

# Evaluation of the Antibacterial Efficiency of a Combination of 1% Alexidine and Sodium Hypochlorite on *Enterococcus faecalis* Biofilm Models: An *In Vitro* Study

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## ABSTRACT

**Aim:** The aim of the study was to assess the antibacterial efficiency of a combination of 1% alexidine (ALX) and 5.25% sodium hypochlorite (NaOCl) against *E. faecalis* biofilm using a confocal scanning electron microscopy.

**Materials and methods:** An estimated 120 human root dentin disks were prepared, sterilized, and inoculated with *E. faecalis* strain (ATCC 29212) to develop a 3-weeks-old biofilm. The dentin discs were exposed to group I—control group: 5.25% sodium hypochlorite (NaOCl) ( $n = 20$ ); group II—1% ALX + 5.25% NaOCl ( $n = 40$ ); group III—1% alexidine (ALX) ( $n = 40$ ) (Sigma-Aldrich, Mumbai, India); group IV—negative control: saline ( $n = 20$ ). After exposure, the dentin disks were stained with the fluorescent live/dead dye and evaluated with a confocal scanning electron microscope to calculate the proportion of dead cells. Statistical analysis was done using the Kruskal–Wallis and Mann–Whitney  $U$  test ( $p < 0.05$ ).

**Results:** The maximum proportion of dead cells were seen in the groups treated with the combination of 1% ALX + 5.25% NaOCl (94.89%) and in the control group 5.25% NaOCl (93.14%). The proportion of dead cells presented in the 1% ALX group (51.79%) and negative control group saline (15.10%) were comparatively less.

**Conclusion:** The antibacterial efficiency of a combination of 1% ALX and 5.25% NaOCl was more effective when compared with 1% ALX alone.

**Clinical significance:** Alexidine at 1% could be used as an alternative endodontic irrigant to chlorhexidine, as alexidine does not form any toxic precipitates with sodium hypochlorite. The disinfection regimen comprising a combination of 1% ALX and 5.25% NaOCl is effective in eliminating *E. faecalis* biofilms.

**Keywords:** Alexidine, Biofilm, Confocal laser scanning microscope, *Enterococcus faecalis*.

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## INTRODUCTION

Microorganisms play a primordial role in the pathogenesis of pulpal and periapical diseases. The success of endodontic treatment depends on complete elimination of microorganisms and their by-products from the root canal system through the combined action of mechanical instrumentation and chemical debridement.<sup>1–3</sup> It has been observed that aerobic and facultative anaerobic microorganisms are found in higher counts in endodontic flare-ups and retreatment cases. *Enterococcus faecalis* has been found predominantly in failed cases than in primary infection.<sup>4</sup> *E. faecalis* is highly resistant during the starvation phase and it is probable that the physiologic state of the cells, distinctly in retreatment cases, bear a resemblance to the starvation phase.<sup>5</sup>

It is certain that biomechanical debridement of the root canal leads to a significant reduction in the bacterial count; however, the complex anatomy of the root canal system often makes it difficult to achieve complete debridement.<sup>6</sup>

Several root canal irrigants have been used in endodontic practice to minimize the residual debris, necrotic tissue, and bacteria, during cleaning and shaping of the dentin,<sup>7</sup> of which the most commonly used irrigating solution is sodium hypochlorite (NaOCl) because of its tissue-dissolving capability as well as its broad antimicrobial action.<sup>9,20</sup> However, NaOCl has a major drawback that it does not impart antimicrobial substantivity.<sup>10</sup>

Chlorhexidine gluconate (CHX) is another significant irrigant used widely; its structure comprises a cationic bisbiguanide with antimicrobial efficacy against certain NaOCl-resistant bacteria and its virulence factor. Most importantly, CHX has antimicrobial substantivity. Therefore, CHX can be considered an advantageous

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conjunctive root canal irrigant, even though it lacks tissue-dissolving properties.<sup>10–12</sup>

For this reason, many studies were conducted to analyse the effect of a root canal disinfection regimen with a combination of CHX and NaOCl. However, it was seen that a dense-brown precipitate para-chloroaniline (PCA) was formed, which was found to hamper the seal of obturation.<sup>13,14</sup>

Alexidine (ALX) (belonging to the bis-biguanide family), similar to chlorhexidine, is also an effective disinfectant that helps inhibiting the immune response of the major virulence factors

(lipopolysaccharide and lipoteichoic acid) of bacteria<sup>15-17</sup> and has been used as antimicrobial agent in mouthwashes and in contact lens' solution.<sup>18</sup> The combination of ALX/NaOCl did not produce any harmful precipitate; ALX might be used sequentially as an effective root canal irrigant or in combination with NaOCl.<sup>19,33</sup> Hence, the aim of the study was to compare the antimicrobial activity of a combination of 1% alexidine with 5.25% sodium hypochlorite and 1% alexidine against *E. faecalis* biofilm on human dentin blocks using a confocal laser scanning microscope (CLSM).

