ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2011, p. 3338–3344 Vol. 55, No. 7
0066-4804/11/$12.00 doi:10.1128/AAC.00206-11

Antimicrobial Penetration and Efficacy in an *In Vitro* Oral Biofilm Model▽†

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Received 15 February 2011/Returned for modification 25 March 2011/Accepted 20 April 2011

The penetration and overall efficacy of six mouthrinse actives was evaluated by using an *in vitro* flow cell oral biofilm model. The technique involved preloading biofilm cells with a green fluorescent dye that leaked out as the cells were permeabilized by a treatment. The loss of green color, and of biomass, was observed by time-lapse microscopy during 60 min of treatment under continuous flow conditions. The six actives analyzed were ethanol, sodium lauryl sulfate, triclosan, chlorhexidine digluconate (CHX), cetylpyridinium chloride, and nisin. Each of these agents effected loss of green fluorescence throughout biofilm cell clusters, with faster action at the edge of a cell cluster and slower action in the cluster center. The time to reach half of the initial fluorescent intensity at the center of a cell cluster, which can be viewed as a combined penetration and biological action time, ranged from 0.6 to 19 min for the various agents. These times are much longer than the predicted penetration time based on diffusion alone, suggesting that anti-biofilm action was controlled more by the biological action time than by the penetration time of the active. None of the agents tested caused any removal of the biofilm. The extent of fluorescence loss after 1 h of exposure to an active ranged from 87 to 99.5%, with CHX being the most effective. The extent of fluorescence loss *in vitro*, but not penetration and action time, correlated well with the relative efficacy data from published clinical trials.

The control of oral biofilms (i.e., dental plaque) depends in part on the use of chemical actives that kill or remove plaque. Actives that kill microorganisms presumably reduce bacterial virulence and retard the rate of plaque accumulation. Actives that aid in removing biofilm, whether or not killing occurs, are also expected to be helpful in reducing disease. The penetration of such actives into the microbial biofilm is a fundamental requirement for their efficacy.

The first objective of the work reported here was to investigate three aspects of active function. We asked the following questions. (i) How fast do actives penetrate biofilm? (ii) How effectively do actives kill biofilm bacteria? (iii) Do actives remove biofilm? These three topics were examined by using an *in vitro* flow cell biofilm model (3, 34, 35) to compare six different actives with an untreated control. Actives were used alone rather than in formulations as is typical in commercial mouthrinses. The six actives were ethanol, sodium lauryl sulfate (SLS), triclosan (TRN), cetylpyridinium chloride (CPC), chlorhexidine digluconate (CHX), and nisin (Table 1).

The second objective of the present study was to test whether any of our *in vitro* results correlated in any way with clinical experience. This was done by analyzing published clinical studies reporting on four of the actives we examined *in vitro*: SLS, TRN, CPC, and CHX. There was not sufficient data for the other two actives to permit their inclusion in the analysis. We devised a simple scoring system to quantify the relative efficacy of actives measured in clinical trials and then sought to determine whether this score correlated with any of the properties of the actives measured *in vitro* (penetration time, killing, and removal). As purveyors of *in vitro* experimental data, this is a risky question to pose because if the answer is consistently no, it may mean that the *in vitro* model is not relevant to clinical outcomes. Although this question is of fundamental relevance to every laboratory model, we are not aware of any quantitative attempts to validate *in vitro* oral biofilm models for their ability to predict outcomes of clinical trials of mouthrinse efficacy.

Many *in vitro* models for investigating antimicrobial efficacy in the context of oral biofilms have been described (2, 4, 6, 7, 11, 15, 16, 19, 20, 28–30, 36, 40, 41). These studies often use plate counts to enumerate viable cells and calculate antimicrobial efficacy (2, 4, 11, 15, 19, 20, 28, 29, 36, 40), although some analyses have been performed based on fluorescence or luminescence assays (7, 16, 19). Very few studies have addressed the issue of active penetration into dental plaque or oral biofilm (14, 18, 34, 38). There are also few studies that reported measurements of oral biofilm removal by chemical treatments (7, 41).

MATERIALS AND METHODS

Bacteria and media. *Streptococcus oralis* ATCC 10557, *Streptococcus gordonii* ATCC 10558, and *Actinomyces naeslundii* ATCC 19039 were used in the present study. These organisms, which initiate colonization and coaggregate with each other on the tooth surface (13), were grown from frozen stocks in tryptic soy broth (TSB) containing 0.5% sucrose overnight at 37°C under anaerobic conditions with the GasPak EZ system (Becton Dickinson, New Jersey). Starter cultures were transferred into 10 ml of fresh, anaerobic sucrose-amended TSB and grown for 2 h (*S. gordonii*) or 4 h (other strains) at 37°C under aerobic conditions. The absorbance at 600 nm of all bacterial suspensions was adjusted to 0.05 prior to preparing inocula.

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▽ Published ahead of print on 2 May 2011.

