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The Effect of Various Irrigants on Mixed Biofilms in Dentinal Tubules: A Confocal Laser Scanning Microscopy Study

Dide Tekinarslan[1,2] | Kürşat Er[2] | Mine Eğin[3] | Tuba Dinçer[4] | Ali Osman Kiliç[3]

[1]Department of Endodontics, Faculty of Dentistry, Antalya Bilim University, Antalya, Turkey | [2]Department of Endodontics, Faculty of Dentistry, Akdeniz University, Antalya, Türkiye | [3]Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Türkiye | [4]Department of Medical Biology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Türkiye

Correspondence: Dide Tekinarslan (didetekinarslan@hotmail.com)

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ABSTRACT

This study evaluated the bacterial disinfection efficacy of three different irrigation solutions and their combined usage in dentin tubules contaminated with mixed biofilms. A total of 60 single-rooted mandibular premolars were prepared with ProTaper Next X4, sterilized, and then inoculated with mixed biofilm for 7 days. After biofilm formation, the teeth were randomly divided into six groups ($n = 10$) based on the irrigation solution used: Group 1: sodium hypochlorite (NaOCl), Group 2: chlorhexidine gluconate (CHX), Group 3: polyhexamethylene biguanide (PHMB), Group 4: NaOCl + CHX, Group 5: NaOCl + PHMB, Group 6: distilled water (control group). The teeth were sectioned at the coronal, middle, and apical thirds and stained with LIVE/DEAD BackLight and examined under a confocal laser scanning microscope (CLSM) to determine the depth of dead bacterial penetration into the dentinal tubules. Additionally, one tooth from each group was subjected to bacterial viable counting before and after irrigation. The CLSM analysis revealed that the greatest penetration of dead bacteria occurred in the coronal third, followed by the apical and middle third ($p < 0.05$). Combined solutions demonstrated significantly higher disinfection efficacy compared with PHMB alone ($p < 0.001$). Viable count analysis revealed that Group 4 and Group 5 exhibited the greatest reduction in bacterial proliferation, respectively. The combined use of solutions was found to be more effective than the sole use of solutions. Therefore, it is recommended to combine irrigation solutions during final irrigation in clinical settings.

1 | Introduction

The primary aim of root canal treatment is to thoroughly disinfect the root canal system and prevent reinfection (Hülsmann et al. 2005). Due to the complex anatomical structures of the root canal, mechanical debridement alone is insufficient, often leaving over 35% of the canal system untouched (Peters et al. 2001). Residual microorganisms in these complex areas can cause persistent infections and treatment failure (McGurkin-Smith et al. 2005). Consequently, chemomechanical treatment should complement mechanical debridement.

Sodium hypochlorite (NaOCl) is the commonly used irrigant in endodontics, known for its unique ability to dissolve

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Summary

- Combined irrigation solutions demonstrated superior disinfection efficacy against mixed biofilms in dentinal tubules compared with single solutions.
- This study supports the clinical use of combined irrigation solutions for enhanced root canal disinfection.
- Findings provide evidence for optimizing irrigation protocols to improve endodontic treatment outcomes.

organic tissues within the root canal (Stojicic et al. 2010). Chlorhexidine gluconate (CHX) has strong antibacterial activity but lacks tissue-dissolving capabilities, making it more effective when combined with NaOCl (Mohammadi and Abbott 2009). Polyhexamethylene biguanide (PHMB) hydrochloride is characterized by lower toxicity compared with CHX and exhibits high antimicrobial effectiveness against bacteria, as well as fungi and viruses (Mashat 2016). PHMB has been widely used for many years in the cosmetic, industrial, and food sectors as a disinfectant. It binds to phospholipids in cell membranes, disrupting ion pumps and causing the cell to collapse. Its strong binding affinity to polysaccharides provides an antiseptic effect within the bacterial extracellular biofilm matrix (Müller et al. 2011).