## MATERIALS AND METHODS

### Preparation of the Specimen

This study was approved by institutional review board of SRM Dental College, Kattankulathur, Tamil Nadu, India. Totally, 120 single-rooted human incisors were selected. The apical and coronal portion of the tooth specimens were sectioned using a diamond disk, following which the middle third was sectioned with a low-speed sectioning saw (Censico International Private, Agra, India) to obtain dentin disks of standard dimension 5 × 6 × 0.5 mm (length × width × thickness). The specimens were prepared according to the methodology proposed by Bukhary et al.<sup>36</sup>

The dentin disks were immersed in 17% EDTA (Prime dental products, Mumbai, India) for 1 minute, followed by rinsing in an ultrasonic bath to remove the smear layer and finally autoclaved. Following this, two dentin disks were arbitrarily chosen from each group and incubated in a brain heart infusion broth (BHI) for 24 hours at 37°C to rule out any bacterial contamination.

### Biofilm Preparation

The dentin disks were inoculated with *E. faecalis* under anaerobic conditions using 12-well tissue culture plates. Aliquots of 100 µL of *E. faecalis* (ATCC 29212) suspension were inoculated in each well (1 × 10<sup>8</sup> colony-forming unit/mL) under anaerobic conditions for 21 days at 37°C. New aliquots of 100 µL of *E. faecalis* suspension were replaced every 72 hours to remove dead cells and ensure bacterial viability. After the specified incubation period, the specimens were removed gently from the culture plates and washed with sterile phosphate-buffered saline (PBS) for 2 minutes. This procedure ensures adequate removal of loosely attached planktonic bacteria from the dentin disks. From each group, two dentin disks were selected randomly and examined under a confocal laser scanning microscope (CLSM) (Carl Zeiss Microscopy, Chennai, India) to assess the growth and viability of the biofilm.

The dentin disks were randomly divided into four groups:

- Group I—positive control group: 5.25% NaOCl (*n* = 20) (Prime dental products, Mumbai, India)
- Group II—1% alexidine + 5.25% NaOCl (*n* = 40)
- Group III—1% alexidine (*n* = 40) (Sigma-Aldrich, Mumbai, India)
- Group IV—negative control: sterile saline (*n* = 20) (Prime dental products, Mumbai, India)

The dentin disks were immersed in 2 mL of experimental irrigant for 10 minutes. The samples were finally rinsed with 5 mL of phosphate buffered saline for 5 minutes in order to neutralise the irrigant and prevent any further antimicrobial activity.

The dentin disks were stained with live/dead BacLight fluorescence dye (Invitrogen Molecular Probes, Eugene, OR) for a period of 30 minutes, which differentiates live and dead cells. The dye stains green for bacteria with intact cell membranes, whereas

damaged membranes stain red. A fresh mix of the dye was prepared immediately before microscopic evaluation of each root section.

The dentin disks were rinsed with 2 mL PBS to remove excess dye. The samples were then mounted on the confocal laser scanning microscope and observed using the 25× magnification, at a resolution of 512 × 512 pixels. The green fluorescence representing the live cells and red fluorescence representing the dead cells were displayed using simultaneous dual-channel imaging. Biofilm images were viewed and quantitated with AxioVision Rel.4.8.Ink (Carl Zeiss Microscopy). The percentage of dead cells were calculated by evaluating the volume ratio of red to green fluorescence in each sample.

### Exclusion Criteria

Multiple roots, roots with curvature, roots with fracture lines, previously treated root canals, complex root morphology.

### Statistical Analysis

Statistical analysis was performed using the Kruskal–Wallis test to evaluate the mean value of the proportion of dead cells on exposure to different solutions. The Mann–Whitney *U* test was used for comparisons between irrigant solutions (*p* < 0.05).

## RESULTS

Three-hundred CLSM operative fields 3D stacks were assessed for each sample. The CLSM images of the dentin disks presented the growth and formation of *E. faecalis* biofilm in a homogenous dense manner (Fig. 1).

The maximum proportion of dead cells were in the samples treated with a combination of 1% ALX + 5.25% NaOCl (94.89%) followed by the positive control group 5.25% NaOCl (93.14%) and 1% ALX (51.79%); the lowest proportion was observed with the control saline group (15.10%). There was no statistically significant difference between group I and group II (*p* > 0.05) (Table 1 and Fig. 2).

