Original Article

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Dentistry Section

Comparative Evaluation of Antimicrobial Efficacy of Chlorhexidine, MTAD and Chitosan as Root Canal Irrigant against *Enterococcus faecalis*

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ABSTRACT

Introduction: Endodontic irrigants play a role in disinfection of root canal. Inspite of wider options on selection of irrigant, there is always a search on ideal antibacterial irrigant against recurrent infections.

Aim: To comparatively evaluate the antibacterial efficacy of chlorhexidine, Mixture of Tetracycline, Acid and Detergent (MTAD) and Chitosan against *Enterococcus faecalis* when used as a root canal irrigant.

Materials and Methods: The bacterial *E. faecalis* culture was grown overnight in Brain Heart Infusion (BHI) broth and inoculated in Mueller-Hinton agar plates. The root canal irrigants were divided into four groups as follows. (Group I)-4% Sodium hypochlorite solutions; (Group II)-2% Chlorhexidine (CHX); (Group III)-MTAD; (Group IV)-2% Chitosan. Bacterial inhibition was assessed using agar well diffusion method. All four study irrigants were added to respective wells in agar plate (n=10) and incubated at 37°C for 24 hour. Diameter of bacterial inhibition zone around each well was recorded. The results obtained were statistically evaluated using one-way ANOVA test and the intergroup comparison was done using student's t-test.

Results: All the materials had statistically significant difference in zone of bacterial inhibition when compared to other materials. Based on the mean diameters, 4% sodium hypochlorite had the least zone of inhibition and MTAD had the highest zone of inhibition. 2% Chitosan polymer had greater zone of inhibition than 4% sodium hypochlorite, but less than 2% CHX and MTAD.

Conclusion: MTAD showed the highest antibacterial efficacy against *Enterococcus faecalis* followed by CHX and Chitosan.

Keywords: Antibacterial, Bacterial inhibition, Disinfectant, Endodontic irrigants

INTRODUCTION

The success of endodontic therapy requires the complete debridement of the root canal followed by adequate elimination of microorganisms and their irritants and toxins [1]. To achieve this goal, the endodontic treatment should be based on sound biological rationale by disrupting and destroying the microbial ecosystem through mechanical and chemical methods [2]. The mechanical instrumentation alone cannot reach the complex root canal anatomy to completely eradicate microorganisms. It should be accompanied with chemical disinfection of the root canal with ideal root canal irrigant to completely clean even in uninstrumented root canal surface [3].

Even though the endodontic treatment is performed under aseptic conditions according to accepted clinical principles, the success rate is generally between 86% and 98% [4]. Despite the optimal endodontic therapy, few cases have undesirable outcome which were described as treatment failures. The failure of the treatment is generally attributed to either residual or resistant intra-radicular microorganisms surviving after chemo-mechanical cleaning procedures. *Enterococcus faecalis* has been reported to be seen with increasing frequency in relation to teeth with persistent post treatment disease. Its virulence may be related to resistance to intracanal medicaments and irrigants and an ability to survive in the root canal as a single organism without the support of others [5]. A variety of irrigant solutions have been used in endodontic practice in an attempt to eliminate or reduce this bacterial count.

Sodium hypochlorite has been widely accepted as a root canal irrigant and it is an effective tissue solvent for vital, necrotic pulpal tissue and a potent antimicrobial agent [6]. Other than its desirable properties including antimicrobial property, availability and low cost, it has few drawbacks such as unpleasant taste and noxious effects if concentrated solutions were inadvertently forced into the periapical tissues during irrigation or leaked through the rubber dam [7]. Decreasing the concentration in an attempt to reduce its toxic reactions, the antimicrobial activity also is being reduced.

Chlorhexidine Gluconate (CH) is also another widely used endodontic irrigant and medicament due to its wide range of antimicrobial activity. Furthermore, because of its cationic structure, CHX has a unique property of substantivity against some resistant bacteria such as *Enterococcus faecalis* [8,9] and lower cytotoxicity than sodium hypochlorite [10]. It also impairs the X ability of the regenerative potential of the periapical tissues [11].

