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In vitro antibacterial action of Tetraclean, MTAD and five experimental irrigation solutions

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Abstract

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Aim To investigate the antibacterial effect of Tetraclean, MTAD and five experimental irrigants using both direct exposure test with planktonic cultures and mixed-species *in vitro* biofilm model.

Methodology Tetraclean, MTAD and five experimental solutions that were modifications of existing formulae including MTAD + 0.01% cetrimide (CTR), MTAD + 0.1% CTR, MTAC-1 (Tween 80 replaced by 0.01% CTR in MTAD), MTAC-2 (Tween 80 replaced by 0.1% CTR) and MTAD-D (MTAD without the Tween 80 and no CTR added) were used as disinfectants in the experiments. In the direct exposure test, a suspension of *Enterococcus faecalis* was mixed with each of the solutions. After 0.5, 1, 3 and 10 min, an inactivator was added and the number of surviving bacteria was calculated. A mixed-species biofilm from subgingival plaque bacteria was grown in brain heart infusion broth in anaerobic conditions on synthetic hydroxyapatite discs. Two-week-old biofilms were exposed to the solutions for 0.5, 1 and 3 min. The samples were observed by confocal laser scanning microscopy after bacterial viability staining. The scans were quantitatively analysed, and the volume of killed cells of all cells was calculated for each medicament.

Results Tetraclean and MTAC-2 (0.1% CTR) killed planktonic *E. faecalis* in <30 s. Complete killing of bacteria required 1 min by MTAC-1, 3 min by MTAD + 0.1% CTR and 10 min by MTAD, MTAD-D and MTAD + 0.01% CTR. In the biofilm test, there were significant differences in microbial killing between the different solutions and times of exposure ($P < 0.005$). MTAC-2 showed the best performance, killing 71% of the biofilm bacteria in 3 min, followed by MTAC-1 and Tetraclean. MTAD and the three MTAD modifications demonstrated the lowest antibacterial activity.

Conclusion Tetraclean was more effective than MTAD against *E. faecalis* in planktonic culture and in mixed-species *in vitro* biofilm. CTR improved the antimicrobial properties of the solutions, whereas Tween 80 seemed to have a neutral or negative impact on their antimicrobial effectiveness.

Keywords: antimicrobial, biofilm, endodontics, *Enterococcus faecalis*, irrigants.

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Introduction

Bacteria organized as biofilms are known to be present in inaccessible areas of the root canal system, and the microbes organized in communities generally have a low metabolic rate and tend to be resistant to antimicrobial agents (Stewart *et al.* 2001, Tachikawa *et al.* 2005). Substances that can rapidly kill free-floating microorganisms have not shown the same effectiveness on biofilms. Moreover, the biofilm structure seems to provide protection to resident bacteria from the host's immune defenses (Ward *et al.* 1992). Together, these properties of biofilm help to explain the resistance to treatment of some endodontic infections.

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As microbial infection of the root canal system has been documented as the aetiology of apical periodontitis (Kakehashi *et al.* 1965, Möller *et al.* 1981), the elimination of microbes has been the main goal of root canal treatment. While chemomechanical preparation is the most important step in endodontic disinfection, it cannot predictably achieve total elimination of bacteria (Byström & Sundqvist 1985, Ørstavik *et al.* 1991, Dalton *et al.* 1998). Because of the continuing challenges in endodontic disinfection, new methods and materials are constantly being developed to more predictably reach the treatment goals. A new irrigant, Tetraclean (Ogna Laboratori Farmaceutici, Milan, Italy) has recently been developed for smear layer removal and canal disinfection. It is a mixture of doxycycline hyclate at a lower concentration than MTAD, an acid, and detergents (Torabinejad *et al.* 2003a, Giardino *et al.* 2006). According to the manufacturers, these two irrigation solutions should be used after irrigation with 1.3% sodium hypochlorite (Shabahang & Torabinejad 2003, Shabahang *et al.* 2003, Giardino *et al.* 2006, 2007). Some *in vitro* and *ex vivo* studies have indicated that MTAD has equal or superior antibacterial activity compared to 5.25% hypochlorite against *Enterococcus faecalis* (Shabahang & Torabinejad 2003, Shabahang *et al.* 2003, Torabinejad *et al.* 2003b). However, conflicting results have also been reported (Kho & Baumgartner 2006, Johal *et al.* 2007). A study using *E. faecalis* biofilm revealed that BioPure MTAD was less effective than hypochlorite against biofilm bacteria (Giardino *et al.* 2007). The same study indicated that a new doxycycline-containing root canal irrigant was also more effective against *E. faecalis* biofilm than MTAD. Few studies have evaluated the efficacy of endodontic irrigants against microorganisms grown as a mixed-species biofilm. The purpose of this study was to examine the antimicrobial efficacy of Tetraclean, MTAD and five experimental solutions on *E. faecalis,* and to compare the antibacterial effect of the solutions on mixed-species *in vitro* biofilm using three-dimensional quantitative analysis.

