

Original Article

Antimicrobial assay of combination surfactant irrigant regimen on vancomycin-resistant *Enterococcus faecalis*. An *in vitro* direct contact test

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ABSTRACT

Background: Vancomycin-resistant enterococci (VRE) are on the rise globally in primary intraradicular infections and resistant to most intracanal irrigants and medicaments. The aim of this study was to evaluate the efficacy of irrigants and identify a cost-effective regimen to eradicate VRE.

Materials and Methods: In this *in vitro* study irrigants were categorized as primary and surfactant groups with individual concentrations consisting of 10 samples each. Primary irrigants; sodium hypochlorite (NaOCl), chlorhexidine (CHX), and iodine potassium iodide (IKI) were prepared in concentrations of 5%, 2.5%, 2%, and 1%. Surfactants cetrimide (CTR) and sodium dodecyl sulfate (SDS) were prepared in concentrations of 0.25%, 0.5%, 1%, and 2%. Biopure MTAD was chosen as the control group. ATCC 51299 (VRE) was evaluated for antimicrobial susceptibility to the above irrigants by direct contact test for 5 min. The effect of each test irrigant was determined by calculating the percentage kill of viable bacteria by spectrophotometer. Statistical analysis was done by means of a one-way ANOVA and Mann–Whitney U-test ($P < 0.05$ consider significant).

Results: About 2.5% and 5% CHX were significant over mixture of tetracycline, acid and detergent (MTAD) ($P < 0.05$). 5% CHX could achieve 100% elimination while 2.5% CHX and 5% IKI had 99.90%. 2% CHX and 2.5% IKI had 99% effective kill percentage. All concentrations of NaOCl were ineffective (90%) as compared to MTAD (95%). CTR (0.5%, 1% and 2%) and SDS (2%) were significant ($P < 0.05$) over MTAD. Combination surfactant regimens of 2% CHX +0.5% CTR and 2% CHX +1% SDS achieved 99.90% eradication potential and were significant ($P < 0.05$) over MTAD.

Conclusion: Surfactant regimens were highly effective and superior to MTAD. CTR and SDS by their organic solvent property enhanced the antibacterial action of CHX.

Key Words: Enterococci, root canal irrigants, vancomycin

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INTRODUCTION

Primary root canal infections are polymicrobial, typically dominated by obligate anaerobic bacteria. The obligate anaerobes are easily eradicated during endodontic therapy. On the contrary, facultative

bacteria are more likely to survive chemomechanical instrumentation with irrigants and root canal medication.^[1] Internal dental anatomy (apical deltas,

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lateral canals, accessory canals, etc.) being complex; depend primarily on the irrigant for disinfection. Although removal of vital noninfected pulps is significant, dissolution of necrotic-infected pulps and disinfection of root canal system present greater challenges.^[1,2]

Enterococcus faecalis is a Gram-positive, Group D Streptococci, and facultative anaerobic bacteria that can survive extreme environmental conditions. They have a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation, and cause toxin-mediated damage.^[3] *E. faecalis* has been implicated in persistent root canal infection and more recently has been identified as the species most commonly recovered from root canals of teeth with posttreatment disease and hence gained attention in endodontic literature.^[4]

Recent studies have implicated that *Enterococcus* species have become multidrug resistant including vancomycin and hence termed as vancomycin-resistant enterococci (VRE). Clinical isolates of VRE isolated from primary and failed root canal cases have caused alarming concerns due to their resistance to most intracanal irrigants and medicaments.^[5] Globally, the prevalence of VRE is on the rise due to indiscriminate use of antibiotics both systemically and locally.^[6]

Sodium hypochlorite (NaOCl) and chlorhexidine (CHX) irrigants have certain limitations despite their routine application in endodontics. Although only higher concentrations of NaOCl have been reported to be effective against *E. faecalis*, resistance against the same has been reported.^[7] CHX, on the contrary, has been used as an adjunct irrigant only; thus questioning the endodontic disinfection protocol. Ethylenediamine tetraacetic acid (EDTA) has limited or no antimicrobial activity and is used in combination with NaOCl. Iodine potassium iodide (IKI) being a potent irrigant has limited application in clinical endodontics.^[8]