MTAD, recommended final endodontic irrigant, is a mixture of doxycycline, citric acid and a detergent (Tween 80) with both antibacterial and smear layer removal abilities. Extensive research has been done on its antibacterial properties. The bactericidal effect of MTAD was inferior to 1%-6% sodium hypochlorite against *E faecalis* biofilms [12]. On contrary, the antibacterial activity of MTAD might also be inhibited by the buffering effect of dentin and the serum albumin present in the root canal [13]. So, current research still focuses on search of an ideal antibacterial irrigant to eradicate *Enterococcus faecalis* effectively.

Recently Chitosan, a natural polysaccharide, the deacetylated derivative of chitin, has gained popularity for its effective antibacterial and biodegradability [14]. These are the main structural components of the cuticles of crustaceans, insects and molluscus and it is useful for various biological activities such as antimicrobial activity, antitumour activity, haemostatic activity and acceleration of wound healing. Kishen A et al., evaluated the efficacy of various cationic nanoparticles of Chitosan to improve root canal disinfection [15].

The antibacterial efficacy of Chitosan and zinc oxide in disinfecting and disrupting *E. faecalis* eliminated biofilms in a concentration- and time-dependent manner. It also effectively removes the smear layer after root canal instrumentation [16].

However, the literature search resulted in very few comparative studies of Chitosan with commonly used intracanal irrigants such as MTAD and CHX against *Enterococcus faecalis*. Hence, this study was undertaken to compare the antimicrobial activity of 4% Sodium hypochlorite, 2% CHX, MTAD and 2% Chitosan against *Enterococcus faecalis.*

MATERIALS AND METHODS

This agar diffusion in-vitro study was carried out in Department of Microbiology, Rajah Muthiah Dental College, Annamalai University during the period from October 2016-November 2016.

Preparation of media and culture plates

A 5.8 gm of agar was mixed with 100 mL of distilled water in a mixing jar and agitated in circular motion. After thorough mixing of the ingredients it was sterilised in the autoclave for 15 minutes at 15 lbs and 121° C. The contents were poured into the sterile culture plates to a height of 5 mm and allowed to cool at room temperature. The culture media began to solidify below 58-60°C and got completely solidified in 30 minutes. The plates were then placed in an incubator for removal of moisture.

Preparation of Brain Heart Infusion (BHI) broth for *Enterococcus faecalis*

A 20 mL of BHI broth was taken in a test tube and heated in the Bunsen burner for 60 seconds and allowed to cool at room temperature. *Enterococcus faecalis* (ATCC 29212) strain was taken in a loop and mixed in the prepared BHI broth by shaking the loop into the broth followed by shaking the test tube in circular motion. The test tube was then kept in the incubator for four hours before inoculation.

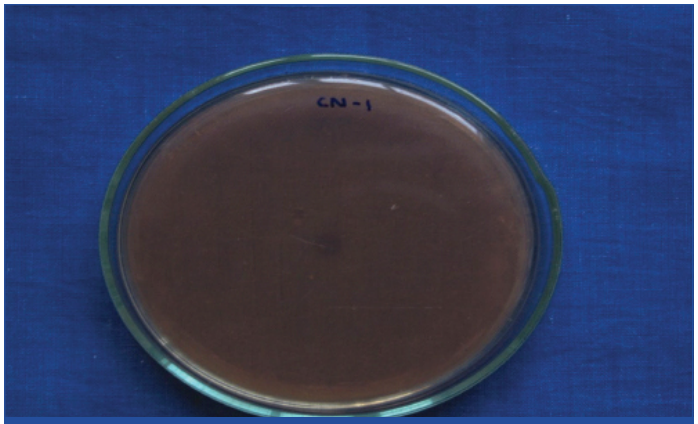
Inoculation of *Enterococcus faecalis* and agar diffusion test

Round wells of around 6 mm diameter were created in the centre of the Mueller-Hinton agar plates by using a sterile conventional punch. Each agar plate was inscribed with the group information on the back side of the plate by using a permanent marker for proper identification. Lawn culture method was performed in the study by dipping a sterile swab in the *Enterococcus faecalis* broth followed by flooding the surface of the plate by brushing across the culture media. The root canal irrigants were divided in to four groups as follows with ten agar plates for each group. (Group I)-4% Sodium hypochlorite solution; (Group II)-2% CHX; (Group III)-MTAD; Group IV-2% Chitosan; were the groups involved.

