Aim: This study evaluated the antimicrobial efficacy of irrigants during the cleaning and shaping of the canal system. This in vitro study evaluated quantitatively and qualitatively, the antimicrobial activity of 2% chlorhexidine gluconate, 5.25% sodium hypochlorite and MTAD against E. faecalis.

Materials and Methods: Eighty freshly extracted human single-rooted mandibular premolars were selected and sectioned below the cement-enamel junction at 15 mm length. E. faecalis ATCC 29212 strain and clinical strains were subcultured from nutrient agar plates in the laboratory. Single colonies of both strains were inoculated from nutrient agar cultures into 100 mL Tryptone Soya Broth (TSB) and incubated at 37°C and 150 rpm for 2 h. After 2 h, when the culture entered the logarithmic phase, the prepared teeth specimens were added and incubated at 37°C and 60 rpm for 7 days. Every 48 h, 75 mL of the culture was replaced with fresh TSB. The 80 samples were divided into two sets of 40 each: the first set of 40 specimens for ATCC 29212 and the second set of 40 specimens for clinical isolates. Each set was further subdivided into four groups of 10 teeth according to the selected irrigants. For quantitative assessment, six teeth from each group were used for viable count, and, for qualitative analysis, four teeth from each group sectioned vertically were used for fluorescent microscopy. Results: In the agar disc diffusion method, both Chlorhexidine and MTAD exhibited good antibacterial properties. Distilled water had no antimicrobial property. The colony counts in the clinical isolates and ATCC specimens when used with different irrigants were compared using one-way ANOVA. Conclusions: Sodium hypochlorite showed the most effective antimicrobial property among the three irrigants against E. faecalis biofilm. Clinical relevance to interdisciplinary dentistry

- Microorganisms remaining in the root canal space after treatment or recolonizing the filled canal system are the main cause of endodontic failures.
- Failure in Root canal therapy will lead reinfecion which may become a problem which has to be handled with multidisciplinary approach such as endodontic surgery or therapy.
Antimicrobial activity of various irrigants against *E. faecalis* biofilm: An *in vitro* study

A Afzal, V Rajesh Gopal, Rajesh Pillai, ... 11/22/2017

Experimental setup

All procedures were performed under strict aseptic conditions in a biological safety cabinet. Eighty sterilized teeth specimens were divided into two sets of 40 each. Each set of 40 was used for biofilm development with one of the microbial strains, i.e., *E. faecalis* ATCC 29212 or clinical isolate.

Each set was further divided into four groups of 10 teeth and treated with three irrigants and control as distilled water (Pharmaceuticals and Chemicals Travancore Pvt. Ltd.). The irrigants used were 2% Chlorhexidine gluconate (Biochek, PSK Pharma, India), 5.25% sodium hypochlorite (Biobals, Cochín, India) and Biopure MTAD (Dentsply, Tulsa, OK, USA).

Antibacterial sensitivity assay of root canal irrigants by the agar disc diffusion method

Five microliters of irrigant was impregnated into sterile filter paper discs and the test was conducted by the agar disc diffusion method. The test bacteria were inoculated on to Mueller Hinton Agar (MHA) to give a lawn culture. The test and the control sample discs were placed on the media and incubated at 33-37°C for 18-24 h. The standard Vancomycin disc prepared, 10 mcg, was used as the positive control. The negative control was a filter paper disk impregnated with sterile distilled water.

Analysis of *E. faecalis* biofilm removal

The antibiofilm activity of irrigants against *E. faecalis* biofilm in dentinal tubules was assayed qualitatively by fluorescence microscopy and quantitatively with total viable count. The procedures used in this study were a reflection of the clinical treatment methodology. The biofilms on the root canals of mandibular premolars were washed with sterile phosphate buffered saline (PBS) for removing loosely bound bacteria. Cleaning and shaping was done using a step-back technique to an apical enlargement of size 40. The canals were passively irrigated with 1 mL of the selected irrigants at a constant rate of 4-5 s each. At the end of the biomechanical preparation, all root canals were flushed with 3 mL of test irrigants and the canals were then dried with sterile paper points. For quantitative assessment, six teeth from each group were used for viable count, and for the qualitative analysis four teeth from each group were sectioned vertically and imaged by fluorescence microscopy.

Quantitative assessment by the total viable count

The teeth samples were aseptically transferred into a sterile test tube and washed with sterile PBS thrice to remove any loosely bound bacteria. These test samples were then transferred into another sterile test tube containing 1 mL of sterile PBS and subjected to ultrasonication and vortexing for 1 min each. This process was repeated thrice, and each time the supernatant was collected, pooled and centrifuged at 300 rpm for 5 min. The supernatant was discarded and the residual pellet was resuspended in 5 mL PBS, of which 10 μL was pipetted into the Trypsine Soya Agar (TSA) plates. Each sample was further diluted 1:50 at different volumes (10 μL, 25 μL, and 50 μL) plated on to TSA plates. All the test plates were incubated overnight at 37°C. The number of colonies grown was counted and the total viable count in the test samples was calculated by multiplying with the dilution factor.