TABLE 1. Antimicrobial agents used in biofilm penetration and activity experiments[a]

Agent	MW (g mol^{-1})	D_{aq} (μm^2 s^{-1})	Applied concn (%)
Ethanol	46.1	1280	40.0
SLS	288.4	546	0.1
Triclosan	289.5	608	0.03
CPC	340.0	421	0.05
CHX	897.6	273	0.12
Nisin	3354	183	0.005

[a] D_{aq}, the diffusion coefficient in water at 25°C, was determined from the Wilke-Chang correlation, except for ethanol and nisin. The D_{aq} value for ethanol was taken from the experimental consensus reported in Perry and Chilton (22), and that for nisin was estimated from the correlation of Polson (24).

Biofilm reactor. The reactor system consisted of a medium carboy, a peristaltic pump, a glass capillary tube (Friedrich and Dimmock, Millville, NJ), and a waste carboy. These components were connected by silicone tubing. The system has been described in detail in prior publications (25, 34). The nominal inside dimension of the glass tube was 0.9 mm square, and the wall thickness was 0.17 ± 0.01 mm. The tube was approximately 10 cm long.

Equal volumes of absorbance-adjusted suspensions of each of the three microorganisms prepared as described above were mixed, and 0.5 ml of this mixture was inoculated into a sterile glass capillary tube reactor. The inoculated solution was allowed to stand without flow for 45 min at 37°C under aerobic conditions. After this inoculation time, the flow of medium was initiated at a flow rate of 1 ml min^{-1}. The medium was 1/10 strength TSB containing 0.05% sucrose. Biofilms were allowed to develop for 20 h under continuous-flow conditions with incubation at 37°C.

Antimicrobial penetration and action. A mature 20-h biofilm grown in the capillary reactor was removed from the incubator to the benchtop. Medium was flushed from the reactor by introducing a continuous flow of buffer containing 0.25 g of dipotassium phosphate and 0.5 g of sodium chloride per liter (pH 7.3). Calcein-AM (CAM; Molecular Probes/Invitrogen, Eugene, OR) was prepared to a concentration of 10 μg ml^{-1} by diluting a 500-μg ml^{-1} stock solution (in dimethyl sulfoxide) into buffer. The CAM solution was pumped into the capillary at a flow rate of 1 ml min^{-1} until it completely replaced the fluid in the capillary. The flow was stopped, and the biofilm was stained statically for 1 h. Excess stain was washed out by restoring the flow of buffer for 1 h. The system was transferred to the microscope stage. Biofilm was exposed to antimicrobial solution under continuous flow at flow rate of 1 ml min^{-1}. The treatments applied included 40% ethanol, 0.1% SLS, 0.03% TRN, 0.12% CHX, 0.05% CPC, and 0.005% nisin. Actives were used alone rather than in formulations, as is typical in commercial mouthrinses.

For microscope observation of biofilm, the capillary was placed in a holder (Biosurface Technologies, Bozeman, MT) that was mounted on the stage of a Leica TCS-SP2 AOBS confocal scanning laser microscope. A ×63 0.9 NA water-immersion objective lens was used. The plane just under the glass ceiling of the capillary was brought into focus. That plane was also the bottom surface of a biofilm cluster. For imaging antimicrobial action, the 488-nm laser was used for excitation and the fluorescent signal was detected in a green channel. Images were collected every 30 s during the 1-h exposure to flowing antimicrobial solution. Transmitted images of biofilms before and after exposure were collected in transmission mode with excitation from the 488-nm laser.

Two prior publications report results using this technique and provide some additional experimental details (3, 34).

Images were analyzed in MetaMorph software (Universal Imaging Corp., Downington, PA). The overall effect of a treatment on biofilm clusters was assessed by tracking the fluorescence intensity throughout the cluster. Two squares (10 × 10 pixels) were drawn in MetaMorph: one in the center and one along the border of the cluster. The integrated pixel intensity inside these areas was calculated by the software at each time step. These values were normalized by dividing them by the initial value of the integrated cluster intensity. At least three biofilm clusters from separate experiments were analyzed for each treatment condition.

Biofilm removal is difficult to distinguish from contraction, rotation, or elongation of the biofilm. For example, an analysis of biomass area before and after treatment would not differentiate between loss of biofilm and shrinkage of a biofilm cell cluster. For this reason, we assessed biofilm removal by visual examination of video of the treatment experiments. This approach is superior for confirming retention of biomass in experiments with little or no removal but would be inadequate for quantifying biofilm removal if it were to occur.

Estimation of diffusive penetration time. The theoretical time for an active to attain 50% of the bulk fluid concentration at the center of a biofilm cell cluster after introduction of the agent into the bulk fluid was determined from the following equation: $t_{50} = 0.139R^2/D_e$. This calculation assumes that diffusion is the predominant transport process and neglects sorption and reaction (32). D_e denotes the effective diffusion coefficient of the active in the biofilm, and R is the radius of a hemispherical biofilm cell cluster. R was taken as 82 μm. D_e was taken as 25% of the aqueous diffusion coefficient, D_{aq} (32). The D_{aq} values are provided in Table 1.