This study aimed to evaluate the disinfection efficacy of three different irrigants and their combinations in root canals infected with a mixed biofilm using CLSM and viable bacteria counting. To the best of our knowledge, no previous study has investigated the disinfection efficacy of PHMB in combination with NaOCl. The null hypothesis of the study posits that there will be no difference in the penetration of dead bacteria into the dentinal tubules among the irrigants.

2 | Materials and Methods

This study was approved by the Scientific Research Ethics Committee of Akdeniz University (TBAEK-577). Using the G*Power 3.1.9.7 software and taking the study by Vatkar et al. (2016) as a reference, a power analysis was conducted to determine the appropriate sample size for this study. An effect size of $f = 0.50$ was used, with the analysis set at a significance level (α) of 0.05 and a power of 0.80—standard parameters that ensure adequate sensitivity to detect statistically significant differences among the groups. Given the need to compare multiple irrigation solutions, a one-way ANOVA was selected as the statistical method.

2.1 | Tooth Selection and Preparation

A total of 60 mature, single-rooted mandibular premolars without resorption, cracks, or fractures in the root were selected. The teeth were decoronized using a diamond disc under water cooling to standardize their length to 18 mm. The working length (WL) was set to 1-mm short of the root apex, and the root canals were shaped using ProTaper Next rotary files (X1–X4; Dentsply-Maillefer, Ballaigues, Switzerland). The root canals were irrigated with 2.5% NaOCl (Cerkamed, Pawloski, Poland) after each file. After shaping, the root surfaces of the teeth were coated with nail polish and embedded in acrylic resin to create working blocks.

2.2 | Biofilm Formation

The strains used for biofilm formation in root canals were *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, *Streptococcus mutans* (*S. mutans*) ATCC 25175, and *Staphylococcus aureus* (*S. aureus*) ATCC 25923. The strains were cultured at 37°C for 24 h on tryptic soy agar (TSA) medium. A single colony was selected from each plate and inoculated into bottles containing 150 mL of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA), followed by incubation at 37°C for 24 h. From each bacterial culture, 50 mL was taken to adjust the optical density (OD_{600}) to 1.3 with a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany); the cultures were then diluted with 850 mL of fresh BHI medium. Subsequently, 20 mL of the prepared culture medium was dispensed into Falcon tubes, and each sample tooth was immersed in the culture medium for biofilm formation. The samples were incubated at 37°C for 7 days.

2.3 | Irrigation and Preparation of Dentin Discs

The PHMB solution used in the study was obtained as a polymer of hexamethylene biguanide hydrochloride with 85% purity (Abcr GmbH, Karlsruhe, Germany). Following a previous protocol (Chandki et al. 2020), 0.7 g of the powder was mixed with 2.3 g of sterile deionized water to make 300 mL of PHMB at a 0.2% (w/v) concentration.

The tooth samples were randomly divided into six groups ($n = 10$) based on the application of the irrigants:

Group 1: 6 mL of 2.5% NaOCl for 60 s.

Group 2: 6 mL of 2% CHX (Microvem, Altun Sterilizasyon, İstanbul, Türkiye) for 60 s.

Group 3: 6 mL of 0.2% PHMB for 60 s.

Group 4: 3 mL of NaOCl for 60 s, followed by distilled water, and then 3 mL of CHX for 60 s.

Group 5: 3 mL of NaOCl for 60 s, followed by distilled water, and then 3 mL of PHMB for 60 s.

Group 6 (*control*): distilled water.

For each group, the EDDY device with a 25/0.04 flexible polyamide tip was positioned in the canal 2-mm short of the WL, and the irrigants were continuously activated at 6000 Hz for 20 s. The irrigation and activation procedures were repeated three times. To neutralize the reaction of the irrigation solutions, the canals were rinsed with distilled water.

