in the number of viable bacteria in both types of biofilm despite the fact that they continued to be periodically exposed to chlorhexidine. In the case of the S. sanguis biofilms the viable counts did not reach the levels seen before pulsing; the population level under these conditions represents an equilibrium achieved as a consequence of two competing activities—bacterial growth during the 12 h periods between antimicrobial pulsing and killing of bacteria during the short-term pulsing. In the case of the microcosm plaques, the post-pulsing total viable anaerobic count was similar to that found in biofilms before pulsing. However, the bacterial composition of the biofilms had altered substantially, indicating, not surprisingly, a population shift to one containing higher proportions of species displaying reduced susceptibilities to chlorhexidine.

Microcosm plaques are more complex than single species biofilms, and so are better models of the in-vivo situation. Furthermore, the proportions of species detected in the microcosm community were similar to those observed in supra-gingival plaque.16 Although >80% of the saliva inoculum consisted of streptococci, these organisms comprised only 25% of the microcosm plaques, suggesting that colonization of the enamel and subsequent growth of the biofilm community was similar to that which occurs in the oral cavity.

Streptococci appeared to be the most susceptible to chlorhexidine of those organisms comprising the microcosm plaques grown on untreated discs. The effects of chlorhexidine pulsing were comparable to those obtained with the single species biofilms in that the number of viable streptococci also recovered during subsequent pulsing, but did not reach the levels seen before pulsing. It appears, therefore, that, although they were present in a more complex microbial community, the susceptibility of these organisms was not altered. Following pulsing with chlorhexidine, the total aerobic count of the microcosm
plaques soon recovered to pre-pulsing levels. In contrast, the anaerobic count took much longer to reach the same pre-pulsing levels. This may be attributable, in part, to the aerobic atmosphere within the CDFF, growth of anaerobes may have been limited until an appreciable community had developed to allow the establishment of an anaerobic environment within the biofilm.

Work of a similar nature has been carried out on a nine-membered community grown in a conventional chemostat used to inoculate the CDFF over an 8 h period. The biofilms were challenged with eight 10 min pulses of two concentrations of chlorhexidine (0.0125 and 0.125% w/v). The lower concentration had a limited effect on the composition of the biofilms while a differential and substantial inhibition was obtained with the higher concentration. A c tinomycyes naelundii was lost from the biofilm, and the viable counts of streptococci, Fusobacterium nucleatum and Porphyromonas gingivalis were reduced by over three orders of magnitude by 0.125% chlorhexidine, whereas Veillonella dispar was only transiently affected. However, the relative proportions of the species present in the nine-member community (8.1% streptococci, <0.1% A. naelundii and <0.01% V. dispar) were very different from those found in the microcosm plaques used in the present study. Because of the far greater species diversity within the microcosm plaques, detailed analysis of the relative proportions of individual species was not possible in the present study. This greater complexity may account for the ability of the microcosm plaques to withstand (in terms of the total number of viable organisms) repeated chlorhexidine pulsing although it is likely that the relative proportions of the individual species would have been altered.

When the enamel substrata supporting the microcosm plaques were pre-treated with chlorhexidine, the numbers of viable bacteria after 8 h were 1 log_{10} lower than when untreated discs were used, with Veillonella spp. being undetectable. When these biofilms were subsequently pulsed with chlorhexidine, a much greater kill was seen than with biofilms formed on untreated enamel, with the Actinomyces spp. falling below the detection limit. In previous studies of the colonization of chlorhexidine-treated enamel by S. sanguis, after 8 h the viable counts were 2 log_{10} lower than those achieved with the microcosm plaques in this study. This difference may be a consequence of the availability of a greater variety of adhesion sites (due to the greater range of primary colonizers) for secondary colonization in the multi-species biofilms, thereby enabling the more rapid formation of a biofilm community.

It would be of great interest to compare the results of this study with those of clinical trials involving chlorhexidine. However, such comparisons are difficult as the mouthwashes used often contain other agents, such as fluoride, which may increase or reduce their effectiveness, especially when the test compound is used in lower concentrations. Nevertheless, the results from plaque regrowth trials, where patients' teeth are cleaned, treated and subsequently rinsed twice daily, are comparable to those obtained in the pre-treatment and pulsing experiments in this study. This method of delivery has consistently been shown in vivo to be the most effective way of administering chlorhexidine.

The results of this study have demonstrated the inability of chlorhexidine to kill biofilms in vitro after pulsing twice daily over a period of 4 days. The use of microcosm plaques mimics the in-vivo situation more closely than when single species are used, the interactions between bacteria of the same and different species being of great importance in the formation and maturation of dental plaque. Hence, by using microcosm plaques that have a similar composition to supra-gingival plaque, we should be better able to predict reproducibly the antimicrobial and/or anti-plaque effectiveness of test compounds in vitro. One of the advantages of this model is that it enables pulsing of these agents into the system and pre-treatment of the substratum with the same or different agents. Plaque regrowth clinical trials with chlorhexidine have shown this to be the most effective way of preventing plaque formation and this was also found to be the case in this study.

Acknowledgement

This project was funded by a grant from SmithKline Beecham Consumer Healthcare, Oral Care Research, Weybridge, U.K.

References

Effect of chlorhexidine on oral biofilms


Received 17 December 1997; returned 9 March 1998; revised 6 April 1998; accepted 28 April 1998