Analysis of clinical efficacy data. We devised a simple, quantitative scoring system to compile results from several clinical studies. The score is based on the relative performance of mouthrinse actives in published clinical trials. Within a particular study, a treatment that did not perform statistically significantly better (as determined by the study authors) than the placebo control received a score of 0. A treatment that outperformed the control with statistical significance, but was not better than other treatments received a 1. A treatment that performed statistically significantly better than another treatment, which itself performed better than the control, received a score of 2. Higher scores than 2 are conceivable in such a scheme but were not observed. Ten investigations that included a control and two or more treatments with either CHX, CPC, SLS, or TRN were analyzed.

RESULTS

Antimicrobial penetration and action. The penetration and activity of various antimicrobial agents in model oral biofilms was investigated. The properties and application concentrations of the six agents tested are summarized in Table 1. The mean radius and standard deviation of the biofilm cell clusters was 82 ± 19 μm. The biofilms were prestained with CAM, which loads the cells with an unbound, green fluorescent dye. When subjected to a subsequent treatment that causes permeabilization of the cell membrane, the green color is lost from the cells as the dye leaks out. Where the green color diminishes, one can infer that the antimicrobial has both penetrated to this location in the biofilm and has acted biologically on the cells in that region. Although membrane integrity has been widely used as a surrogate for bacterial viability, the loss of green color does not prove that the cell was killed. Loss of green color indicates that cell membranes have been compromised. Representative results for each of the six agents and an untreated control are presented in Fig. 1 (videos can be found in the supplemental material). None of the agents tested caused any disruption or removal of the biofilm. A few of the treatments, namely, CHX, CPC, and ethanol, caused some minor (a few percent) contraction of the biofilm. These effects are evident in comparing the before and after treatment transmission images (grayscale in Fig. 1), which reveal stable biomass structures.

Figure 1 is suitable for getting a sense of the time scale of antimicrobial action but is not useful for judging the overall extent of fluorescence loss. The overall fluorescence loss was determined after 1 h of treatment, whereas Fig. 1 only shows results for a fraction of this interval.

Image analysis was performed to quantify the kinetics of fluorescence loss. We determined the T_{50}, the time elapsed from the initiation of treatment until the fluorescence intensity fell to half of its initial value at a particular spot. This measurement was performed at the center of cell clusters and also at the edges of the same clusters (Table 2). This time presumably represents a summation of the penetration time to a particular location and the biological action time at that loca-