## DISCUSSION

A biofilm is a complex aggregation of microorganisms that secrete a protective and adhesive exo-polymeric matrix called extracellular polymeric substance or exopolysaccharide (EPS). The EPS protects

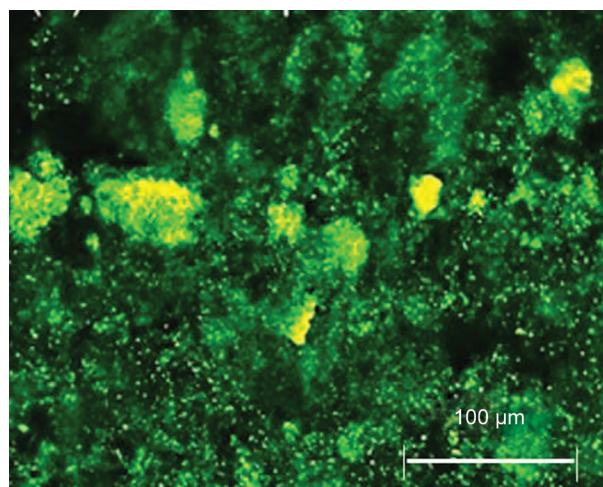


Fig. 1: Homogeneous dense 3-weeks-old *E. faecalis* biofilm grown on dentin disks

**Table 1:** Percentage of dead cells (%) after 10 minutes of exposure to the endodontic irrigants: ( $p < 0.05$ )

Irrigant solution	Median (range%)
Control group: 5.25% NaOCl	93.14 (89.13–95.34)
1% ALX + 5.25% NaOCl	94.89 (91.09–96.22)
1% ALX	51.79 (30.14–64.13)
Negative control: saline	15.10 (10.11–15.45)

the biofilm cells and facilitates communication as well as nutrient distribution among them. Organizations of microorganisms within biofilms are characterized by surface attachment, structural heterogeneity, complex community interactions, and the presence of EPS.<sup>21–24</sup>

Bacteria growing in biofilms may survive starvation periods and recover rapidly, and also may exhibit new and more virulent types. Furthermore, bacteria within biofilms have inherently increased resistance to antimicrobial agents, compared with the same bacteria grown under planktonic conditions.<sup>25,26</sup>

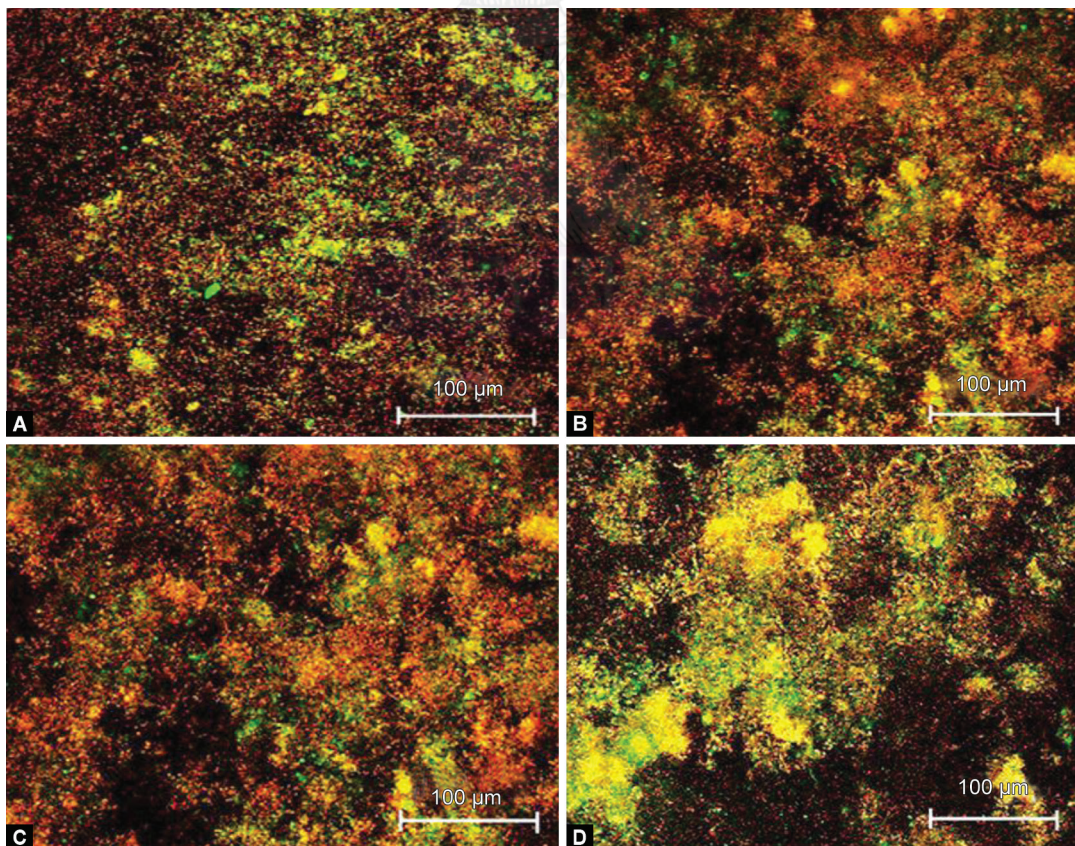
In this study, we used a three-week-old *E. faecalis* biofilm, ensuring the maturation of the biofilm. Previous research showed that a mature biofilm is more resistant to endodontic irrigants than a young biofilm.<sup>34,35</sup> *E. faecalis* was chosen, as it is the most common bacterial strain used to evaluate the efficiency of endodontic medicaments and irrigants. Furthermore, it possesses the ability to invade the dentinal tubules, attributed to an active process mediated by cell division, and has been researched to be the most prevalent strain in persistent root canal infections.<sup>38</sup> Although

