An *in vitro* evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates

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Abstract

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Aim The bactericidal effect of four antimicrobial agents was investigated against single-species biofilms derived from a range of root canal isolates.

Methodology Single-species biofilms of *Prevotella intermedia*, *Peptostreptococcus micros*, *Streptococcus intermedius*, *Fusobacterium nucleatum* and *Enterococcus faecalis* were generated on membrane filter discs and subjected to 15 min or 1 h incubation with 5 p.p.m. colloidal silver, 2.25% sodium hypochlorite (NaOCl), 0.2% chlorhexidine, 10% iodine or phosphate buffered saline (PBS) as a control. The antimicrobial activity of the agents was neutralized and the bacterial cells were harvested from the discs by vortexing, serially diluted in reduced transport fluid, plated on fastidious anaerobe agar containing 5% horse blood, incubated anaerobically and colony-forming units calculated.

Results Iodine and NaOCl were more effective than chlorhexidine except against *P. micros* and *P. intermedia* where they were all 100% effective. Iodine and NaOCl elicited a 100% kill after 1 h incubation for all strains used. However, after 15 min, they showed differing bactericidal effects depending on the strain. None of the agents were effective against *F. nucleatum* after 15 min but NaOCl, iodine and chlorhexidine were all effective after 1 h. Colloidal silver was generally ineffective.

Conclusions The effectiveness of a particular agent was dependent on the nature of the organism in the biofilm and on the contact time. NaOCl was generally the most effective agent tested, followed by iodine. However the clinical efficacy of these agents must be considered in light of the complex root canal anatomy and polymicrobial nature of root canal infections.

Keywords: antimicrobial, irrigant, root canal.

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Introduction

It is well recognized that the primary aim of the treatment of periapical disease consists of eradication of polymicrobial infections from the involved root canal system. The validity of this has been demonstrated by studies that have shown that the prognosis of successful outcome of treatment is improved by between 10 and 26% when a negative culture test is obtained prior to obturation (Zeldow & Ingle 1963, Engstrom 1964, Heling & Shapira 1978, Sjogren *et al.* 1997). Numerous clinical studies have sought to evaluate the antimicrobial effectiveness of treatment strategies. Mechanical instrumentation alone appears not to reduce the bacterial load effectively or permanently (Ingle & Zeldow 1958, Byström & Sundqvist 1981). The use of antimicrobial agents as adjuncts for irrigation and medication of root canals has been shown to help reduce the bacterial counts further (Grahnen & Krasse 1963, Goldman & Pearson 1969, Olgart 1969, Byström & Sundqvist 1983, Gomes *et al.* 1996, Molander *et al.* 1999). These studies also demonstrate that despite the use of such antimicrobial agents, bacteria may still persist. The genera most frequently implicated as persistent are streptococci, enterococci, staphyloccocci, fusobacteria, peptostreptococci and lactobacilli. Revealing as these studies are, they are

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relatively time-consuming and labour-intensive to carry out. Hence, the use of a simpler laboratory test to predict the effectiveness of antimicrobial agents is indicated for initial assessment of irrigants and medicaments.

A number of approaches have been used to test the effectiveness of antimicrobial agents in the laboratory. These include: incubation of broth cultures of selected bacteria with the antimicrobial agent (O'Hara *et al.* 1993, D'Arcangelo *et al.* 1999), growth of selected bacteria as 'lawns' on agar surfaces and use of the disc diffusion method (Siqueira *et al.* 1998), the artificial infection of extracted teeth with selected bacteria and in-use irrigation with the test antimicrobial agents (Briseno *et al.* 1992, Sen *et al.* 1999). A combination of such approaches has sometimes been adopted but with discrepant findings (Shih *et al.* 1970, Foley *et al.* 1983). Shih *et al.* (1970) used two renowned resistant strains, *Enterococcus faecalis* (formerly *Streptococcus faecalis*) and *Staphylococcus aureus*, and found the bacteria were easily killed when incubated in broth culture with a concentration of sodium hypochlorite as low as 1 : 1000. However, in the same study, when the infected tooth model was used, even full strength sodium hypochlorite failed to eradicate the bacteria completely in all canals. The difference was attributed to the opportunity for contact between microorganisms and the irrigant. Foley *et al.* (1983) used *Prevotella melanogenica* and *Peptostreptococcus anaerobius* as the test organisms and found them to be easily killed in both models.