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Afterwards, the teeth were dried using sterile paper points (Pearl Endo, Ho Chi Minh City, Vietnam). Acrylic blocks were marked at the apical (2 mm), middle (5 mm), and coronal third (8 mm) regions from the apex. Horizontal sections, approximately 1 ± 0.1 mm in thickness, were cut from these marked regions using a 0.3-mm thick diamond-cutting disk in the Microcut device (Metkon Microcut 125, Bursa, Türkiye) under water cooling.

2.4 | Bacterial Sampling and Viable Count

Initial bacterial sampling was performed before applying the irrigant, using a randomly selected tooth from each group. A vigorous scraping motion inside the root canal was conducted for 30 s using a sterile #30 H-type file. This was followed by pipetting 200 μL of PBS into the canal to collect the suspension, which was recorded as S1.

After the irrigation, the same sampling method was applied to the root canals, with the resultant sample recorded as S2. The bacterial suspensions in the S1 and S2 tubes were vortexed and serially diluted with PBS. Aliquots of 100 μL were plated on BHI agar and incubated at 37°C for 48 h, followed by CFU counting.

2.5 | Confocal Laser Scanning Microscopy

Each dentin disc was stained according to the manufacturer's instructions using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR), which consists of SYTO 9 and propidium iodide (PI) for live and dead bacteria, respectively. The staining solution was applied to the dentin discs and incubated at room temperature in the dark for 20 min. The samples were rinsed with 1 mL of PBS to halt the dye reaction and to remove unbound bacteria.

The samples were examined using CLSM (Leica TCS SP8 MP, Mannheim, Germany) at the wavelengths of 480/500 nm for SYTO 9 and 490/635 nm for PI at 4× and 20× magnification. The images were analyzed using the Leica Application Suite-Advanced Fluorescence software (version 2.6.0.7266; LAS AF, Leica Microsystems GmbH, Wetzlar, Germany) at a resolution of 4096 × 4096 pixels.

The analysis focused on quantifying the penetration of red fluorescence, representing dead bacteria, into the dentinal tubules. Measurements assessed the distance from the canal lumen to the endpoint of the red fluorescence in the dentinal tubules, as illustrated in Figure 1.

2.6 | Statistical Analysis

The statistical analysis was performed using SPSS 2017 software (SPSS Inc.; Chicago, Illinois, USA). The Wilcoxon test was applied to analyze data when the normality assumption was not met in the comparison of two dependent groups. For comparisons involving more than two independent groups, the Kruskal–Wallis *H* test was employed if the data did not follow a normal distribution or if the sample size in at least one group was less than 30. Post hoc analyses with Bonferroni correction were performed following Kruskal–Wallis *H* test results. A value of $p < 0.05$ was considered statistically significant.

3 | Results

The study analyzed CLSM images to determine the maximum depth of bacterial penetration in a dead state. The analysis of different regions within the root canal, as shown in Table 1, revealed that the maximum penetration depth of dead bacteria in the coronal third was significantly greater than that in the middle third ($p < 0.05$).

The efficacy of various irrigants in disinfecting dentinal tubules was also assessed and is summarized in Table 2. The control group exhibited significantly lower disinfection efficacy compared to all other solutions. The combination groups