culture techniques do not entirely simulate clinical conditions, they offer an adequate method to evaluate antimicrobial activity of endodontic irrigants on bacterial biofilm over dentin surface. Also, they allow systematic comparisons among different solutions at different exposure times, and are practical and easy to reproduce. The substrate for biofilm growth and formation chosen was the dentin disk, as *E. faecalis* has shown to have an excellent ability to bind to the dentin surface.<sup>39</sup>

This article analyses the antimicrobial efficacy of a combination of 1% alexidine and 5.25% sodium hypochlorite and 1% alexidine on the *E. faecalis* biofilm grown on human root dentin sections using the CLSM. The results of the study indicated that there was no statistical difference between the percentage of dead cells in the positive control and the combination of ALX + NaOCl. The combination of sodium hypochlorite and alexidine performed better than the alexidine group.

The assessment of live/dead organisms in this study was carried out using the CLSM, despite the ascensions in technology with polymerase chain reaction (PCR), which enables the detection of culture difficult species, PCR methods cannot differentiate between viable or dead cells. The CLSM utilizes an immunofluorescence technique to identify bacteria and also enables the assessment of viability of distinct bacteria colonizing the root canal.<sup>38</sup>

ALX has cationic molecules that exert their antibacterial effects by disrupting the integrity of the bacterial cytoplasmic membrane, causing the leakage of the intracellular contents.<sup>30</sup> It has been observed that Gram-positive bacteria are more sensitive to cations because they are more negatively charged.<sup>31</sup> ALX has greater affinity for the major virulence factors of bacteria than



**Figs 2A to D:** A representation of the proportion of live/dead organisms in the biofilm treated with the following endodontic irrigants; (A) Control group 5.25% NaOCl; (B) 1% ALX + NaOCl; (C) 1% ALX; (D) Negative control saline

CHX. The difference in the hydrophobic moieties between these two compounds is believed to be responsible for the more rapid bactericidal action of ALX.<sup>27,30</sup> Previous research shows that 2% and 1% ALX used for 1 minute provide longer antimicrobial substantivity against *E. faecalis* than CHX when applied to 2% and 0.5%.<sup>28,32,37</sup>

In this study, 1% ALX solution was used because it was seen that concentrations higher than 1% caused moderate cytotoxicity against human gingival fibroblasts.<sup>29</sup> The result of this study is in accordance with a previous study that ALX should be in direct contact with the infected dentinal surface for a prolonged time (>5 minute) in order to achieve their maximum antibacterial effect against *E. faecalis*.<sup>29</sup> The findings of the current study are in accordance to the previous research that 1% ALX did not perform better than 5.25% NaOCl (Bukhary et al.).<sup>36</sup> The reason for the ALX group not performing better than the sodium hypochlorite or the combination could be because it lacks the ability to dissolve organic tissues.<sup>40</sup> Another reason could be attributed to the fact that the cationic bisbiguanides were inactive in the presence of organic matter and have limited ability to penetrate the EPS.<sup>40</sup> It could be used as an alternative endodontic irrigant to chlorhexidine, as alexidine does not form any toxic precipitates with sodium hypochlorite.

## LIMITATIONS

- The culturing technique allows the root canal irrigants to be exposed to the biofilm directly, which is far from the clinical situation, where the root canal anatomy is complex comprising isthmuses, fins, and lateral extensions, and the biofilm maybe enclosed in these irregularities, posing a challenge for achieving efficient disinfection.<sup>41</sup>
- Though the CLSM is a popular noninvasive imaging technique, it only assesses a portion of the canal surface and does not give the entire picture.<sup>42</sup>
- To simulate a more relatable clinical condition, a polymicrobial biofilm is grown; that consisting of typical root canal isolates would have been preferable.<sup>43</sup>

## CONCLUSION

It can be concluded within the limitations of this study that a disinfection regimen comprising a combination of alexidine and sodium hypochlorite is effective in the elimination of *E. Faecalis* biofilm. However, this is a preliminary study and further studies need to be conducted to assess the efficacy of this combination of endodontic irrigants.

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