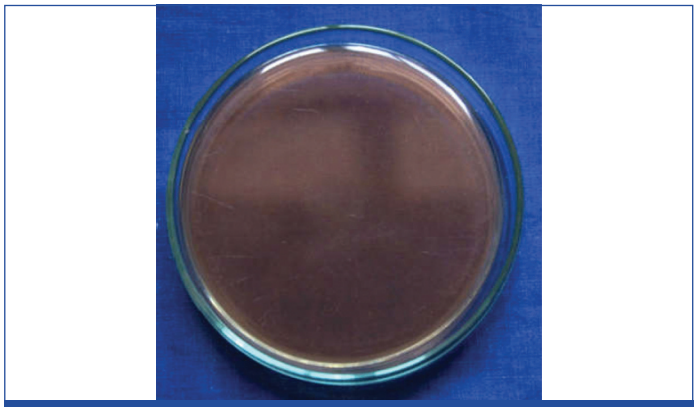
Each agar plate was loaded with only one irrigant. For each irrigant, 10 plates were used for the test. By using a sterile micropipette tips, the created wells of each culture plates were filled completely with the irrigant solution. Then, the inoculated agar plates were incubated aerobically for 24 hours at 37°C. One control plate had the inoculated *Enterococcus faecalis* without any test material and the other control plate had the particular test material but no *Enterococcus faecalis* to prevent any false positive or false negative results respectively [Table/Fig-1,2].

Measurement of zone of inhibition

After 24 hours incubation at 37°C, the inoculated agar culture plates were analysed for zone of inhibition. For measuring the diameter of zone of inhibition for each culture plate, the following method was used. By using the divider and ruler, the shortest diameter of the inhibition zone was measured as D1 and the longest diameter was measured as D2 and the average of the two was recorded as the "Diameter of zone of bacterial inhibition" for that culture plates.



[Table/Fig-1]: Control-with only material and no *Enterococcus faecalis*.



[Table/Fig-2]: Control-with only *Enterococcus faecalis* and no materials.

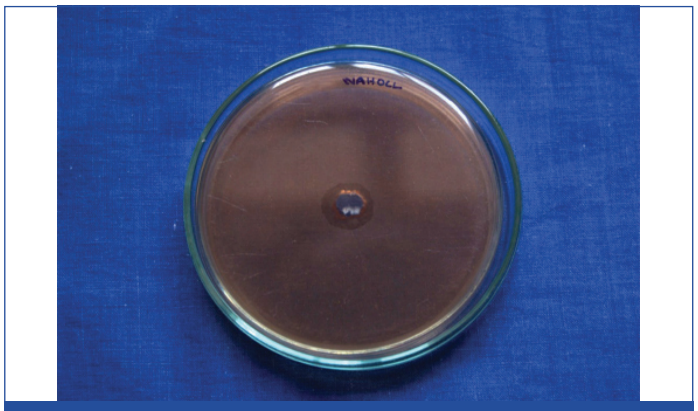
Following 24 hours incubation at 37°C, the culture plates were examined in a well-lit area for zone of bacterial inhibition. The zone of inhibition was seen as a round to oval clear area around the central well devoid of any bacterial growth.

STATISTICAL ANALYSIS

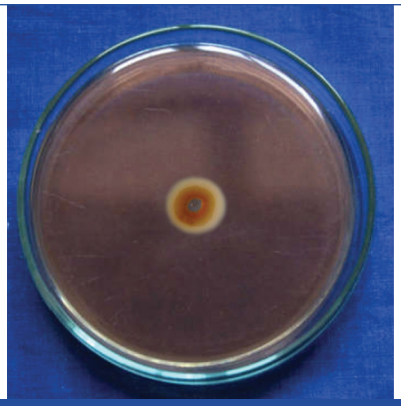
The statistical package SPSS (Statistical Package for Social Science) version 10.5 software was used for statistical analysis. Mean values were estimated from the sample for each study group and analysed using one-way Anova test. Separate student's t-test was used to compare two groups among the various groups. For this study p-value of <0.01 was considered as statistically significant.