Material and methods

Antimicrobial solutions

The disinfecting solutions tested were BioPureMTAD (Dentsply Tulsa Dental, Tulsa, OK, USA); Tetraclean (Ogna Laboratori Farmaceutici); and five experimental solutions, where 0.1% or 0.01% cetrimide (CTR; SigmaUltra, St. Louis, MO, USA) was either added to MTAD, or replaced Tween 80 (SigmaUltra), or MTAD without the Tween 80 and no additional CTR in the original MTAD composition which was similar to the commercially available product (Torabinejad & Johnson 2003). Saline was used as a control solution. Table 1 shows the composition of the solutions used in the experiments.

Direct exposure test

Bacteria and experimental conditions

The *E. faecalis* strain (VP3-181) was isolated from a root-filled tooth associated with chronic apical periodontitis (Peciuliene *et al.* 2000). Cultures of the *E. faecalis* strain were grown overnight at 37 °C on tryptic-soy-agar plates (TSA; Difco Laboratories, Detroit, MI, USA) in air. Identification of the strain has been described previously (Peciuliene *et al.* 2000).

Bacterial suspensions were prepared in filtered and heat-sterilized water and adjusted spectrophotometrically to give a cell density of 3×10^7 colony-forming units (CFU) per mL for the stock solution. Fifty-microlitre samples of *E. faecalis* suspension were mixed with 1.45 mL of the disinfecting solutions for experimental times of 0.5, 1, 3 and 10 min. After indicated times of exposure, 100-μL samples were obtained and mixed with 900 μL TSB broth and serially 10-fold diluted. The first two tubes in the dilution series contained inactivator (3% Tween 80, 0.3% α-lecithin) to reduce the carry-over effect of the medicaments. Droplets of 20 μL from the various dilutions were cultured on TSA plates for 24 h at 37 °C. Bacterial colonies were counted using a stereomicroscope. Purity of the growth was checked. The results were expressed as the percentage of surviving bacteria from the initial inoculum. All experiments were performed in triplicate.

Table 1 Formulae of solutions included in the study

Solution	Doxycycline (%)	Citric acid (%)	Tween 80 (%)	CTR (%)
MTAD	3	4.25	0.5	–
MTAD-D	3	4.25	–	–
MTAD + 0.01% CTR	3	4.25	0.5	0.01
MTAD + 0.1% CTR	3	4.25	0.5	0.1
MTAC-1 (w. 0.01% CTR)	3	4.25	–	0.01
MTAC-2 (w. 0.1% CTR)	3	4.25	–	0.1
Tetraclean	1	10	–	0.2

CTR, cetrimide.

Evaluation of carry-over effect and detection limit

When no inactivator was used, the carry-over of the active medicament(s) prevented the growth of *E. faecalis* on the TSA plates from the first two dilutions, while growth was detected from the third, fourth and fifth dilutions after short contact with the medicaments. With inactivators present, only the first dilution gave no growth (false negative) on the plate culture. For further verification of the results, additional control experiments were performed when no growth was found on the plates: 10-μL samples were taken from the first two dilution tubes and transferred into 10 mL of TSB broth each, giving a final dilution ratio of 1 : 10 000 from the original experiment. At this dilution it has been previously shown that MTAD carry-over no longer affects the growth of *E. faecalis*. In separate, parallel experiments 5 μL of microorganism suspension (3×10^7 CFU mL^{-1} for the bacteria) was also added to the 10 mL TSB tubes. Bacterial growth was detected in each tube confirming that negative growth when observed in the medicament experiments was not a consequence of carry-over effect by MTAD, MTAC or Tetraclean. Purity of growth was also checked.

The detection limit of the test was calculated and tested by serial ten-fold dilutions, showing that negative growth indicated killing of 99.99% or more of the microbial cells. All the tests were performed in triplicate.