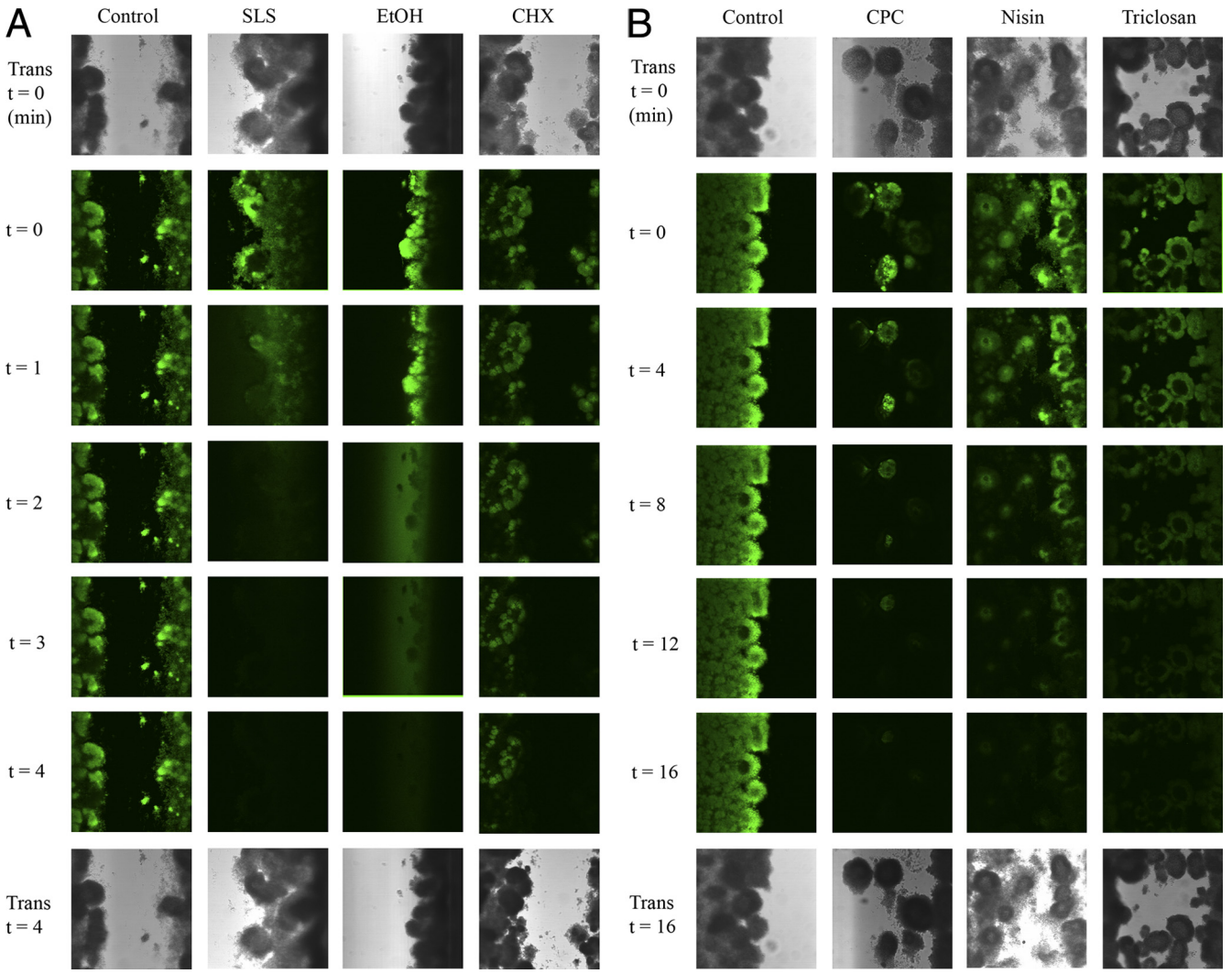

FIG. 1. (A) Representative antimicrobial action of control, SLS, ethanol (EtOH), and CHX on model oral biofilm stained with CAM (green). The loss of green color indicates permeabilization of cells. The top and bottom rows are transmission images showing biofilm structure before and after treatment, respectively. Corresponding videos can be found in the supplemental material. (B) Representative antimicrobial action of CPC, nisin, and triclosan on model oral biofilm stained with CAM (green). The loss of green color indicates permeabilization of cells. The top and bottom rows are transmission images showing biofilm structure before and after treatment, respectively. Corresponding videos can be found in the supplemental material.

TABLE 2. Penetration and action times of antimicrobial agents in biofilms[a]

Agent (concn [%])	t_{50} (min)	Mean time (min) ± SD		$T_{50,C}/T_{50,E}$	No. of replicates
		$T_{50,C}$	$T_{50,E}$		
Control		NM[b]	NM	NM	3
SLS (0.1)	0.11	1.18 ± 0.30	0.69 ± 0.52	1.71	2
Ethanol (40)	0.05	1.28 ± 0.11	0.73 ± 0.14	1.75	2
CHX (0.12)	0.23	2.32 ± 1.30	0.98 ± 0.36	2.36	3
Nisin (0.005)	0.34	10.2 ± 1.9	8.18 ± 0.93	1.25	3
CPC (0.05)	0.15	15.7 ± 8.9	7.58 ± 5.0	2.07	3
TRN (0.03)	0.10	19.1 ± 13.0	13.08 ± 11.4	1.46	5

[a] The parameter t_{50} is the calculated penetration time of the agent in the absence of reaction or sorption (see the text). T_{50} denotes the time elapsed after introduction of the antimicrobial agent into the flow cell until the CAM fluorescence intensity dropped to half of its pretreatment value. The two values correspond to measurements made either at the center of cell clusters ($T_{50,C}$) or at the periphery or edge of the cell clusters ($T_{50,E}$). The CAM fluorescence remained above 50% of its pretreatment value for the entire 60-min observation period.

[b] NM, not measurable.

tion. As anticipated, the T_{50} was longer at the center of cell clusters than at the edge. The overall loss of CAM fluorescence in cell clusters after 1 h of exposure to the treatment agent was also recorded (Table 3).

None of the measures of antimicrobial agent penetration time responses (Table 2) correlated with molecular weight or diffusion coefficient in water ($P > 0.35$) as determined by separate analyses of variance. A multiple-linear-regression model indicated that there was no significant correlation between the log reduction of overall fluorescence (Table 3) and any of the predictors, including molecular weight, diffusion coefficient in water, and the log-transform of the concentration of the antimicrobial ($P = 0.57$). Neither did the antimicrobial agent penetration time correlate with the log reduction in overall fluorescence by a linear regression ($R^2 = 0.19$, $P = 0.37$). This indicates that the initial speed and final extent of antimicrobial action are independent measures.

TABLE 3. Overall efficacy of antimicrobial agents in biofilms expressed as the mean log reduction in the total (average of cluster center and edge) CAM fluorescence after 1 h[a]

Agent (concn [%])	Mean log reduction ± SEM	% Loss	No. of replicates
CHX (0.12)	2.33 ± 1.28	99.5	3
Ethanol (40)	1.18 ± 0.14	93.4	3
Nisin (0.005)	1.11 ± 0.14	92.2	3
CPC (0.05)	1.00 ± 0.21	90.0	3
SLS (0.1)	0.89 ± 0.49	87.1	3
TRN (0.03)	0.88 ± 0.30	86.8	5
Control	0.11 ± 0.07	22.4	3

[a] This loss is alternatively expressed as a percentage for the mean log reduction.