Numerous commercial irrigants are being marketed with modifiers/surfactants and claim to have superior antimicrobial efficacy than their conventional counterparts. Biopure MTAD (Tulsa, Dentsply) contains 3% doxycycline (tetracycline isomer) 150 mg/5 ml, 4.25% citric acid, 0.5% polysorbate 80 (surfactant).^[9] Although most studies have claimed MTAD being superior to NaOCl/EDTA regimen and CHX,^[10] controversial reports claim no such

difference exists.^[11] Although clinical isolates have shown resistance to tetracycline,^[12] limited data exist on the antimicrobial efficacy of MTAD on VRE.

Surfactants sodium dodecyl sulfate (SDS) and cetrimide (CTR) are surface active agents that enhance the wetting ability of the irrigants by reducing the surface tension, thereby promoting their close contact with the microorganisms to exert their antimicrobial effect.^[13] Although we hypothesize there will be no significant difference in the antimicrobial efficacy of conventional irrigants, surfactants, and MTAD. The aim of this study was to evaluate the antimicrobial efficacy of endodontic irrigants and their surfactant regimens on VRE by direct contact assay.

MATERIALS AND METHODS

Place of study and bacterial strain used

This *in vitro* study was performed in the Central Research laboratory, A. B Shetty Memorial Institute of Dental Sciences, NITTE University. *E. faecalis* (VRE) ATCC 51299 (Himedia) was used in the study. Bacteria were subcultured from the stock culture. The suspension culture of the test microorganism was prepared in Brain Heart Infusion broth.

Standardization of microorganisms

Brain heart infusion broth was inoculated with *E. faecalis* and incubated for 6–7 h to get a mean optical density of 0.5 McFarland constant; equivalent to 1.5×10^8 CFU/ml (negative control). Then, 1 ml of each suspension culture was transferred to the required number of sterile screw cap tubes (HIMEDIA). All procedures were performed using sterilized instruments and materials.

Irrigants and surfactants used

About 5% NaOCl (Prevest Denpro Limited), 5% CHX (Sigma), 5% IKI was prepared by dissolving 5 g of iodine (Merck) and 10 g of potassium iodide (Himedia) in distilled water. Concentrations 1%, 2%, 2.5%, and 5% of the irrigants were prepared for the study by serial dilution. Surfactants CTR from Himedia and SDS from Merck were prepared in concentrations ranging from 0.25%–2%. Biopure MTAD (Tulsa, Dentsply) was used as per manufacturer's instructions served as positive control. All prepared irrigants were stored in sterile bottles.

Evaluation of antimicrobial efficacy

One milliliter of suspension culture of *E. faecalis* was treated with 20 μ l of each of NaOCl, CHX and

IKI at varying concentrations of 5%, 2.5%, 2%, and 1%, respectively. The irrigant concentrations consisted of 10 samples each, and the same was done for other primary groups. After adding the irrigants to the microtiter plates, the suspension cultures were added later and mean optical density was recorded in a spectrophotometer (Lisa plus) at 630 nm after 5 min. Simultaneously streaking was performed on already prepared Mueller Hinton agar plates for bacterial colony count. These plates were incubated overnight in an incubator at 37°C. The same was repeated for surfactant group CTR and SDS (0.25%–2%), combination regimens, and Biopure MTAD (control).

The number of CFU (colony-forming unit) per ml of culture was determined. The effect of each test agent on microbes was determined by calculating the percentage kill of viable bacteria with the test agent. The percentage of kill for each test agent was calculated by the formula: $1 - (\text{average CFU [test agent]} / \text{average CFU [negative control]}) \times 100$. Statistical analysis was done by means of a one way ANOVA and Mann–Whitney U-test.