Biofilm development

Subgingival plaque on the first or second upper molars of each of three healthy volunteers was collected. Ethical approval for the study was obtained from the University Research Ethics Board. Three volunteers participated after giving informed consent to the protocol. Sterile ceramic calcium hydroxyapatite discs, 10-mm diameter and 2-mm thickness, (Clarkson Chromatography Products, Williamsport, PA, USA) were placed in the wells of a 12-well tissue culture plate containing 1.80 mL of brain heart infusion broth (BHI; Difco). Each well was inoculated with 0.2 mL of dispersed dental biofilm, in a cell concentration of 3.2×10^7 CFU mL^{-1}. The discs were incubated in anaerobic conditions (AnaeroGen, OXOID, Basingstoke, UK) at 37 °C for 14 days with a change of fresh medium at 72-h intervals.

Scanning electron microscopy of the biofilm

Specimens evaluated under scanning electron microscopy (SEM) were washed in phosphate-buffered saline two times for 5 min, fixed in 2.5% glutaraldehyde solution for 10 min, and fixed in 1% buffered osmium tetroxide for 1 h under hooded conditions. The samples were then buffer-washed twice for 5 min each time and dehydrated in a graded ethanol series: 50%, 70%, 80% and 100%, for 5 min each. The samples were dried in a critical point dryer (Tousimis Research Corporation, Rockville, MD, USA) and mounted, sputter-coated with gold/palladium, and viewed with a Hitachi S-3000N scanning electron microscope with light element (EDX; Hitachi, Boston, MA, USA) at 1000× to 5000× magnifications at 6–12 kV.

Bacterial viability assay in biofilm

Bacterial viability after exposure of the biofilm to the disinfecting agents was analysed using viability staining and confocal laser scanning microscopy (CLSM) (Nikon Eclipse 600; Nikon Inc., Melville, NY, USA). After 14 days of incubation, biofilm samples were rinsed in 0.85% NaCl to remove the culture broth and exposed to the disinfecting solutions (Table 1) for 0.5, 1 or 3 min. As a negative control, the samples were exposed to sterile saline for the same periods.

LIVE/DEAD BacLight bacterial viability kit L-7012 for microscopy and quantitative assays (Molecular Probes, Eugene, OR, USA) containing separate vials of the two component dyes in solution, was used for staining the biofilm bacteria, following the manufacturer's instructions. BacLight consists of Syto 9 and propidium iodide (excitation/emission spectra 480/530 and 520/580 nm, respectively) and stains viable bacteria green while those with damaged membranes stain red. The two colours are viewed and recorded as separate images. Individual biofilm images covered an area of 1.64 mm^2 per field of view. The scanned images for each disinfecting agent were obtained by using ten randomly chosen areas of each sample. The samples were observed with a 40× magnification lens, and the scanning was performed at 0.5-μm thick sequential sections through the entire thickness of the biofilm. The images were achieved using EZ-C1 software for Nikon (ver. 3.2; Nikon Inc.), and transferred for quantitative analysis for the The MeVisLab package (available from http://www.mevislab.de/). The volume ratio of red fluorescence (dead cells) to green-and-red fluorescence (all cells) was calculated for each medicament. The method of evaluation of the antimicrobial effect against biofilm bacteria was described in detail in a previous study (Shen *et al.* 2009).

Data analysis

Each solution in each experimental time was considered an experimental group. The results were analyzed using Univariate and *post hoc* analysis, when necessary, at a significance level of $P < 0.05$.

Results

Direct exposure test

MTAD, Tetraclean, and the experimental solutions killed all planktonic *E. faecalis* cells within the 10 min time frame in the direct exposure test. However, there were great differences in the time required for complete killing of the bacteria (Table 2). Tetraclean and MTAC-2 (0.1% CTR) killed 100% of *E. faecalis* cells at 30 s. The second most effective solutions in the planktonic killing test were MTAC-1 and MTAD + 0.1% CTR, while MTAD + 0.01% CTR, MTAD and MTAD-D required the longest time for complete elimination of the test bacteria (Table 2).

Killing of biofilm bacteria

Scanning electron microscopy images demonstrated the presence of biofilm on the HA discs, mostly consisting of cocci and filamentous bacteria (Fig. 1). The biofilms were ca. 20 μm thick, and after exposure to solutions no biofilm detachment was observed. Figure 2 shows CLSM images of biofilm prior to and after exposure to the antimicrobial solutions.