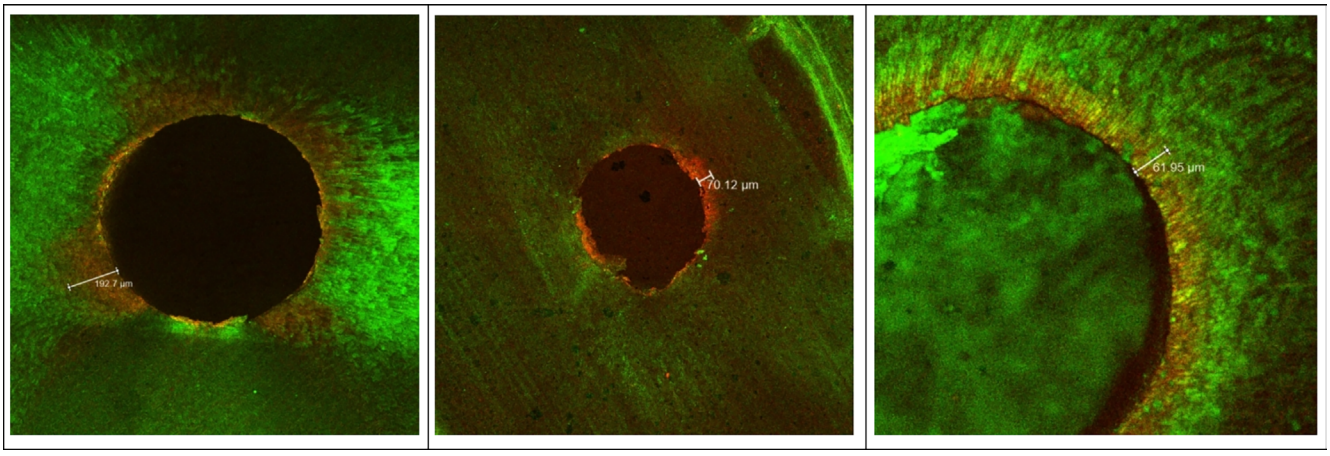


FIGURE 1 | Measurements of dead bacterial penetration by the distance from the canal lumen to the endpoint of the red fluorescence in the dentinal tubules.

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TABLE 1 | The maximum dead bacterial penetration values in the apical, middle, and coronal thirds of the sample teeth.

	Root canal region				Post Hoc analysis results		
	Apical ($n=60$)	Middle ($n=60$)	Coronal ($n=60$)	p[a]	p^{1-2}	p^{1-3}	p^{2-3}
7 days	39.4 (28.14–65.03)	28.92 (20.6–53.3)	45.61 (28.37–68.86)	**0.028**	0.127	>0.999	**0.035**

Note: The corresponding values used in the presentation of post hoc analysis results are as follows. 1: apical, 2: middle, 3: coronal.
[a]Kruskal–Wallis *H* Test, post hoc Bonferroni correction, and Med(IQR).

TABLE 2 | Bacterial elimination values according to the irrigation solutions used.

	Irrigation solutions						
Biofilm	Distilled water ($n=10$)	NaOCl ($n=10$)	CHX ($n=10$)	PHMB ($n=10$)	NaOCl + CHX ($n=10$)	NaOCl + PHMB ($n=10$)	Post Hoc analysis results
7 days	18.06 (14.1–25.21)[a]	97.35 (44.19–192.7)[A]	41.77 (29.8–62.8)[A]	31.45 (25.1–46.6)[A,b]	57.57 (37.47–70.12)[A,B]	36.08 (29.03–51.28)[A,B]	**1 < 2, 3, 4, 5, 6** **4 < 5, 6**

Note: Different superscript letters indicate a significant difference among the groups created on the basis of the irrigation protocol ($p<0.001$). The corresponding values used in the presentation of Post Hoc analysis results are as follows. 1: Distilled water, 2: NaOCl, 3: CHX, 4: PHMB, 5: NaOCl + CHX, 6: NaOCl + PHMB.

TABLE 3 | Log reduction of viable bacterial cell number in biofilms with different irrigation solutions.

	CFU before irrigation	CFU after irrigation	Log reduction
Distilled water	1.3×10^7	3×10^6	0.64
NaOCl	5×10^6	1.16×10^4	2.63
CHX	1.9×10^7	3×10^2	4.8
PHMB	2.6×10^7	4.8×10^2	4.73
NaOCl + CHX	3.2×10^7	1×10^2	5.50
NaOCl + PHMB	2.6×10^7	1×10^2	5.41

demonstrated significantly greater disinfection efficacy than the sole use of solutions. Although the NaOCl group showed superior performance compared with the CHX and PHMB groups, the differences were not significant ($p>0.05$).