RESULTS

In the primary irrigants, only 5% and 2.5% CHX were significant ($P < 0.05$) in eliminating *E. faecalis* with MTAD (concentration 100%) as seen in Table 1. In the surfactant group, all concentrations of CTR (except 0.25%) and 2% SDS were significant ($P < 0.05$) in killing *E. faecalis* over MTAD as seen in Table 2. The combination regimens (2% CHX +0.5% CTR and 2% CHX +1% SDS) were significant ($P < 0.05$) and highly effective than MTAD as seen in Table 3.

With respect to kill percentage in the primary irrigant group, only 5% CHX could achieve 100%; 2.5% CHX, and 5% IKI had 99.90%. About 2% CHX and 2.5% IKI had 99% effective kill percentage. All concentrations of NaOCl were ineffective (90%) as compared to MTAD (95%) as seen in Figure 1. In the surfactant group, all concentrations of CTR and SDS were significantly effective and superior than MTAD as seen in Figure 2. The combination regimens comprising of 2% CHX +0.5% CTR and 2% CHX +1% SDS achieved an effective kill percentage of 99.9%, respectively, in contrast to 2% CHX (99%) and MTAD (95%) as seen in Figure 3.

Table 1: Group comparison between primary irrigants with MTAD (100%)

Concentration (I) (%)	Concentration (J) (%)	Mann-Whitney test (P)		
		Parameters, mean CFU		
		NaOCl	CHX	IKI
1.00	2.00	1.000	0.051	1.000
	2.50	1.000	0.000*	1.000
	5.00	1.000	0.000*	0.081
2.00	100	0.756	0.051	1.000
	1.00	1.000	0.051	1.000
	2.50	1.000	0.002*	1.000
	5.00	1.000	0.001*	0.166
2.50	100	1.000	1.000	1.000
	1.00	1.000	0.000*	1.000
	2.00	1.000	0.002*	1.000
5.00	5.00	1.000	0.250	0.387
	100	1.000	0.002*	1.000
	1.00	1.000	0.000*	0.081
	2.00	1.000	0.001*	0.166
100	2.50	1.000	0.250	0.387
	100	0.756	0.001*	0.387
	1.00	0.756	0.051	1.000
100	2.00	1.000	1.000	1.000
	2.50	1.000	0.002*	1.000
	5.00	0.756	0.001*	0.387
	5.00	0.756	0.001*	0.387

*Significant at the 0.05 level. NaOCl: Sodium hypochlorite; CHX: Chlorhexidine; IKI: Iodine potassium iodide; CFU: Colony-forming unit; MTAD: Mixture of tetracycline, Acid and detergent

Table 2: Group comparison between surfactants with MTAD (100%)

Concentration (I) (%)	Concentration (J) (%)	Mann-Whitney test (P)	
		Parameters	
		CTR, mean CFU	SDS, mean CFU
1.00	2.00	0.250	1.000
	100	0.002*	0.051
	0.25	0.005*	0.051
	0.50	1.000	0.250
2.00	1.00	0.250	1.000
	100	0.001*	0.015*
	0.25	0.001*	0.015*
	0.50	0.250	0.051
100	1.00	0.002*	0.051
	2.00	0.001*	0.015*
	0.25	1.000	1.000
	0.50	0.002*	1.000
0.25	1.00	0.005*	0.051
	2.00	0.001*	0.015*
	100	1.000	1.000
	0.50	0.005*	1.000
0.50	1.00	1.000	0.250
	2.00	0.250	0.051
	100	0.002*	1.000
	0.25	0.005*	1.000

*Significant at the 0.05 level. CTR: Cetrimide; SDS: Sodium dodecyl sulfate; CFU: Colony-forming unit; MTAD: Mixture of tetracycline, Acid and detergent