RESULTS

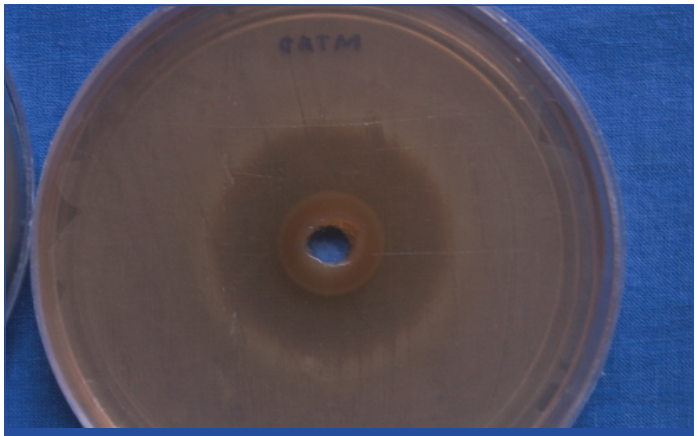
Based on the mean diameters, 4% sodium hypochlorite had the least zone of inhibition and MTAD had the highest zone of inhibition. 2% Chitosan polymer had greater zone of inhibition than 4% sodium hypochlorite, but less than 2% CHX and MTAD [Table/Fig-3-6]. As shown in [Table/Fig-7,8], all the irrigants had statistically significant difference in zone of bacterial inhibition when compared to irrigants (p<0.01).



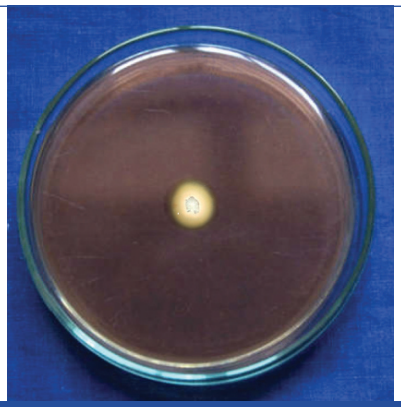
[Table/Fig-3]: (Group 1)-4% Sodium hypochlorite.



[Table/Fig-4]: Zone of inhibition in 2% Chlorhexidine digluconate.



[Table/Fig-5]: Zone of inhibition in MTAD.



[Table/Fig-6]: Zone of inhibition in 2% Chitosan.

Variable	4% Sodium hypochlorite		2% Chlorhexidine digluconate		MTAD		2% Chitosan		F-value	p-value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
Inhibition Zone (mm)	12.8	0.33	26.0	0.75	z36.5	0.29	18.2	0.68	349.13	p<0.001

[Table/Fig-7]: Comparison of mean diameter (Zone of inhibition) by various irrigants.
p<0.01-Significant at 1% level

Variable	4% Sodium hypochlorite		2% Chlorhexidine digluconate		MTAD		2% Chitosan		t-value	p-value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
Inhibition Zone (mm)	12.8	0.33	26.0	0.75					16.09	<0.01
	12.8	0.33			36.5	0.29			54.37	<0.01
	12.8	0.33					18.2	0.68	7.19	<0.01
			26.0	0.75	36.5	0.29			13.02	<0.01
			26.0	0.75			18.2	0.68	7.71	<0.01
					36.5	0.29	18.2	0.68	24.90	<0.01

[Table/Fig-8]: Comparison of mean diameter (Zone of inhibition) between two groups by individual student's t-test.
p<0.01-Significant at 1% level

DISCUSSION

The antibacterial efficacy of various irrigants with Chitosan had been tested against *Enterococcus faecalis* in this present study. The experimental model used in this study was similar to previous studies done by Basrani B et al., who compared the efficacy of CHX and calcium hydroxide as root canal medicaments and Yesilsoy C et al., who studied the antimicrobial effect of sodium hypochlorite, CHX and other potential irrigants against four different microorganisms [17,18].

Chitosan is a natural biopolymer on earth after cellulose. It is a partially N-deacetylated derivative of chitin and consists of polymeric (1→4) linked 2-amino-2-deoxy-β-D-glucopyranose units. It is active against many gram negative and gram positive bacteria and has the advantage of low toxicity towards mammalian cells [19]. Tarsi R et al., proved that low molecular weight Chitosan inhibit adsorption of *Streptococcus mutans* to hydroxyapatite and thus it impairs the colonization of microorganisms over the tooth surface [20]. The concentration of 2% Chitosan was used in the present study as this concentration is water soluble ingredient in the available disinfectant and it was proved to be effective in its original form against a vast number of pathogens [21].