3.1 | Bacterial Sampling and Viable Count Analyses

Bacterial sampling and analysis showed that the combined use of the irrigants reduced the number of viable bacteria by more than five logs, which is consistent with the CLSM result, whereas CHX alone showed a reduction of almost five logs, which is inconsistent with the CLSM result. Distilled water has less than one log reduction in viable bacteria, which is also consistent with the CLSM result (Table 3 and Figure 2).

4 | Discussion

This study compared three different irrigants and their combinations to eliminate bacteria in root canals. It utilized a novel method for analyzing CLSM images, enabling the quantification of dead bacteria penetration within the dentinal tubules at different portions of the root canal. The in vivo infected dentine model adapted from the Haapasalo and Ørstavik (1987)'s design with modifications was employed to assess the antibacterial efficacy of root canal disinfectants, medicaments, and sealers. In this study, clinical settings were replicated by using human teeth rather than bovine teeth, and the teeth were cultured with bacteria in a liquid medium to mimic the oral environment.

In this study, three bacterial species were used in biofilm formation: *E. faecalis*, known for its deep penetration into dentin tubules and association with persistent infections; *S. mutans* for its ability to initiate biofilm formation and cause caries, a major cause of pulpal necrosis; and *S. aureus* for its resistance to antibacterial agents and association with chronic infections (Zan et al. 2015).

Literature reports biofilm incubation period up to 70 days (Swimberghe et al. 2019). In this study, biofilm was formed for 7 days. Previous studies (Halford et al. 2012; Kasić et al. 2017; Shrestha and Kishen 2012) that used a 7-day inoculation period demonstrated in scanning electron microscope (SEM) analysis that this duration is sufficient for biofilm formation and for comparing the antimicrobial efficacy of agents. Shen et al. (Shen et al. 2011) propose that a mature biofilm demonstrates higher resistance to antimicrobial treatments, making it a better model

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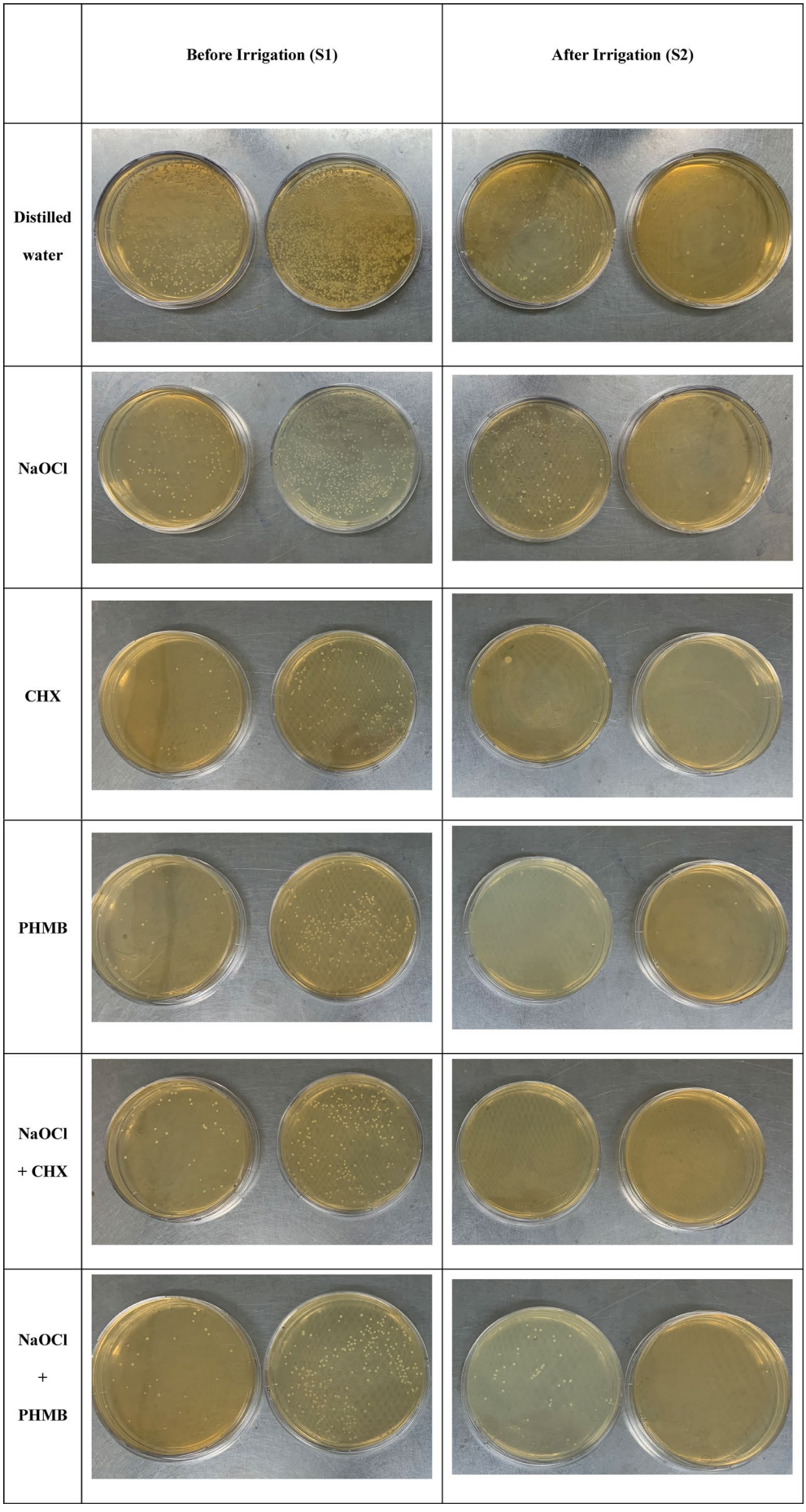