More recent revelations about the influence of growth conditions on the bacterial phenotype and its effect on resistance to antibacterial agents sheds further light on these observations (Nichols 1989, Anwar *et al.* 1990). The conditions used for determining minimum inhibitory concentrations (MIC) for bacteria in fluid suspension do not reflect the *in vivo* conditions where bacteria grow as biofilms on tooth surfaces (Wilson 1996). A biofilm is defined as an aggregation of bacteria associated with a surface, embedded in an extra-cellular matrix of polysaccharide. These biofilms differ greatly in phenotype when compared with their planktonic counterparts and they are far less susceptible to antimicrobial killing (Wilson 1996). However, this has not been widely appreciated in endodontics and only Sen *et al.* (1999) have made reference to it. A clinically valid and simple assay for microbial susceptibility testing could therefore be based on a biofilm model. Generation of such biofilms in root canals of extracted teeth provides a more realistic scenario (Shih *et al.* 1970, Foley *et al.* 1983) but the results may be confounded by the variation in root canal anatomy between teeth. In addition, the collection and preparation of the teeth prior to experimentation is laborious and very labour intensive. However, growing biofilms on standardized readily available surfaces eliminates these problems and allows a more accurate assessment of antimicrobial efficacy. The model consists of a membrane filter in contact with an agar surface, the filter is inoculated with the test strain and once the biofilm has formed it can be removed intact and exposed to the test antimicrobial agent. This model has been successfully used in previous studies to assess the effect of a variety of antimicrobial agents on a range of bacterial species, including *Streptococcus sanguis* (Milward & Wilson 1989), *Actinobacillus actinomycetemcomitans* (Thrower *et al.* 1997), *Pseudomonas aeruginosa* (Nichols 1989) and a number of periodontal pathogens (Caufield *et al.* 1987). Once the choice of antimicrobial agent has been rationalized using such a model, it may be tested in an 'infected tooth' model.

The purpose of the present study was to use the simple biofilm model to evaluate the effectiveness of a range of commonly recommended antimicrobial irrigants against five root canal isolates.

Materials and methods

The selected bacterial species were previously isolated from teeth with infected root canals associated with periapical disease. The bacteria were taken from frozen stock (brain heart infusion, Lab M Ltd; Bury, UK plus 10 v/v glycerol, Merck, Poole, UK), recultivated by inoculation on fastidious anaerobe agar (FAA) plus 5% v/v defibrinated horse blood and checked for purity by colony morphology and gram staining. The isolates were originally identified by 16S rRNA gene sequencing and analysis.

Single-species biofilms of *Prevotella intermedia*, *Peptostreptococcus micros*, *Streptococcus intermedius*, *Fusobacterium nucleatum* and *Enterococcus faecalis* were generated on cellulose nitrate membrane filters (0.2 μm pore size, 13 mm diameter – Whatman International Ltd, Maidstone, UK). The membranes were placed on the surface of FAA blood agar and inoculated with a suspension of bacteria in brain heart infusion broth (Lab M). The plates, each containing 10 membrane filters, were incubated for 48 h in an anaerobic cabinet (Don Whitley Ltd, Shipley, UK) at 37 °C in an atmosphere of nitrogen (80%), hydrogen (10%) and carbon dioxide (10%).

Following incubation, the membrane filters were removed aseptically from the agar plate and transferred slowly, so as to avoid any disruption of the biofilm, into 5 mL of the selected antimicrobial test agent or control and incubated for 15 or 60 min at 20 °C.

The antimicrobial agents used were 2.25% v/v sodium hypochlorite (Thin Bleach, J. Sainsbury PLC, London, UK), 0.2% chlorhexidine gluconate (Adam Health Care Ltd, Leeds, UK), 10% povidone iodine (Betadine, Seton Health Care PLC, Oldham, UK) and 5 p.p.m. colloidal silver (Changes International, Fort Walton Beach, Florida, USA). All agents were used at packaged concentrations except the sodium hypochlorite (NaOCl), which was diluted in deionized water to 2.25% available chlorine, validated by iodometric titration. Phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) was used as a control agent.