The tabulated data were statistically analyzed with one-way analysis of variance (ANOVA), with the help of SPSS software.

Qualitative assessment by fluorescence microscopy

Four teeth from each set after treatment with irrigants were sectioned vertically with the help of a diamond disc and kept in a bijoux bottle and washed in PBS to remove loosely bound bacteria, after which they were fixed with 5% glutaraldehyde for 1 h. The sectioned samples were then washed and stained with 0.1% 5 mL Acridine orange for 15 min. Excess stains were washed off with PBS, air-dried and examined with a fluorescent microscope (Leica DMR) under 50x oil immersion objective.

Results

Agar disc diffusion method for analysis of antimicrobial activity of the different irrigants

From this method, it was observed that [Figure 1] and [Table 1] both Chlorhexidine and MTAD exhibited good antibacterial properties, while 5.25% sodium hypochlorite had a zone of inhibition of 8 mm and 7 mm for *E. faecalis* ATCC 29212 and the clinical isolate, respectively. The antibacterial activity of the irrigants, evaluated by the vial count, showed that the most effective agent against *E. faecalis* was 5.25% sodium hypochlorite, followed by 2% Chlorhexidine. MTAD showed only marginal reduction in viable count, and distilled water was the least effective ([Figure 1]).

Microscopic evaluation for qualitative analysis

The samples with biofilm that were subjected to the various irrigants were stained with Acridine orange and examined using a fluorescence microscope. The bacterial DNA takes up the fluorescent dye and gives an orange-red fluorescence, indicating the presence of bacterial biofilm as seen in [Figure 2] and [Figure 3]. The yellowish-green background shows the absence of biofilm, as seen in [Figure 4] and [Figure 5] ([Figure 2],[Figure 3],[Figure 4],[Figure 5]).

From the figures showing the order of antibacterial efficacy, it was inferred that 5.25% sodium hypochlorite showed the highest antibacterial efficacy, followed by 2% Chlorhexidine, MTAD and distilled water.

Discussion

Microorganisms remaining in the root canal space after treatment or recolonizing the filled canal system are the main cause of endodontic failures. [4] Irrigation is an important procedure in eliminating both forms of microorganisms - planktonic and biofilms - from the root canal system. The efficacy of irrigation depends on the ability to bring the irrigant in contact with those elements, materials and structures within the canal system that have to be removed. [5],[6],[7] Root canal irrigants ideally should have a broad antimicrobial spectrum, especially against anaerobic and facultative microorganisms. The irrigant should dissolve necrotic pulp tissue remnants, inactivate endotoxins and prevent the formation or eliminate the smear layer during instrumentation. When irrigants come in contact with vital tissues, they should be systemically non-toxic, non-caustic to periodontal tissues and have minimal potential for an anaphylactic reaction. [8],[9],[10]

The persistence of *E. faecalis* in treated root canals has been attributed to its ability to resist the high pH of the antimicrobial agents used during the root canal treatment. *E. faecalis* has been demonstrated to synthesize a variety of stress proteins when exposed to acids and alkali. Acid resistance to *E. faecalis* is the result of activity of the cell membrane-bound proton-translocating ATPase, which maintains pH by excreting protons from the cells. [10],[11],[12] The *E. faecalis* biofilm within the dentinal tubules are able to survive for more than 6 months. [13],[14]

A variety of irrigant solutions have been used in endodontics to eliminate or reduce the number of bacteria before obturation of the canal systems. [15] In the present study, the efficacy of 2% Chlorhexidine gluconate, Biopure MTAD, 5.25% sodium hypochlorite and distilled water as irrigants was analyzed.

Chlorhexidine gluconate has a broad spectrum of antibacterial effect. It permeates into the cell wall and causes osmotic imbalance, resulting in leakage of intracellular components. [15] Two percent liquid concentration is effective at reducing or completely eliminating *E. faecalis* from the canal space and dentinal tubules (up to 100 μm) depth and contact of 15 s. [15],[24] The bactericidal ability of sodium hypochlorite results from the formation of Hypochlorous acid (HOCl) when in contact with organic debris. Hypochlorous acid exerts its effects by oxidizing sulfhydryl groups within the bacterial enzyme system, thereby disrupting the metabolism of the microorganisms resulting in killing of the bacterial cells. [1],[18] Sodium hypochlorite is recommended for endodontic purposes, particularly at higher concentrations because of its greater antibacterial effect than diluted concentration. [12] Higher concentrations have increased toxicity and can irritate the periapical tissues; therefore, sodium hypochlorite should be used with caution in endodontic therapy, preferably using rubber dam isolation. Sodium hypochlorite 5.25% was efficacious in eliminating the bacterial strains commonly found in infected root canals. [19],[23] MTAD is a biocompatible material and has minimal effects on the physical properties of the tooth. The efficacy of MTAD in disinfecting the internal and external surfaces of the roots is a result of the antibacterial effect of Doxycycline. [17]