FIGURE 2 | Legend on next page.

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FIGURE 2 | Viable bacterial counts were determined using brain heart infusion agar plates incubated at 37°C for 48 h. The S1 column shows bacterial dilutions of 10^{-4} and 10^{-5}, whereas the S2 column represents bacterial dilutions of 10^{0} and 10^{-1}, respectively, following the irrigation procedures.

for reflecting clinical conditions. Nevertheless, prolonging the growth period still results in an in vitro model that remains quite different from real clinical environments. Although there is no established consensus on the ideal inoculation time for intra-articular biofilm studies, our research found that a 7-day incubation period was sufficient to assess the efficacy of various antimicrobial agents.

The coronal third of the root canal showed significantly more disinfection compared with the middle third ($p < 0.05$) (Figure 3). This effect can be attributed to the increased number and diameter of dentinal tubules in the coronal third (Mjör et al. 2001). The higher disinfection in the apical third relative to the middle third may be explained by syringe irrigation. The placement of the syringe tip 2 mm short of the apex, combined with the elevated flow rate of the ejected irrigant, likely enhanced disinfection in the apical region (Boutsioukis et al. 2010). Variations in the acquired results compared to other studies may be due to the use of different tooth types and varying canal diameters.

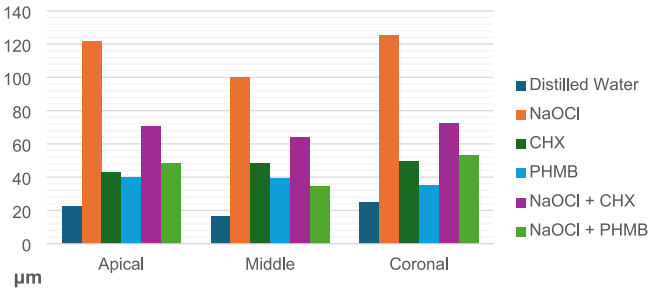


FIGURE 3 | Assessing the disinfection efficacy of various irrigation solutions in eliminating microbial biofilms, applied to different anatomical regions of the tooth.