The membrane filters were then carefully transferred to neutralizing broth (Difco Ltd, East Molesey, UK) and vortexed for 1 min to resuspend the organisms. Ten-fold serial dilutions were generated from the neat bacterial suspension in reduced transport fluid (Syed & Loesche 1972) and plated out on to FAA containing 5% horse blood. The plates were then incubated for 3–4 days and the number of colony forming units (CFU) per disc was calculated. Four replicates were performed for each antimicrobial agent and microorganism combination and the means and standard deviations were calculated.

Results

Comparisons of the susceptibility of the single-species biofilms to the various antimicrobial agents are shown in Figs 1–5.

Sodium hypochlorite, chlorhexidine and iodine were the most effective agents, whilst colloidal silver appeared to have only a marginal antimicrobial effect on all except *P. micros* and *E. faecalis*. However, the effect of the three main antimicrobial agents differed according to the strain tested. Sodium hypochlorite was the most effective agent against *E. faecalis* (Fig. 1) achieving 100% kills at both time intervals. Iodine was only 100% effective after 60 min exposure. Although there were some kills after 15 min with both chlorhexidine and iodine, both agents left in excess of 10^7 CFU. Figure 2 shows the results for *S. intermedius* where iodine was the most effective agent with 100% kills at both time intervals. Sodium hypochlorite was also very effective, achieving 100% kill after 60 min but only reducing the CFU to 10^2 after 15 min. Chlorhexidine was 100% effective after 60 min but at 15 min the counts were only reduced to

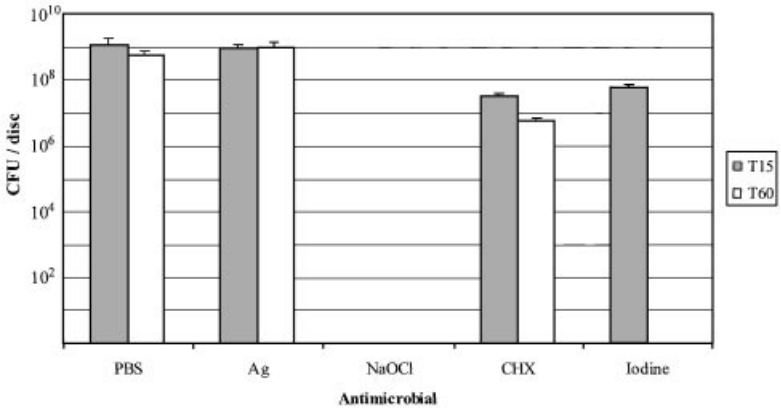

Figure 1 Susceptibility of *E. faecalis* biofilms to a range of antimicrobial agents over 15 and 60 min. Ag, colloidal silver; CHX, chlorhexidine gluconate; PBS, phosphate buffered saline (control). Error bars represent standard deviations. ($n = 4$).

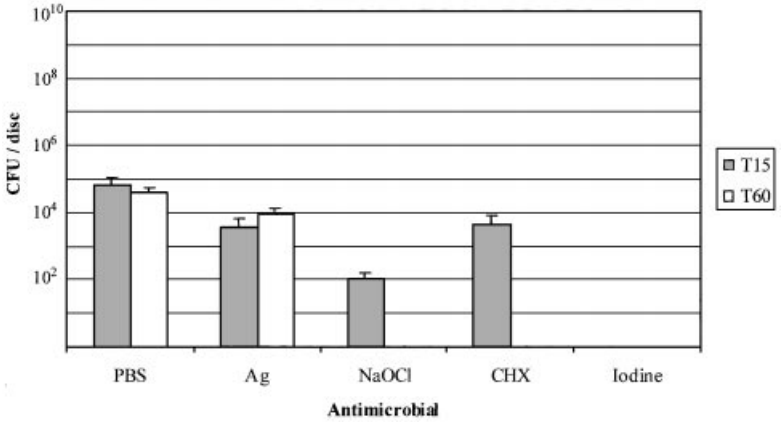

Figure 2 Susceptibility of *S. intermedius* biofilms to a range of antimicrobial agents over 15 and 60 min. Ag, colloidal silver; CHX, chlorhexidine gluconate; PBS, phosphate buffered saline (control). Error bars represent standard deviations. ($n = 4$).