A primary source of uncertainty with this technique is thought to be the experiment-to-experiment variation in biofilm structure (transmission images in Fig. 1). Sometimes the biofilm localizes in corners of the capillary tubes, and sometimes clusters are distributed across the width of the flow cell. Some of the biofilm microcolonies have hollow interiors.

Comparison of *in vitro* results to clinical efficacy data. We sought to make comparisons between our *in vitro* results and published clinical data. Examining the multistudy dose response of the relative plaque index, a statistically significant dose response is evident for CHX ($P = 0.0016$, indicating that the slope of the least-squares regression line for the CHX data plotted in Fig. 2 was statistically significantly different from zero). Both CPC and TRN also exhibit plots with negative slopes, but the slopes are not statistically significantly different from zero.

Next, we tested whether the relative clinical efficacy scores summarized in Table 4 for CHX, CPC, SLS, and TRN correlated them with either of the two key *in vitro* measurements made in this investigation. The clinical scores correlated poorly with penetration time, $T_{50,C}$ ($R^2 = 0.35$, $P = 0.37$), indicating that the slope of the least-squares regressed line for this plot (data not shown) was not statistically significantly different from zero). Clinical scores did correlate with log reduction in fluorescence measured *in vitro* ($R^2 = 0.94$, $P = 0.002$), indicating that the slope of the least-squares regressed line through the data plotted in Fig. 3 is statistically significantly different from zero.

DISCUSSION

***In vitro* time for activity against biofilm is much longer than diffusive penetration time.** Penetration and action times for the six actives for which $T_{50,C}$ was measurable ranged from 1.2 to 19 min (Table 2). These times are much longer than the predicted penetration time based on diffusion alone (t_{50} in Table 2), by factors ranging from 10 to 191. The penetration and action times measured here are also much longer than the reported diffusive penetration times in biofilms (24, 35). For example, using the identical experimental system Takenaka et al. (35) found that fluorescein (molecular weight [MW] of 376) had a t_{90} penetration time of just 10 s, which is similar to the diffusive penetration times calculated in the present study and reported in Table 2. One obvious explanation for this discrepancy is that $T_{50,C}$ is controlled more by the biological action time than by the penetration time of the active. This interpretation is supported by the lack of correlation of $T_{50,C}$ with physical properties of the solute such as MW or aqueous diffusion coefficient. The relatively long action times measured at the cluster edge ($T_{50,E}$, Table 2), where penetration time should be negligible, also support this conjecture. According to this interpretation, one can hypothesize that the cells in the interior of a biofilm cluster are less susceptible to antimicrobial treatments than are the cells near the cluster edge. This difference is reflected in the longer action times measured at the center of cell clusters ($T_{50,C}$) compared to the edge ($T_{50,E}$). The ratio $T_{50,C}/T_{50,E}$, which ranged from 1.3 to 2.4 (mean 1.8; Table 2), suggests that the difference in intrinsic susceptibility at the two locations in the biofilm is not more than about a factor of 2.

Another phenomenon that could contribute to the discrepancy between calculated diffusive penetration times and the measured combined penetration and action time is sorption of the active to the biofilm matrix. Sorption retards penetration (31). The penetration time tabulated in Table 2 neglects any sorption or reaction of the active in the biofilm. Agents such as

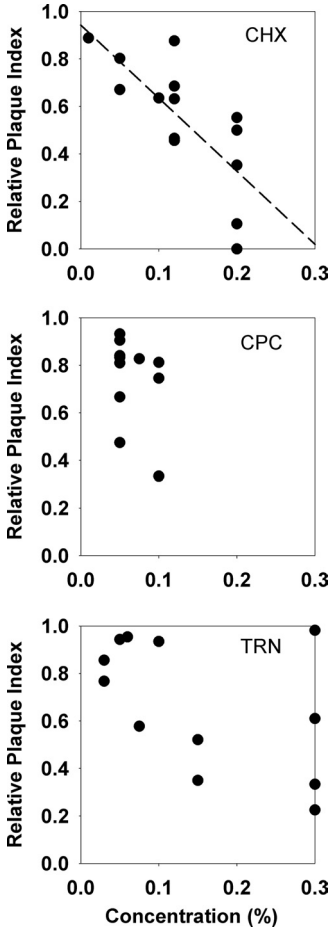

FIG. 2. Dose response for three mouthrinse actives. The relative plaque index is the plaque index measured with the active at the specified concentration divided by the plaque index reported for the appropriate placebo control. The data are from Table 4 and four additional sources (1, 9, 19, 30). The least-squares regressed line is shown as a dashed line.