In the control group, 90% and 89% of the bacterial cells were viable after 30 s and 3 min of exposure, respectively (Table 3). The proportion of killed bacteria was dependent on the type of disinfecting agent and the exposure time ($F = 14.67$, $P = 0.00$). There were significant differences in the killing ratio between different times ($F = 103.96$, $P = 0.00$) and solutions ($F = 260.18$, $P = 0.00$).

MTAC-2 was the most effective solution against the biofilm as ca. 71% of the biofilm volume was stained red after 3 min. MTAC-2 was followed by MTAC-1 and Tetraclean. MTAD in its original formula and MTAD-D were the least effective against biofilm bacteria: more than 50% of the bacteria were viable after 3 min in contact with MTAD and MTAD-D (Tables 3 and 4).

Discussion

A number of different *in vitro*, *ex vivo* and *in vivo* approaches have been used in an effort to determine the efficacy of the various disinfecting agents against the involved organisms (Shih *et al.* 1970, Foley *et al.* 1983, Siqueira *et al.* 1998, Sen *et al.* 1999, Siqueira 2001, Spratt *et al.* 2001, Vianna *et al.* 2004, Dametto *et al.* 2005). Bacteria in the various locations of the root canal system are typically organized as polymicrobial biofilms; however, studies of endodontic disinfection have only recently employed biofilm models to examine the eradication of the microbes. For example, a search in Medline database reveals over 200 studies with 'chlorhexidine' and 'endodontics', while only three original studies are found when 'biofilm' is added to the search. In two of these studies biofilm composed of a single species, *E. faecalis*, was used while in one a polymicrobial biofilm was used (Clegg *et al.* 2006, Dunavant *et al.* 2006, Williamson *et al.* 2009). Regarding MTAD, of the thirty published studies two involved bacteria in biofilm (Clegg *et al.* 2006, Dunavant *et al.* 2006). Two different models were used in the present study: in one planktonic *E. faecalis* cells were challenged by a number of irrigants/disinfecting agents for 0.5–10 min, in another polymicrobial biofilms grown on hydroxyapatite substrate were used and exposed to the same chemicals for 0.5–3 min. The reason for

Table 2 Survival (%) of *Enterococcus faecalis* VP3-181 cells after exposure to the disinfecting solutions

	% Survival of *E. faecalis* cells			
Solution	0.5 min	1 min	3 min	10 min
Negative Control	100	100	100	100
MTAD	26.49 ± 15.28	9.96 ± 6.30	3.47 ± 1.83	0
MTAD-D	55.17 ± 12.43	46.15 ± 16.70	4.29 ± 2.47	0
MTAD + 0.01% CTR	15.43 ± 4.73	9.65 ± 6.84	2.84 ± 2.03	0
MTAD + 0.1% CTR	2.80 ± 1.24	0.59 ± 0.52	0	0
MTAC-1 (w. 0.01% CTR)	2.19 ± 3.61	0	0	0
MTAC-2 (w. 0.1% CTR)	0	0	0	0
Tetraclean	0	0	0	0

CTR, cetrimide.

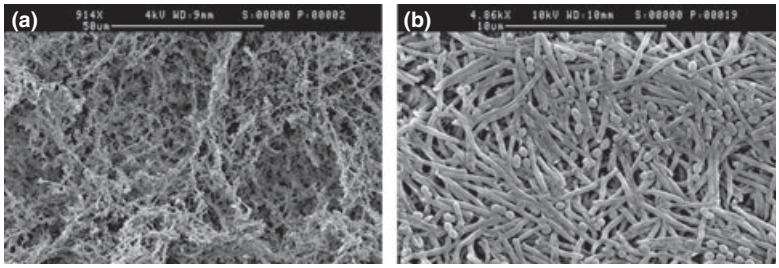

Figure 1 Scanning electron micrograph of 2-week-old biofilms in low (a) and high-magnification view (b).

using these two methods was to make it possible to compare the results with earlier literature (planktonic killing), and to examine if the performance of the chemicals against planktonic *E. faecalis* had any predictive value for their effectiveness against mixed bacterial growth in a polymicrobial biofilm grown under anaerobic conditions. Furthermore, it can be assumed that the polymicrobial biofilm model gives a more realistic picture of the true performance of the disinfecting agents *in vivo* (Wilson 1996, Abdullah *et al.* 2005).