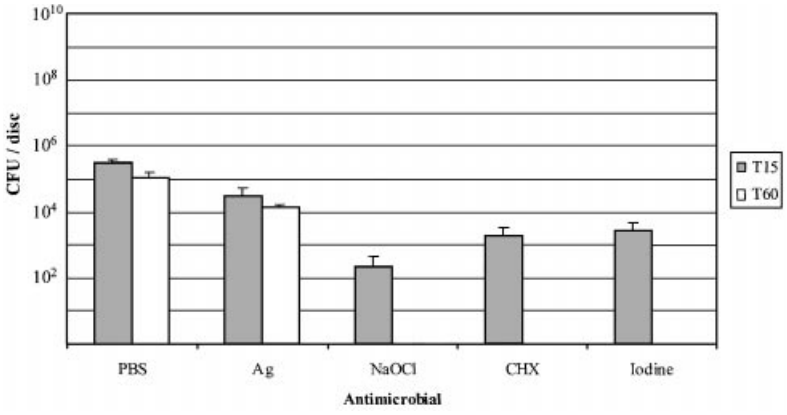

Figure 3 Susceptibility of *F. nucleatum* biofilms to a range of antimicrobial agents over 15 and 60 min. Ag, colloidal silver; CHX, chlorhexidine gluconate; PBS, phosphate buffered saline (control). Error bars represent standard deviations. ($n = 4$).

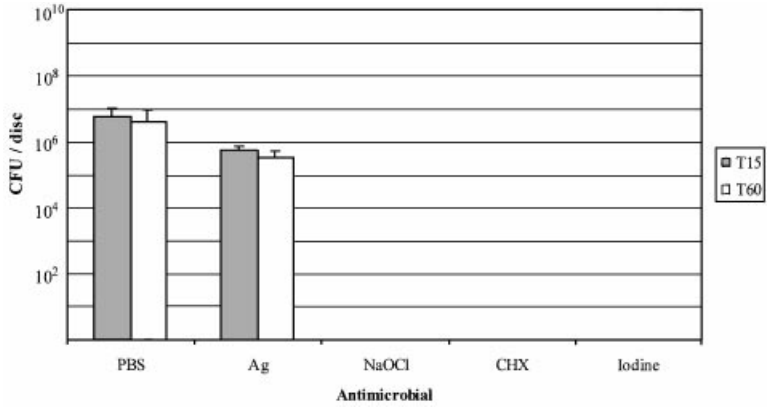

Figure 4 Susceptibility of *P. intermedia* biofilms to a range of antimicrobial agents over 15 and 60 min. Ag, colloidal silver; CHX, chlorhexidine gluconate; PBS, phosphate buffered saline (control). Error bars represent standard deviations. ($n = 4$).

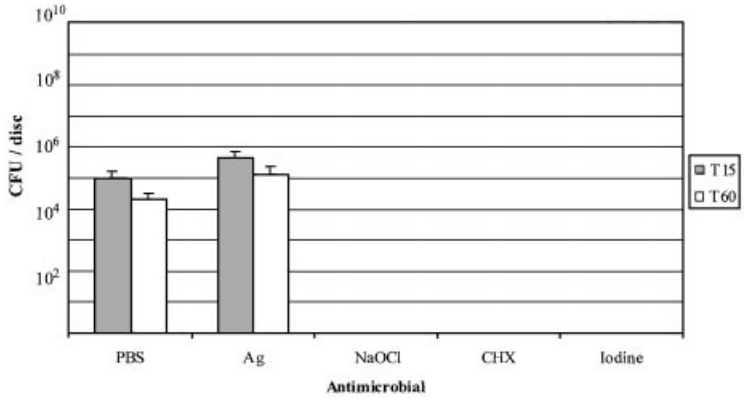

Figure 5 Susceptibility of *P. micros* biofilms to a range of antimicrobial agents over 15 and 60 min. Ag, colloidal silver; CHX, chlorhexidine gluconate; PBS, phosphate buffered saline (control). Error bars represent standard deviations. ($n = 4$).

between 10^3 and 10^4. *F. nucleatum* (Fig. 3) was 100% susceptible to iodine, NaOCl and chlorhexidine at 60 min but these compounds only managed to reduce CFU to between 10^2 and 10^4 at the 15-minute time-point. For both *P. intermedia* (Fig. 4) and *P. micros* (Fig. 5) iodine, NaOCl and chlorhexidine were 100% effective in killing these organisms at both time-points.