Table 3: Group comparison of combination surfactant irrigant regimens with MTAD

Parameter (I)	Parameter (J)	Mann-Whitney test (P)
2% CHX + 0.5% CTR	2% CHX + 1% SDS	0.924
	MTAD	0.000*
2% CHX + 1% SDS	2% CHX + 0.5% CTR	0.924
	MTAD	0.000*
MTAD (100%)	2% CHX + 0.5% CTR	0.000*
	2% CHX + 1% SDS	0.000*

*Significant at the 0.05 level. CHX: Chlorhexidine; CHX: Cetrimide; SDS: Sodium dodecyl sulfate; MTAD: Mixture of tetracycline, Acid and detergent

DISCUSSION

Although it has been suggested that nonmicrobial factors may be implicated in endodontic treatment failure; current literature suggests that persistent intraradicular or secondary infections are the major causes of failure of both poorly treated and well-treated root canals.^[14] Various survival and virulence factors possessed by *E. faecalis*, possibly explain its high prevalence rates ranging from 24%–77%. Use of good aseptic technique increased apical preparation sizes, and the inclusion of 2% CHX in combination with NaOCl is currently the most effective method to combat *E. faecalis* within the root canal systems of teeth.^[15]

Due to the emergence of bacterial resistance to most known antibiotics; their use in endodontics should be restricted as per their requisite.^[16] The prevalence of vancomycin-resistant *E. faecalis* (VRE) was significantly higher in diabetic patients and nonhealing endodontic cases as detected by polymerase chain reaction (PCR).^[17] According to Macovei and Zurek enterococcal isolates, *E. faecalis* (88.2%), *E. faecium* (6.8%), and *Enterococcus casseliflavus* (4.9%) accounted for antibiotic resistance. The phenotypic drug resistance to antibiotics was as follows; tetracycline (66.3%), erythromycin (23.8%), streptomycin (11.6%), ciprofloxacin (9.9%), and kanamycin (8.3%). Tetracycline resistance was encoded by conjugative transposon Tn 916 (30.2%) and Tn 916/Tn1545 (34.6%). The tetracycline resistance genes encoded were tet M (65.8%), tet O (1.7%), and tet W (0.8%).^[18]

Although the presence of VRE was found to vary (0%–30%) among clinical isolates obtained in India; occurrence of VRE along with high-level aminoglycoside resistance needs for regular

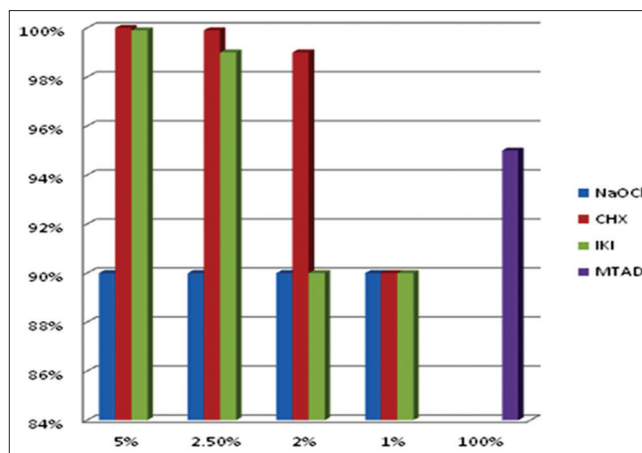


Figure 1: Bar graph showing the kill percentage of primary irrigant groups at different concentrations with MTAD.

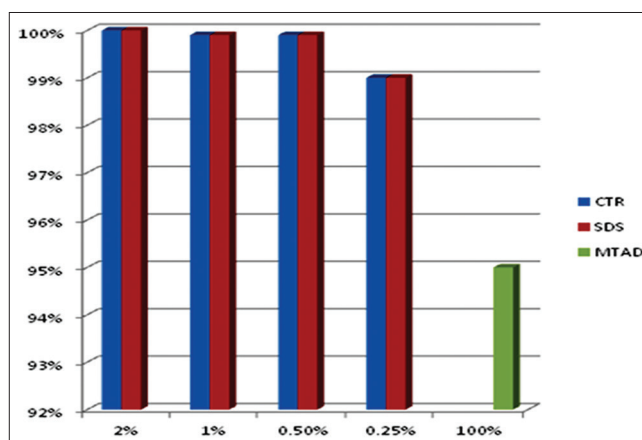


Figure 2: Bar graph depicting the kill percentage of surfactant irrigant group at different concentrations with MTAD.