One of the challenges in experiments with planktonic killing is the possibility of carry-over of the active medicament to the final culture medium, plate or broth culture (Krause *et al.* 2007). The risk for carry-over is particularly real when antibiotics are used in high concentrations in locally used disinfecting agents (Ciarlone *et al.* 1988). It has been previously shown that even 1 : 1000 dilution of MTAD may contain enough doxycycline to inhibit the growth of the surviving bacteria in subsequent culture (Portenier *et al.* 2006). Several precautions were taken in the present study to prevent the possibility of false negative results (=no growth detected although the bacteria are not killed). First, the original inoculum of bacteria contained a high number of cells to allow serial dilutions down to $1 : 10^4$–$1 : 10^6$. Also using an inactivator in the dilution series improved the recovery of the organisms. Tween 80-α-lecithin has been recommended for inactivation of chlorhexidine (Zamany & Spångberg 2002). Although there is no earlier literature supporting the use of Tween 80-α-lecithin in inactivation of tetracyclines, it seemed to have a moderate effect. An additional 1 : 1000 dilution into TSB broth from the first dilution tube in cases where no growth was present on the plates gave a final dilution

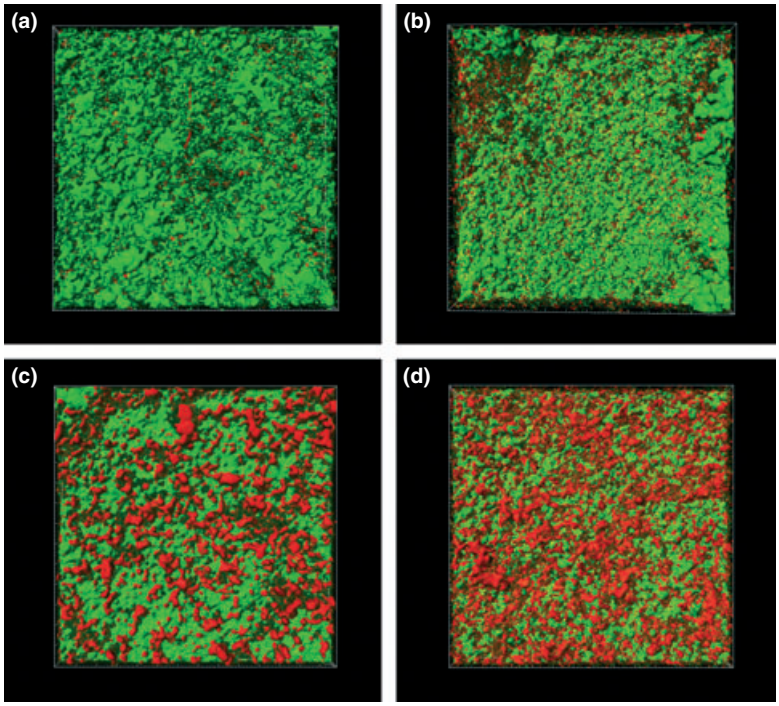

Figure 2 Three-dimensional constructions of confocal laser scanning microscopy (CLSM) scans of 2-week-old multispecies biofilms after treatment with disinfecting agents for 1 min. (a) Biofilm treated with physiological saline (control); (b) MTAD treatment; (c) MTAC-2 [(0.1% cetrimide (CTR)] treatment; (d) Tetraclean treatment. Green, viable cells; red, dead cells.

Table 3 The proportion of the dead cell volume (red) of the entire biofilm volume after exposure to different solutions (mean ± SD)

	0.5 min*	1 min*	3 min*
MTAD**	0.15 ± 0.01	0.31 ± 0.03	0.43 ± 0.04
MTAD-D**	0.16 ± 0.01	0.33 ± 0.03	0.44 ± 0.03
MTAD + 0.01% CTR**	0.23 ± 0.02	0.32 ± 0.03	0.45 ± 0.03
MTAD + 0.1% CTR**	0.24 ± 0.03	0.34 ± 0.06	0.48 ± 0.07
MTAC-1 (w. 0.01% CTR)**	0.26 ± 0.03	0.34 ± 0.02	0.62 ± 0.11
MTAC-2 (w. 0.1% CTR)**	0.33 ± 0.05	0.38 ± 0.03	0.71 ± 0.03
Tetraclean**	0.36 ± 0.10	0.41 ± 0.07	0.56 ± 0.04
Saline**	0.10 ± 0.02	0.10 ± 0.01	0.11 ± 0.01

CTR, cetrimide.
*Significant difference among groups at different times (Univariate analysis, $P < 0.005$).
**Significant difference among groups (Univariate analysis, $P < 0.005$).

of 1 : 10 000 of the medicament. Together with additional control experiments, this verified the negative growth (when detected) and improved the detection level to 99.99% killing.