TABLE 4. Summary of published clinical mouthrinse efficacy studies used for comparison with *in vitro* results

Clinical score[a]				Concn (%)	No. of subjects	Duration (days)	Endpoint(s)[b]	RPI[c]	Reference
CHX	CPC	SLS	TRN						
2				0.2	15	4	PI, PA	0.50	21
		1		0.25				0.920.96	
			0	0.06					
		1		1.5	11	4	PI	0.49	37
			0	0.3				0.33	
			0	0.15				0.35	
2				0.05	20	4	PI, PA	0.80	9
	2			0.1				0.75	
	2			0.05				0.81	
			1	0.05				0.94	
2				0.01	26	4	PI, PA	0.89	10
2				0.05				0.67	
2				0.1				0.64	
2				0.2				0.55	
			1	0.2				0.93	
2				0.2	12	4	PI	0.11	12
			1	0.3				0.23	
			1	0.15				0.52	
			1	0.075				0.58	
2				0.12	20	4	PI, PA	0.63	27
	1			0.05				0.83	
			1	0.03				0.86	
2				0.2	76	35	PI, BOP, MGI	0.00	42
	1			0.05				0.47	
			1	0.3				0.61	
2				0.12	298	157	PI, GI	0.69	33
	1			0.1				0.81	
	1			0.075				0.83	
2				0.12	16	4	PI	0.88	39
			0	0.3				0.98	
2				0.2	15	4	PI	0.35	23
2				0.12				0.46	
	0			0.05				0.93	
			1	0.03				0.77	

[a] See Materials and Methods for an explanation of the clinical score.
[b] PI, plaque index; PA, plaque area; BOP, bleeding on probing; MGI, modified gingival index; GI, gingival index; VAB, viable anaerobic bacteria.
[c] RPI, relative plaque index. The RPI is the PI value measured for a treatment divided by the PI value of the corresponding control.

CHX and CPC in particular are thought to sorb to matrix constituents (5, 34).

We sought to address the question of how fast do actives penetrate biofilm? In the end, we believe that we have not answered this question very well. Instead, we have demonstrated that common mouthrinse actives have relatively slow biological action at both the edges and the centers of biofilm cell clusters. We suggest that penetration is not the critical phenomenon controlling efficacy, but rather it is the low intrinsic susceptibility of biofilm bacteria (2–4, 20). This interpretation is supported by the fact the differences in the speed of action (Table 2) do not correlate with clinical efficacy.

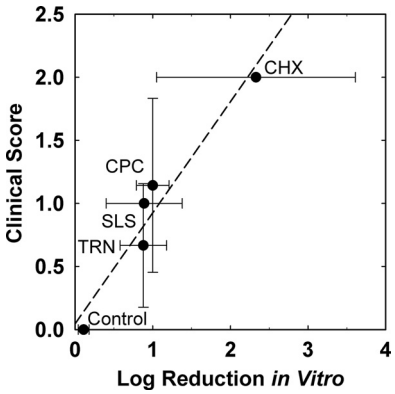

FIG. 3. Correlation of a simple clinical efficacy score (see the text) with log reduction in fluorescence measured in the *in vitro* capillary oral biofilm model. The errors indicated are the standard error of the mean. The least-squares regressed line is shown as a dashed line.

All agents exhibit some antimicrobial efficacy. Using loss of membrane integrity as a surrogate endpoint for viability, we addressed the question of how effectively do actives kill biofilm bacteria? All of the actives exhibited some efficacy. Ethanol, nisin, CPC, SLS, and TRN all achieved log reductions (in CAM fluorescence) of ~1.0. CHX achieved a log reduction of ~2.3. These modest log reductions, after a full hour of antimicrobial exposure, suggest that the biofilms grown in the capillary flow cell model capture the antimicrobial tolerance that is a hallmark of the biofilm mode of growth.

All agents fail to remove biofilm. We also posed the question of whether actives remove biofilm. Our clear answer for the six agents tested here is that they do not. This result is the same as that from another study in which no removal was discerned upon treatment with ethanol (11.6%), chlorhexidine (0.12%),

or the commercial mouthrinse Biotene (34). The study of Takenaka et al. (34) did report biofilm removal by a toothpaste suspension, which was attributed to the high shear associated with flowing this viscous material into the capillary tube. In a recent investigation that used methods similar to those of the current study, no biofilm removal was measured when *Staphylococcus epidermidis* biofilms were treated with nisin, glutaraldehyde, or a quaternary ammonium compound (3). The only biocide that effected some removal was chlorine, whose action was attributed to reactive attack on the biofilm matrix.

Comparison of *in vitro* results to published clinical efficacy reveals a correlation. A correlation between the log reduction in fluorescence measured *in vitro* and the relative efficacy measured in clinical trials was demonstrated (Fig. 3). This partially validates this *in vitro* model as a reasonable tool for assessing the bactericidal efficacy of potential mouthrinse antimicrobial agents. Penetration and action time measured *in vitro* failed to correlate with the relative efficacy of actives measured in clinical trials. This result further reinforces the interpretation suggested above that penetration is not the critical phenomenon controlling active efficacy.