Discussion

In this study, a biofilm model that has been previously tested on strains isolated from other oral infections, was used to evaluate the antimicrobial efficacy of several endodontic irrigants against selected root canal isolates. The use of broth or planktonic cultures for such tests

usually gives highly effective kills that do not correlate with clinical findings (Shih *et al.* 1970, O'Hara *et al.* 1993, Thrower *et al.* 1997, Desai *et al.* 1998, D'Arcangelo *et al.* 1999). The behaviour of bacteria in biofilms is notably different from their planktonic counterparts and should be accounted for in any laboratory tests (Wilson 1996). Bacteria in biofilms also respond differently depending on their growth phase, the dose and the frequency of exposure to the antimicrobial agent (Desai *et al.* 1998, Pratten & Wilson 1999) which may be relevant to clinical endodontics.

The model used in the present study is a modification of that adopted by Siqueira *et al.* (1998) in that it allows the film to be grown on a membrane and then removed intact to be placed in a defined amount of the antimicrobial agent. Probably the most relevant of the *in vitro* tests is the one in which extracted teeth are used to generate biofilms on the canal wall which can then be subjected to the antimicrobial agent (Shih *et al.* 1970, Briseno *et al.* 1992, Sen *et al.* 1999). This method has the potential difficulty in achieving contact between the entire biofilm and the antimicrobial agent because of the complexity and variability of the root canal system. This is an uncontrolled variable and therefore may not provide an accurate representation of the antimicrobial efficacy of the agent itself, but rather a combination of this and access of the antimicrobial agent to the biofilm. In this respect, the model recommended in this study has its merits, to which may also be added the lack of a need for extracted teeth and their time-consuming preparation. However, this model does not account for the anatomical variations in tooth models. The protocol used in this study is simple and straightforward to perform. It also has the advantage of allowing a large number of variables to be tested quickly and easily. This protocol may therefore be useful as a rapid primary screen to test the antimicrobial effect against biofilms. Further studies using 'infected tooth' models could then be targeted at the most promising combinations of antimicrobial agents identified, thus rationalizing the combinations tested on teeth. This approach should allow more focused studies on the 'infected tooth' model.

The antimicrobial agents selected included sodium hypochlorite (NaOCl), povidone iodine and chlorhexidine. Colloidal silver was included as it has recently been recommended as an antimicrobial root canal irrigant by alternative therapists (Bauman 1999, personal communication).

The strains chosen for the study were *Prevotella intermedia*, *Peptostreptococcus micros*, *Streptococcus intermedius*, *Fusobacterium nucleatum* and *Enterococcus faecalis*, which have been frequently isolated after completion of treatment measures *in vivo* (Grahnen & Krasse 1963, Goldman & Pearson 1969, Olgart 1969, Cvek *et al.* 1976, Byström & Sundqvist 1983, Byström & Sundqvist 1985, Gomes *et al.* 1996, Sirén *et al.* 1997, Molander *et al.* 1998, 1999).

This study showed that different bacteria are susceptible, in varying degrees, to the range of antimicrobial agents tested and that the duration of exposure could be crucial. Overall, colloidal silver was ineffective, whilst sodium hypochlorite seemed the most effective followed by iodine and chlorhexidine. The latter three were completely (100% kills) effective against *P. micros* and *P. intermedia* at both time intervals and the iodine was effective against *S. intermedius.* However, with the exception of the latter, none were 100% effective against *S. intermedius* and *F. nucleatum* at 15 min but were effective after 60 min. *E. faecalis* proved more resistant than the other organisms and it was only killed effectively by sodium hypochlorite. Iodine was only 100% effective after 60 min and although chlorhexidine had some antimicrobial effect, it did not achieve 100% kill at either time interval. It is difficult to systematically compare these results to other studies because the available data are not comprehensive nor homogenous. Nevertheless, useful inferences can be drawn even by comparison with studies using diverse methodologies.