The study compared the disinfection of dentinal tubules using various irrigants, finding that the control group had significantly lower disinfection than all other irrigants ($p < 0.05$). Maximum dead bacteria penetration was observed with NaOCl, followed by CHX and PHMB, but the differences were not significant (Figure 4). Although Koburger et al. (2010) reported that PHMB exhibits greater antibacterial activity than CHX, Dammaschke et al. (2013) and Zaugg et al. (2014) reported that CHX is more effective than PHMB. These discrepancies may arise from variations in PHMB concentrations and purity across studies, as well as differences in experimental methodologies. PHMB is available in various commercial formulations, often combined with additives such as macrogolum and undecylenamidopropyl betaine. These substances act as surfactants, reducing liquid surface tension and promoting better spread, which is believed to enhance PHMB's antimicrobial effectiveness and biocompatibility (Alblas et al. 2011; Wiegand et al. 2017). In this study, the preparation of a solution from powdered PHMB may have contributed to the observed differences.

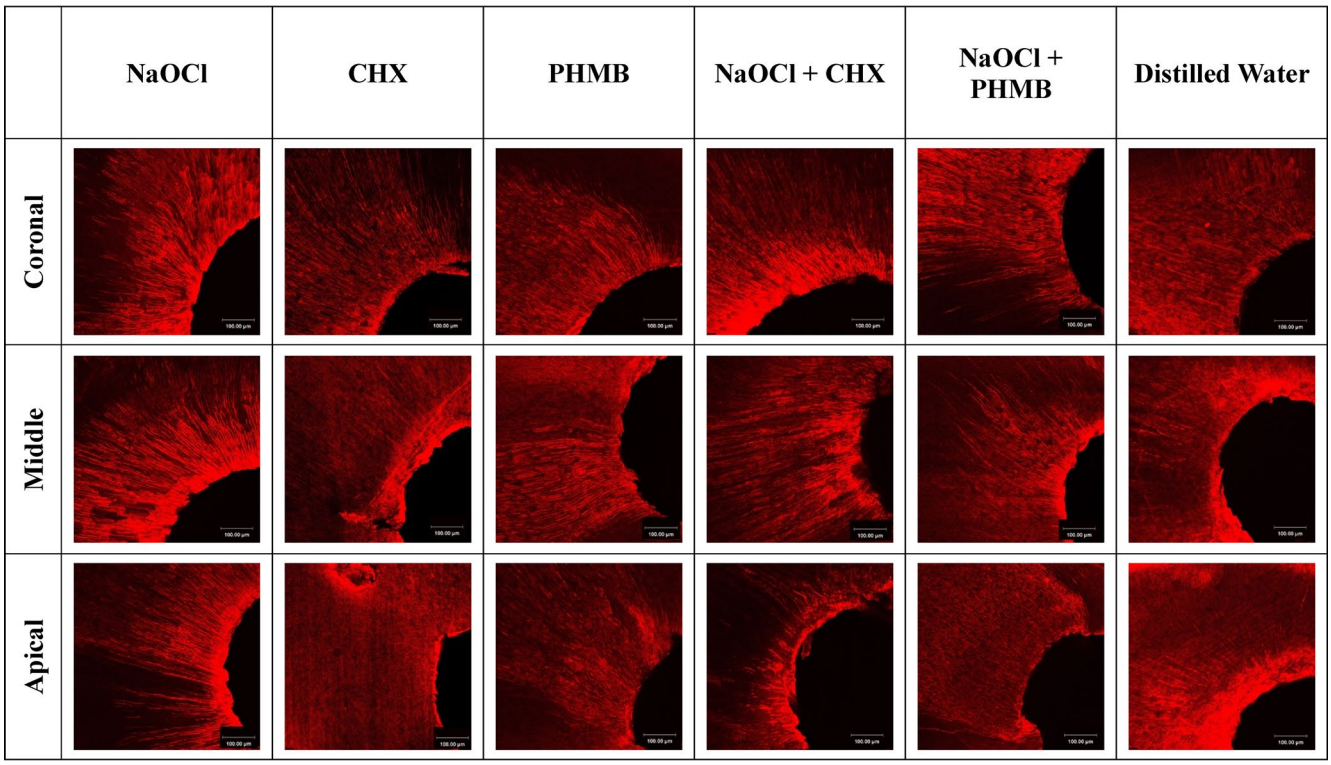


FIGURE 4 | Red fluorescence penetration that demonstrates dead bacterial penetration in dentinal tubules within various irrigation solutions.

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The combined use of NaOCl and CHX was significantly more effective than the use of PHMB alone ($p < 0.05$). Similar to our result, Kuruvilla and Kamath (1998) showed that the combination of NaOCl and CHX was more effective compared with their individual use. The combined use of solutions enhances disinfection by leveraging the organic solvent properties of NaOCls along with the antibacterial effects of CHX and PHMB. Notably, no prior studies have examined the combined use of NaOCl and PHMB.

It is well established that NaOCl can react with CHX, leading to the formation of an orange–brown precipitate. This precipitate is recognized for its carcinogenic properties and its potential to obstruct dentinal tubules, which may interfere with the penetration of sealers (Metri et al. 2015). Previous studies in the literature (Bui et al. 2008; Prado et al. 2013) recommend using distilled water, EDTA, or alcohol between irrigants to prevent this occurrence. Although EDTA is often suggested, the risk of precipitate formation with CHX remains (González-López et al. 2006). Therefore, in this study, distilled water was used between irrigants to minimize the potential for precipitate formation.