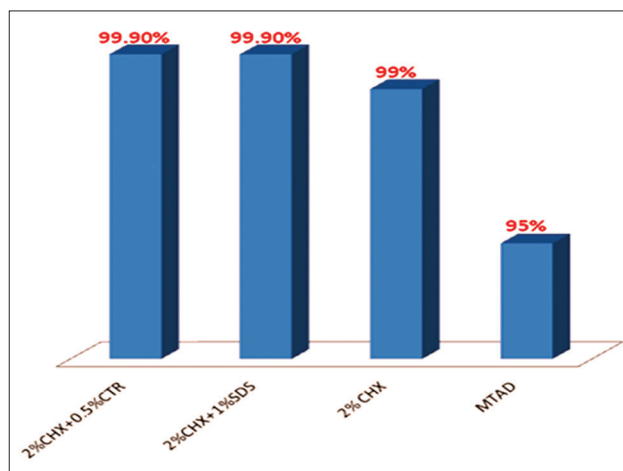


Figure 3: Bar graph displaying the mean effective kill percentage of combination surfactant irrigant regimens with 2% chlorhexidine and MTAD.

detection of vancomycin resistance which will help in preventing establishment and spread of

multidrug-resistant *Enterococcus* species.^[19] Mathur *et al.* characterized vancomycin resistant *E. faecalis* phenotypes by PCR indicated; 115 isolates (26%) high level resistance to aminoglycoside, 293 (66%) to ampicillin, 391 (88%) to ciprofloxacin, 377 (85%) to erythromycin, and 5 (1%) to vancomycin (4 had van A phenotype, and 1 had van B phenotype).^[20]

We employed quality control strain *E. faecalis* ATCC 51299 (vanB positive – low-level vancomycin resistant)^[21] to evaluate its susceptibility to endodontic irrigants. MTAD was chosen as it contains doxycycline which potentiates for its antibacterial action. Surfactants CTR and SDS were experimental irrigants chosen to evaluate their antibacterial activity.

Vianna et al. reported that the time required for 1% and 2% CHX liquid to eliminate all microorganisms was the same required for 5.25% NaOCl.^[22] Estrela *et al.* reported 1% NaOCl was effective on all microorganisms; however, 2% CHX was ineffective.^[23]

In our study, we found NaOCl was ineffective and could not eliminate VRE even after a contact of 5 min. Radcliffe *et al.*^[24] and Retamozo *et al.*^[25] also found higher concentrations of NaOCl and longer exposure times are required to eliminate *E. faecalis*. On the contrary, Sassone *et al.* reported that 1% and 5% NaOCl were effective in eliminating *E. faecalis* immediately and even after 5, 15, and 30 min interval.^[26]

In the CHX group, both 2.5% and 5% were highly effective against VRE. The possible explanation for this finding could be related to the nature of CHX liquid that mixes well with the bacterial suspension, thus immediately exerting its bacterial action. CHX is a cationic bis-guanide, positively charged hydrophobic and lipophilic molecule that interacts with phospholipids and lipopolysaccharides (LPS) on the cell membrane of bacteria and enters the cell through active or passive transport. At a higher concentration of 2%, it is bactericidal to bacteria resulting in precipitation of the cytoplasmic contents, which results in cell death.^[27]