Enterococcus faecalis has gained notoriety as a persistent organism that can survive as a monoculture in root canals (Grahnen & Krasse 1963, Engstrom 1964, Goldman & Pearson 1969, Myers *et al.* 1969, Olgart 1969, Gomes *et al.* 1996, Sirén *et al.* 1997, Molander *et al.* 1998). A number of laboratory studies have compared the effectiveness of sodium hypochlorite and chlorhexidine in eliminating *E. faecalis*. For example NaOCl has been shown to be more effective than chlorhexidine in a 'bovine root dentine infection' model (Vahdaty *et al.* 1993), but no differences were shown by Heling & Chandler (1998) or Siqueira *et al.* (1998). In an earlier study Siqueira *et al.* (1997), using an 'infected extracted tooth' model, showed that 4% (v/v) NaOCl was completely effective in only 60% of cases. This relatively low figure may be explained by the lack of adequate contact of the antimicrobial agent. The importance of achieving 100% kill has been highlighted by Shih *et al.* (1970) who used an 'infected tooth' model. After irrigation with 5.25% NaOCl (v/v), no positive cultures were obtained following immediate sampling, but after 2 days they showed a culture reversal rate of 80%. Vahdaty *et al.* (1993) used bovine root dentine infected with *E. faecalis* over 6 days and evaluated the effectiveness of two-minute applications

of 0.2% chlorhexidine and 2% NaOCl, in eliminating the organism at various depths into the dentine. They showed a reduction in the number of colony forming units after NaOCl irrigation at the dentine surface compared with chlorhexidine. The differences were less marked with increasing depth. Heling & Chandler (1998) used a similar model with 1% NaOCl and 0.2% chlorhexidine applied for 10 min and showed a large reduction in optical density (indicating cell density) with sodium hypochlorite although the difference between the two agents was not statistically significant. Siqueira *et al.* (1998) used an agar diffusion test with 0.2% chlorhexidine and 2.25% NaOCl and found no difference between their effect on *E. faecalis*. Their earlier study (Siqueira *et al.* 1997), using an infected extracted tooth model, evaluated 4% NaOCl applied for 5 min and found that it was completly effective in 12 out of 20 cases. The discrepancy may be explained by the lack of adequate contact or duration of application of the antimicrobial agent. Shih *et al.* (1970) also used an extracted tooth model infected with *E. faecalis* and found that no cultures were obtained when sampled immediately after irrigation with 5.25% NaOCl. However, there were eight culture reversals in the sample of 20 teeth when they were sampled after 2 days and 16 reversals when sampled after 7 days. These results illustrate the ability of the species to survive as a monoculture and rapidly reinfect the root canal system. A total initial kill is therefore desirable or, alternatively, the use of an effective medicament.

Streptococcus intermedius, together with other *Streptococcus* species, has been routinely implicated as a survivor of root canal treatment regimes (Grahnen & Krasse 1963, Goldman & Pearson 1969, Myers *et al.* 1969, Olgart 1969, Byström & Sundqvist 1983, 1985, Gomes *et al.* 1996). Despite the prevalence of this genus in persistent infections, it is not commonly utilized in antimicrobial efficacy tests. Briseno *et al.* (1992) used *Streptococcus mutans* in an infected extracted tooth model and compared the effectiveness of different concentrations of sodium hypochlorite with and without ultrasonic activation. Although there were large reductions in numbers, the bacteria were not completely eliminated and the total irrigation time was not given. Siqueira *et al.* (1998) compared the effectiveness of 2.25% NaOCl and 0.2% chlorhexidine using an agar diffusion test against three species of streptococci. Their overall conclusions were that NaOCl was marginally more effective but it was dependent on the species being tested. The effect of iodine irrigation cannot be substantiated because no studies have been reported. Nevertheless, Molander *et al.* (1999) used 5% iodine potassium iodide as a dressing and found that the most predominant group of persistent organisms were streptococci, although *S. intermedius* was not specifically identified amongst them. The inference is that individual species may respond differently to various antimicrobial agents.

Fusobacterium nucleatum and other species of the genus have also been found to persist after root canal cleaning (Grahnen & Krasse 1963, Olgart 1969, Cvek *et al.* 1976, Byström & Sundqvist 1983, 1985, Sirén *et al.* 1997, Gomes *et al.* 1996, Molander *et al.* 1998). It is difficult to compare the data from the current study with other studies since this species has not been used in other antimicrobial efficacy tests. The finding in this study that this species is quite resistant to all of the irrigants at 15 min suggests that the duration of contact is crucial for its eradication. This species may also be important due to its ability to bind to the acquired pellicle or coating present on dentine walls (Kolenbrander *et al.* 1999). Furthermore, it has strong associations with other bacteria, both nutritionally and structurally as a major bridging component in biofilms (Sundqvist 1992, Kolenbrander *et al.* 1999). These features may contribute to its ability to survive in root canals.