Commercial BioPure MTAD contains 3% doxycycline hyclate, 4.25% citric acid and 0.5% Tween 80 (Torabinejad & Johnson 2003). The antibacterial effect in planktonic killing and biofilm experiments of MTAD and a simulated solution prepared for this study which also contained 3% doxycycline hyclate, 4.25% citric acid and 0.5% Tween 80 was almost identical (data not shown). The planktonic killing experiments revealed interesting differences between MTAD, Tetraclean and the five experimental solutions (Table 2). Leaving out Tween 80 from MTAD (MTAD-D) made no difference in planktonic and biofilm killing at 3 min (Tables 2 and 3). However, when Tween 80 was replaced by CTR, the dynamics of killing was greatly improved. Tween 80 seemed to inhibit the activity of CTR in a dose-dependent manner. No or only slight difference in activity was measured when 0.01% CTR was added to MTAD, while addition of 0.1% CTR resulted in marked increase in the killing of planktonic *E. faecalis* cells. However, the greatest improvement in performance was gained when CTR substituted Tween 80 (both MTAC formulas and Tetraclean; Table 2). Concentrations of doxycycline and citric acid differ in MTAD and Tetraclean (30 and 10 mg mL^{-1}, respectively), but the present results indicate that the use of CTR instead of Tween 80 is more important for the improved antibacterial activity against planktonic *E. faecalis*. El-Nima (1984) demonstrated higher effectiveness of antibiotics when 0.01% to 0.04% CTR was associated to these formulations. Botelho (2003, 2005) and Portenier *et al.* (2006) also described the excellent antibacterial activity of CTR when used together with other compounds. Although not directly comparable, the results are in agreement with several previous studies on MTAD (Clegg *et al.* 2006, Dunavant *et al.* 2006, Kho & Baumgartner 2006, Ruff *et al.* 2006, Johal *et al.* 2007, Krause *et al.* 2007).

While killing of planktonic bacteria was fast and effective, it is unlikely that it reflects the true effectiveness of the solutions in the root canal. Experiments with biofilm, not surprisingly, showed much slower progress of killing of the microbes with the same disinfecting agents. After 1 min of exposure, less than half of the microbes were killed by all of the tested solutions, as opposed to complete killing of the bacteria in planktonic culture by Tetraclean and MTAC-2 in <30 s. After three minutes of challenge, these solutions had killed 56–71% of the biofilm bacteria, while the other three solutions had killed <50% (Table 3). Previously, Giardino *et al.* (2006) described better antimicrobial activity against *E. faecalis* biofilm by Tetraclean than by MTAD.

Although the results of the two experiments with planktonic and biofilm bacteria revealed great differences in the dynamics of killing, it is worth noticing that the results of the planktonic killing experiments had some predictive value regarding the effectiveness of the same disinfecting solutions against microbes in the

Table 4 Comparison of the effectiveness of the different disinfecting solutions in the biofilm model

	MTAD-D	MTAD + 0.01C	MTAD + 0.1C	MTAC-1	MTAC-2	Tetraclean	Saline
MTAD	N.S.	N.S.	N.S.	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
MTAD-D		N.S.	N.S.	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
MTAD + 0.01C			N.S.	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$
MATD + 0.1C				$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$
MTAC-1					$P < 0.01$	N.S.	$P < 0.01$
MTAC-2						N.S.	$P < 0.01$
Tetraclean							$P < 0.01$

MTAD-D, MTAD without Tween 80 and no cetrimide (CTR) added; MTAD + 0.01C, MTAD + 0.01% CTR; MTAD + 0.1C, MTAD + 0.1% CTR; MTAC-1, MTAC with 0.01% CTR, no Tween 80; MTAC-2, MTAC with 0.1% CTR, no Tween 80; N.S., non-significant.

biofilm. There is no doubt that the results from planktonic killing studies must be interpreted with caution and direct extrapolation to the agents' performance in complex *in vivo* systems is not possible. However, comparison of the two test settings in the present study indicates that planktonic killing tests may be useful for preliminary screening of new disinfecting agents before proceeding into more complex experimental designs.

Conclusions

Under the conditions of this study, Tetraclean was more effective against *E. faecalis* and polymicrobial biofilm than MTAD. Modifications of MTAD formula where CTR replaced Tween 80 were also more effective against *E. faecalis* and bacteria in biofilm than MTAD.

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