Although this analysis of clinical efficacy data is admittedly primitive, it offers a check on the face validity of our *in vitro* results. Short of conducting a new and large clinical comparative trial, there is no way to know whether the results of an *in vitro* model translate to the clinic. Intuitively one might anticipate that speed of action would be clinically important given the short dose duration of an antimicrobial mouthrinse. This expectation is contradicted by our finding that the speed of antimicrobial penetration and action measured *in vitro* failed to correlate with relative clinical efficacy. On a cautionary note, our finding that reduction in CAM fluorescence does correlate with relative clinical efficacy should not be viewed as a guarantee that *in vivo* performance can be predicted from this single *in vitro* model.

ACKNOWLEDGMENTS

This study was funded in part by Colgate-Palmolive. Microscopy was facilitated by equipment obtained through an award from the M. J. Murdock Charitable Trust.

REFERENCES

1. **Amini, P., M. W. B. Araujo, M.-M. Wu, and N. C. Sharma.** 2009. Comparative antiplaque and antigingivitis efficacy of three antiseptic mouthrinses: a two week randomized clinical trial. Braz. Oral Res. **23:**319–325.
2. **Bercy, P., and J. Lasserre.** 2007. Susceptibility to various oral antiseptics of *Porphyromonas gingivalis* W83 within a biofilm. Adv. Ther. **24:**1181–1191.
3. **Davison, W. M., B. Pitts, and P. S. Stewart.** 2010. Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. **54:**2920–2927.
4. **Fine, D. H., D. Furgang, and M. L. Barnett.** 2001. Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of *Actinobacillus actinomycetemcomitans*. J. Clin. Periodontol. **28:**697–700.
5. **Ganeshnarayan, K., S. M. Shah, M. R. Libera, A. Santostefano, and J. B. Kaplan.** 2009. Poly-*N*-acetylglucosamine matrix polysaccharide impedes fluid convection and transport of the cationic surfactant cetylpyridinium chloride through bacterial biofilms. Appl. Environ. Microbiol. **75:**1308–1314.
6. **Guggenheim, B., E. Giertsen, P. Schüpbach, and S. Shapiro.** 2001. Validation of an in vitro biofilm model of supragingival plaque. J. Dent. Res. **80:**363–370.
7. **Herles, S., S. Olsen, J. Afflitto, and A. Gaffar.** 1994. Chemostat flow cell system: an in vitro model for the evaluation of antiplaque agents. J. Dent. Res. **73:**1748–1755.
8. **Hernandez-Cott, P. L., A. E. Boneta, B. Stewart, W. DeVizio, and H. M. Proskin.** 2009. Clinical investigation of the efficacy of a commercial mouthrinse containing 0.05% cetylpyridinium chloride in reducing dental plaque. J. Clin. Dent. **20:**39–44.
9. **Jenkins, S., M. Addy, and R. G. Newcombe.** 1994. A comparison of cetylpyridinium chloride, triclosan and chlorhexidine mouthrinse formulations for effects on plaque regrowth. J. Clin. Periodontol. **21:**441–444.
10. **Jenkins, S., M. Addy, and R. G. Newcombe.** 1994. Dose response of chlorhexidine against plaque and comparison with triclosan. J. Clin. Periodontol. **21:**250–255.
11. **Kinniment, S. L., J. W. T. Wimpenny, D. Adams, and P. D. Marsh.** 1996. The effect of chlorhexidine on defined, mixed culture oral biofilms grown in a novel model system. J. Appl. Bacteriol. **81:**120–125.
12. **Kjærheim, V., S. M. Waaler, and G. Rölla.** 1994. Significance of choice of solvents for the clinical effect of triclosan-containing mouthrinses. Scand. J. Dent. Res. **102:**202–205.
13. **Kolenbrander, P. E., et al.** 2002. Communication among oral bacteria. Microbiol. Mol. Biol. Rev. **66:**486–505.
14. **Landa, A. S., H. C. van der Mei, and H. J. Busscher.** 1997. Detachment of linking film bacteria from enamel surfaces by oral rinses and penetration of sodium lauryl sulphate through an artificial oral biofilm. Adv. Dent. Res. **11:**528–538.
15. **Larsen, T., and N. E. Fiehn.** 1995. Development of a flow method for susceptibility testing of oral biofilms in vitro. APMIS **103:**339–344.
16. **Leung, A. P., et al.** 2009. Antimicrobial peptides for plaque control. Adv. Dent. Res. **21:**57–62.
17. **Leyes, B. J. L., et al.** 2002. Efficacy of chlorhexidine mouthrinses with or without alcohol: a clinical study. J. Periodontol. **73:**317–321.