Although 2.5% IKI and 5% IKI were effective in the elimination of VRE, lower concentrations proved to be ineffective. 2% IKI was most effective and required a contact time of 15 min to achieve total elimination in a bovine tooth model; while 10% IKI required 30 min in a planktonic model. Higher concentrations requirement, greater contact time, and delayed availability of free iodine in the aqueous

medium account for the poor antibacterial effect. IKI due to its staining ability has limited application in endodontics.^[28]

Handal *et al.* reported that Gram-positive microorganisms are more susceptible to lower concentrations of tetracycline (doxycycline in MTAD) than Gram-negative ones.^[29] Fujii *et al.* stated the efficacy of doxycycline in endodontic application may be questionable as Gram-negative species dominate established infections.^[30] Mohammadi proposed that the antibacterial effect of MTAD is attributed largely to doxycycline, a primary component that is included to inhibit bacteria. However, resistance to doxycycline (tetracycline isomer) is not uncommon among the bacteria isolated from root canals, and the antibiotic has a narrow spectrum.^[31]

The combination surfactant regimen of 0.5% CTR and 1% SDS with 2% CHX was chosen based on experimental data and previous results obtained.^[32] Both combinations (0.5% CTR +2% CHX) and (1% SDS +2% CHX) had an effective kill of 99.90% as compared to 2% CHX (99%) and MTAD (95%). The possible explanation could be due to surfactants intrinsic antimicrobial activity, lower surface tension, and organic solvent ability that could account for its synergism with CHX.

Surfactants CTR and SDS showed promising antibacterial activity even at lower concentrations.^[33] The cationic environment of the CTR molecule encourages linking with anionic compound at the bacterial surface and is capable of altering the cytoplasmic membrane integrity. Inactivation of the enzymes of cytoplasmic membrane results in protein denaturation and cell death. CTR is noncytotoxic and has been used as endodontic irrigant.^[34] SDS is an anionic alkyl sulfate; has the properties of low-surface tension, can solubilize proteins, increases LPS disaggregation, and inhibits bacterial coaggregation; which could account for its antimicrobial activity.^[35]

The results of our study were in accordance with Jungbluth *et al.*, who stated chlor-xtra (6% NaOCl + surface modifiers) did not result in greater antimicrobial activity or soft-tissue dissolution by the addition of surfactants.^[36] IKI with surfactant combinations yielded a lesser antimicrobial activity as compared with IKI alone. This could be due to interaction of IKI with the organic surfactants limiting its antimicrobial effect.^[37] Giardino *et al.* stated that cetrexedin (0.2% CTR +0.2% CHX) has the lowest

surface tension value; thus increasing the intimate contact of irrigant solution with the dentinal walls, thus permitting deeper penetration of the irrigant.^[38] Baca *et al.* stated that 2% CHX +0.2% CTR would be an effective alternative as final irrigation regimen given its antimicrobial action over time.^[39]

The results of our study were not in accordance with Portenier *et al.*, who compared the antimicrobial activity of MTAD to that of CHX digluconate and found MTAD and CHX were equally effective in killing *E. faecalis*. The presence of dentine or bovine serum albumin delayed killing by both medicaments.^[40] Estrela found 2% NaOCl could eliminate most microorganisms individually and in mixed composition while 2% CHX failed even after an initial contact time after 5 min by direct contact assay.^[41]

MTAD as per manufacturer's instructions requires 1.3% NaOCl to be employed initially followed by a final rinse of MTAD for 5 min.^[42] NaOCl and MTAD have failed individually to achieve complete elimination thus raising concerns about this regimen in the current clinical scenario.^[43,44] Our experimental data are of clinical significance and suggests the use of surfactants 0.5% CTR or 1% SDS with 2% CHX, contribute to the organic solvent property (CHX lacks), and potentiate the substantive antimicrobial activity of CHX.^[45]

CONCLUSION

Our study utilized the quality control strain of VRE to find an alternative to MTAD. The combination regimens proved to be effective over MTAD. In the challenging field of endodontics, the use of clinical strain of VRE and biofilms will enable us to arrive at a concrete picture of this experimental surfactant regimen.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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