When irrigation solutions are used alone, the antibacterial effects of irrigants have been found to differ between CLSM and bacterial viable counting. This discrepancy may be due to the presence of dentine components. Previous studies have also reported that NaOCl exhibits enhanced antibacterial efficacy in the presence of dentine, whereas CHX demonstrates reduced effectiveness under similar conditions (Habelitz et al. 2002; Schmalz et al. 2004). Also, this discrepancy may be due to surfaces stained with SYTO 9 and PI, which do not always clearly differentiate between bacterial viability, often displaying an intermediate cellular form instead (Zotta et al. 2012). Microbiological sampling techniques are effective for estimating the amount of CFU; however, they do not provide detailed information on the placement of bacteria within dentin (Miller 2015). In contrast, CLSM can identify bacterial viability and biofilm distribution within dentinal tubules (Swimberghe et al. 2019).

The null hypothesis was rejected as there was a difference in the penetration of dead bacteria in teeth with mixed biofilm irrigated with different irrigants. The limitation of this study was the use of human teeth without considering the age group and the sample preparation process, which involved cutting with a disc under water cooling.

5 | Conclusion

This study found that irrigants had varying effects on dentinal tubule disinfection, and no solution completely eliminated bacterial penetration. Within the limitations of this study, the combined use of irrigants was more effective than using a single irrigant, making it the preferred method for final irrigation in clinical practice. CLSM imaging with fluorescent dyes was found to be more effective compared with viable bacterial counting for detecting both dead and live bacteria, but using both approaches is important for validating the method. Further studies are needed to investigate the effects of the PHMB solution on dentinal tubule disinfection.

Author Contributions

Dide Tekinarslan: conceptualization, investigation, writing – original draft, methodology, visualization, formal analysis, data curation, project administration, writing – review and editing. **Kürşat Er:** conceptualization, methodology, validation, supervision, resources, writing – review and editing, project administration. **Mine Eğin:** writing – review and editing, investigation, validation, supervision, data curation, formal analysis, resources. **Tuba Dinçer:** software, writing – review and editing, validation, supervision, resources, project administration. **Ali Osman Kiliç:** supervision, validation, writing – review and editing, resources, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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