18. **Melsen, B., O. Kaae, and G. Rölla.** 1983. Penetration of fluoride and chlorhexidine in human dental plaque in vitro. Caries Res. **17:**113–117.
19. **Merritt, J., J. Kreth, F. Qi, R. Sullivan, and W. Shi.** 2005. Non-disruptive, real-time analyses of the metabolic status and viability of *Streptococcus mutans* cells in response to antimicrobial treatments. J. Microbiol. Methods **61:**161–170.
20. **Millward, T. A., and M. Wilson.** 1989. The effect of chlorhexidine on *Streptococcus sanguis* biofilms. Microbios **58:**155–164.
21. **Moran, J., M. Addy, and S. Roberts.** 1992. A comparison of natural product, triclosan and chlorhexidine mouthrinses on 4-day plaque regrowth. J. Clin. Periodontol. **19:**578–582.
22. **Perry, R. H., and C. H. Chilton.** 1973. Chemical engineers' handbook, 5th ed. McGraw-Hill Book Co., New York, NY.
23. **Pizzo, G., et al.** 2006. The effects of antimicrobial sprays and mouthrinses on supragingival plaque regrowth: a comparative study. J. Periodontol. **77:**248–256.
24. **Polson, A.** 1950. Some aspects of diffusion in solution and a definition of a colloidal particle. J. Phys. Colloid Chem. **54:**649–652.
25. **Rani, S. A., B. Pitts, and P. S. Stewart.** 2005. Rapid diffusion of fluorescent tracers into *Staphylococcus epidermidis* biofilms visualized by time lapse microscopy. J. Antimicrob. Chemother. **49:**728–732.
26. **Rawlinson, A., et al.** 2008. Efficacy of two alcohol-free cetylpyridinium chloride mouthwashes: a randomized double-blind crossover study. J. Clin. Periodontol. **35:**230–235.
27. **Renton-Harper, P., M. Addy, J. Moran, F. M. Doherty, and R. G. Newcombe.** 1996. A comparison of chlorhexidine, cetylpyridinium chloride, triclosan, and C31G mouthrinse products for plaque inhibition. J. Periodontol. **67:**486–489.
28. **Sena, N. T., et al.** 2006. In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms. Int. Endod. J. **39:**878–885.
29. **Shen, Y., W. Qian, C. Chung, I. Olsen, and M. Haapasalo.** 2009. Evaluation of the effect of two chlorhexidine preparations on biofilm bacteria in vitro: a three-dimensional quantitative analysis. J. Endod. **35:**981–985.
30. **Sissons, C. H., L. Wong, and T. W. Cutress.** 1996. Inhibition by ethanol of the growth of biofilm and dispersed microcosm dental plaques. Arch. Oral Biol. **41:**27–34.
31. **Stewart, P. S.** 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother. **40:**2517–2522.
32. **Stewart, P. S.** 2003. Diffusion in biofilms. J. Bacteriol. **185:**1485–1491.
33. **Stookey, G. K., et al.** 2005 A 6-month clinical study assessing the safety and efficacy of two cetylpyridinium chloride mouthrinses. Am. J. Dent. **18:**24A–28A.
34. **Takenaka, S., H. M. Trivedi, A. Corbin, B. Pitts, and P. S. Stewart.** 2008. Direct visualization of spatial and temporal patterns of antimicrobial action within model oral biofilms. Appl. Environ. Microbiol. **74:**1869–1875.
35. **Takenaka, S., B. Pitts, H. M. Trivedi, and P. S. Stewart.** 2009. Diffusion of macromolecules in model oral biofilms. Appl. Environ. Microbiol. **75:**1750–1753.
36. **von Ohle, C., et al.** 2010. Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine. Appl. Environ. Microbiol. **76:**2326–2334.
37. **Waaler, S. M., G. Rolla, K. K. Skjorland, and B. Ogaard.** 1993. Effects of oral rinsing with triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. Scand. J. Dent. Res. **101:**192–195.
38. **Watson, P. S., et al.** 2005. Penetration of fluoride into natural plaque biofilms. J. Dent. Res. **84:**451–455.
39. **Welk, A., et al.** 2005. The effect of a polyhexamethylene biguanide mouth-

rinse compared with a triclosan rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth. J. Clin. Periodontol. **32:**499–505.

40. **Wirthin, M. R., P. K. Chen, and C. I. Hoover.** 2005. A laboratory model biofilm fermenter: design and initial trial on a single species biofilm. J. Periodontol. **76:**1443–1449.
41. **Yang, Y., P. K. Sreenivasan, R. Subramanyam, and D. Cummins.** 2006. Muliparameter assessments to determine the effects of sugars and antimicrobials on a polymicrobial oral biofilm. Appl. Environ. Microbiol. **72:**6734–6742.
42. **Yates, R., B. H. Shearer, E. Huntington, and M. Addy.** 2002. A method to compare four mouthrinses. J. Clin. Periodontol. **29:**519–523.