Peptostreptococcus micros, or associated species, have been recovered after treatment procedures in a number of studies (Olgart 1969, Byström & Sundqvist 1983, 1985, Molander *et al.* 1998, 1999). It should however, be stressed that the numbers of recovered colonies are usually small. Gomes *et al.* (1996) found that the species was significantly more susceptible to eradication from the root canal than other organisms. The findings of the present study tend to confirm this impression. Foley *et al.* (1983) used an infected extracted tooth model to test the ability of 5.25% sodium hypochlorite to eradicate *Peptostreptococcus anaerobius*. This procedure achieved total kills after 15 s of contact. Although this work was carried out on a different species in the genus *Peptostreptococcus*, it may be considered as broadly corroborative. One of the reasons may be that the organisms are easily killed but persist because of their strong associations with other organisms, notably *Fusobacterium nucleatum* (Sundqvist 1992).

Prevotella intermedia is a strict anaerobe and a very fastidious organism. It should therefore be easily eradicated, a prediction supported by this study. This organism and associated species are however, occasionally recovered after treatment procedures (Byström & Sundqvist 1983, Gomes *et al.* 1996, Molander *et al.* 1998, 1999). Siqueira *et al.* (1998) compared the ability of 2.25% NaOCl and 0.2% chlorhexidine to kill the species in an agar diffusion test. They found both to be effective but

the NaOCl was more effective with a greater mean diameter of inhibition (24 mm vs. 4 mm). Once again it would appear that this species is not a primary resistant organism but one that may survive because of association with other persistent organisms.

A number of further studies have evaluated or compared the efficacy of sodium hypochlorite and chlorhexidine irrigants on natural root canal infections in extracted teeth (Delany *et al.* 1982, Jeansonne & White 1994) or *in vivo* (Ringel *et al.* 1982, Kuruvilla & Kamath 1998, Leonardo *et al.* 1999). Ringel *et al.* (1982) compared the efficacy of 0.2% chlorhexidine and 2.25% NaOCl in 30 teeth, each with pulp necrosis and periapical lesions. Initial cultures were obtained and the number of appointments required to achieve a negative culture was recorded. The NaOCl was found to be more effective and the difference was attributed to its tissue-dissolving capacity. Kuruvilla & Kamath (1998) evaluated the same concentrations on a total of 40 single-rooted teeth with periapical radiolucencies. They found that chlorhexidine reduced the number of microorganisms by 70% compared with 60% by the NaOCl. The alternate use of both solutions gave an 85% reduction. Jeansonne & White (1994) tested 5.25% NaOCl and 2% chlorhexidine on 20 infected extracted teeth each and found the number of residual CFU after treatment to be marginally lower in the chlorhexidine group but the difference was not significant. Delany *et al.* (1982) and Leonardo *et al.* (1999) reported that 0.2% and 2% chlorhexidine were effective in reducing bacterial counts when used as irrigants. In addition, they reported that these concentrations were useful for interappointment antibacterial activity.

It appears from these studies on the polymicrobial root canal flora, that both NaOCl and chlorhexidine have adequate overall antibacterial activity. The probable effect of disrupting the interactions between bacteria that sustain some species clearly has a beneficial effect beyond that evident from the antimicrobial tests on individual species. Further *in vitro* tests should also evaluate multispecies biofilms. Surprisingly, the effect of iodine as an antibacterial agent appears not to be as widely tested. Given the different modes of activity of the various agents and the different degrees of susceptibility of bacteria to them, there is much to be said for alternating different irrigants in root canals with persistent infections (Kuruvilla & Kamath 1998).

Conclusion

The biofilm model used in this study gave a quick and simple means of determining the *in vitro* antimicrobial efficacy of a variety of root canal irrigants. This method may be more clinically representative than traditional testing methods which do not account for the presence of bacteria in biofilms.

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