In the agar disc diffusion test, antimicrobial property is assayed against planktonic bacteria. It is a common microbiological study used in clinical practice for determining the most suitable choice of antibiotics to be used in the treatment of infectious bacterial diseases. [19] Bacteria in biofilm are a thousand-times more resistant to antibiotic concentration than the planktonic form. Bacterial biofilms and *E. faecalis* are the most common causes for recurrent root canal infections. [20]
In Figure 1, when the zone of inhibition was assayed, it was seen that both MTAD and Chlorhexidine exhibited anti-bacterial properties against E. faecalis [Table 1] when compared with 5.25% sodium hypochlorite. But, this method does not reflect the clinical scenario when the irrigants are exposed to the bacterial biofilm. Therefore, the present study used the tooth model described to evaluate the efficacy of the irrigants to remove the E. faecalis biofilm from the complex architecture of the dentinal tubules. [Table 1]

Among the ATCC strains [Table 2], Group 1 (2% Chlorhexidine) had a mean of 0.004 × 10^6 colony forming units (CFUs) and Group 2 (5.25% sodium hypochlorite) had a mean of zero CFUs. When comparing Group 3 (MTAD) and Group 4 (distilled water), the mean was 2.4 × 10^6 and 4.7 × 10^6, respectively; there was a statistically significant difference between MTAD and distilled water. There was a significant difference in the number of bacteria between Group 1 (2% Chlorhexidine) and Group 3 (MTAD) and Group 1 (2% Chlorhexidine) and Group 4 (distilled water). When comparing Group 2 (5.25% sodium hypochlorite) with all the other groups, it was found to be the most effective irrigant with zero growth. [Table 2] [Table 3]

In the case of clinical isolate [Table 3], Group 1 (2% Chlorhexidine), Group 2 (5.25% sodium hypochlorite), Group 3 (MTAD) and Group 4 (distilled water) had a mean of 0.006 × 10^6, 3.0 × 10^6 and 6.1 × 10^6 CFUs, respectively. There was a statistically significant difference between MTAD and distilled water. When comparing 5.25% sodium hypochlorite, it was the most effective irrigant with zero growth compared with Group 1 (2% Chlorhexidine), Group 3 (MTAD) and Group 4 (distilled water). There was a significant difference in the number of bacteria between Group 1 (2% Chlorhexidine) when compared with Group 3 (MTAD) and Group 4 (distilled water).

When the antimicrobial property of the root canal irrigants was examined using the agar diffusion method, 5.25% sodium hypochlorite had a minimal zone of inhibition, for ATCC 8 mm and for clinical isolate 7 mm, while the zone of inhibition for MTAD, ATCC was 33 mm and for clinical isolate was 31 mm, and ATCC 24 mm and 23 mm for clinical isolate in the case of 2% Chlorhexidine [Figure 1]. This may be due to the fact that in case of the agar diffusion method, we are testing E. faecalis in the planktonic forms, where it is more sensitive to the antibacterial irrigants while in tooth models the efficiency of irrigants is tested against E. faecalis biofilm. Also, when filter paper discs are made for the agar diffusion method, they are dried. The antibacterial property of 5.25% sodium hypochlorite is due to the nascent chlorine, which gets evaporated on drying, leaving the disc free of a major portion of nascent chlorine and reducing the antibacterial property. When testing as irrigant, we use freshly prepared solutions of sodium hypochlorite, which is most active. These facts are also substantiated by Krause et al.’s observations. [21]

Chlorhexidine was effective in reducing of biofilm while MTAD was inefficient in destroying E. faecalis biofilms. Distilled water, which was used as a control, showed no effect on biofilms, or any antimicrobial property [Graph 1] [SUPPORTING INFORMATION].

This study also confirms Clegg et al.’s observations that sodium hypochlorite is the only effective irrigant in endodontic therapy capable of removing E. faecalis biofilm from the complex dentinal tubule architecture of the root canal system. [22] Currently, the use of a good aseptic technique increases apical preparation sizes, and inclusion of full-strength sodium hypochlorite and 2% chlorhexidine irrigants are the most effective methods to eliminate E. faecalis. [25],[26] The result of our study indicates that the effects of irrigants used on both ATCC and clinical isolate strains are almost similar. Our study also clearly illustrated that sodium hypochlorite was equally efficient in destroying E. faecalis biofilm for both ATCC and clinical strains, reinforcing the universal applicability. These results clearly show that sodium hypochlorite is the most efficient irrigant. In spite of the fact that hypochlorite has inherent drawbacks like being caustic and discoloring operator items, there is always a search for newer irrigants with a similar antimicrobial property but lesser side-effects.

**Conclusions**

Two strains of E. faecalis - ATCC 29212 and clinical isolate - were used in our study. Sodium hypochlorite 5.25% showed the highest antibacterial efficacy against the E. faecalis biofilm, followed by 2% Chlorhexidine, MTAD and distilled water. Further studies with more specimens and more parameters may be necessary to further confirm and validate these results.

**References**