Among all tested irrigants, MTAD showed the highest zone of bacterial inhibition (36.5±0.29 mm). The present study corroborates with various studies where MTAD had better action than 5.25% sodium hypochlorite [22,23]. This can be attributed to the fact that MTAD is not a single material, rather a combination of antibiotic, an acid and a detergent tailored for root canal irrigation. Increased zone of inhibition could be partly due to the addition of Tween 80, a potent detergent which reduces the surface tension and improve the diffusion of the material. It directly affects the cell membrane of the bacteria [24]. The effectiveness of MTAD in removal of the smear layer is by citric acid thus allowing the antibacterial agent (tetracycline) to enter the entire root canal system. Doxycycline also has antibacterial activity, chelating ability and substantivity [25].

Sodium hypochlorite showed the least zone of bacterial inhibition (12.8±0.33 mm) in the present study. Its high pH causes biosynthetic alterations in cellular metabolism and phospholipid destruction. The statistically significant difference existed in the antibacterial efficacy between 4% sodium hypochlorite and 2% CHX or MTAD. Similar results were also obtained by other methods such as post irrigation positive culture test and colony forming units count [26]. This, in addition to unfavorable facts such as toxicity, odour and discoloration of the operatory items makes the material least desirable as an antibacterial agent.

On analysing the results of 2% Chitosan, it showed a zone of inhibition of 18.2±0.68 mm (p<0.01) which was higher than 4% sodium hypochlorite. The mechanism of action of N-carboxybutyl Chitosan is a complex process, and it would likely interact and form poly electrolyte complexes with acidic polymers produced at the bacterial cell [27].

On other side, 2% CHX had a better zone of bacterial inhibition (26±0.75 mm) as compared to 2% Chitosan and 4% sodium hypochlorite. CHX is a cationic molecule that acts by adsorbing onto the cell wall of the microorganism. It targets cytoplasmic membrane of the cells, thereby causing generalised membrane damage to the phospholipids bilayer [28]. Thus, it affects the membrane integrity and causes congealing of the cytoplasm. It had been shown to be effective in eliminating bacteria by penetrating up to 500 μm within dentinal tubules [18]. It is unable to dissolve necrotic tissue remnants and is less effective on gram negative bacteria; hence, it cannot be advocated as the main irrigant in standard endodontic cases.

The antibacterial efficacy of CHX when compared to sodium hypochlorite had given contradictory reports earlier. Jeansonne MJ et al., found no significant differences between 5.25% sodium hypochlorite and 2% CHX in colony forming units when tested in root

canal aspirates [29] On contrast, our study showed a statistically better antibacterial activity for CHX against *Enterococcus faecalis*.

The major advantage of MTAD is that the doxycycline present in the solution has a high binding affinity to root dentin [30]. Yet, MTAD fell short of an ideal root canal irrigant where the requirements include sustained antibacterial activity and biocompatibility. Doxycycline is a macrolide antibiotic which doesn't kill the bacteria, rather prevents bacterial growth by inhibiting protein synthesis. The action is bacteriostatic and often reversible upon withdrawal of the drug. When exposed to longer duration, resistance develops as a result of changes in the permeability of the microbial cell envelope [22]. The biodegradability of other constituents Tween 80 and the acid and, the long term association of these to the tissue need further study.

In this context, Chitosan scores over doxycycline as it is a biodegradable material. N-carboxybutyl Chitosan, a derived product of Chitosan was proved to be a very potent antibacterial agent against gram positive bacteria and fungi [27]. It was also proved to stimulate regeneration of oral soft tissue and in specific surgical situations, regeneration of bone tissue. Further research on Chitosan combined with a suitable surfactant and a synergetic compound could possibly prove to be a valuable disinfectant against *Enterococcus faecalis*.

LIMITATION

Limitation of the study includes that bacteriostatic and bactericidal properties of irrigants cannot be distinguished by agar diffusion test and it could not evaluate the viability of bacteria too. As the irrigant action inside root canal may vary on contact of dentin, these in-vitro preliminary antibacterial results have to be validated in clinical environment to prove their efficiency.

CONCLUSION

Within the limitations of the present study it can be concluded that MTAD showed highest antibacterial activity among all irrigants used followed by CHX and Chitosan polymer. Sodium hypochlorite showed the least antibacterial activity.

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