

Comparative evaluation of the concentration-dependent effect of proton-pump inhibitor in association with calcium hydroxide and chlorhexidine on *Enterococcus faecalis*: An *in vitro* study

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Abstract

Background: *Enterococcus faecalis* is the most persistent organism in the root canal which resists most of the intracanal medicaments. There is always a constant attempt to eliminate this endodontic pathogen from the root canal system.

Aim: The aim of this study was to evaluate the efficacy of the association of different concentrations of proton-pump inhibitor (PPI) (Lansoprazole) with calcium hydroxide (CH) and chlorhexidine (CHX) against *E. faecalis* using a broth dilution method.

Materials and Methods: *E. faecalis* was inoculated into brain–heart infusion broth at 37°C for 5 h. The master broth was then treated with CH (Group 1); CH + 2% CHX (Group 2); CH + PPI 6.25 µg/ml (Group 3A); CH + PPI 25 µg/ml (Group 3B); 2% CHX + PPI 6.25 µg/ml (Group 4A); 2% CHX + PPI 25 µg/ml (Group 4B); CH + 2% CHX + PPI 6.25 µg/ml (Group 5A), and CH + 2% CHX + PPI 25 µg/ml (Group 5B). The groups were spectrophotometrically analyzed at 630 nm at 24 h to determine the group with the least optical density.

Statistical Analysis: Comparison between the groups was done by the one-way analysis of variance and Kruskal–Wallis test for multiple comparisons.

Results: The mean percentage inhibition of *E. faecalis* by Group 5A (CH + 2% CHX + PPI 6.25 µg/ml) was the highest compared to other groups. The lowest mean value was observed in Group 3A (CH + PPI 6.25 µg/ml) indicating least efficiency.

Conclusion: There was a concentration-dependent effect of PPI on CH and CHX against *E. faecalis*. The maximum efficacy was found when the lower concentration of PPI was associated with CH/CHX mixture.

Keywords: Calcium hydroxide, chlorhexidine, *Enterococcus faecalis*, intracanal medicaments, proton-pump inhibitors, root canal, spectrophotometry

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INTRODUCTION

Enterococci are Gram-positive, facultative anaerobic cocci, which are resilient by nature and able to survive a wide array of hostile conditions.^[1] *Enterococcus faecalis*, the predominant human *Enterococcus*, is not a normal commensal in the oral cavity; however, it can be conceived directly or indirectly based on the oral status. *E. faecalis* can be commonly retrieved from dorsum of the tongue (55%), gingival sulcus (22%) and oral rinse samples (29%).^[2] It can get incorporated into the oral cavity by food contaminants and also by nosocomial infections. *E. faecalis* has also been related to various oral diseases, such as caries, endodontic infections, chronic periodontitis (41.7%)^[3] and peri-implantitis. It is a persistent organism that plays a major role in the etiology of secondary periradicular lesions after the root canal treatment. Its prevalence ranges from 4% to 40% in primary endodontic infections^[4] and 24% to 77% in persistent and secondary infections.^[4,5] The eradication of *E. faecalis* is challenging in endodontically treated teeth because of its inherent ability to tolerate starvation, extremes of pH, salt concentration, biofilm formation, dentin tubular invasion and emergence of antibiotic resistant strains.^[6,7]

Intracanal medications have a long history of use as interim appointment dressings to reduce the microbial load. Calcium hydroxide (CH) is the most common and widely used intracanal medicament. It has an initial pH of approximately 12, but these values do not commonly exceed 11 throughout the entire length of the canal, especially in the tubules, because of the dentin buffering capacity.^[8,9] According to Evans *et al.*,^[10] when the challenge is to survive in an alkaline medium, as in the presence of CH dressings, the main tactic of *E. faecalis* is to use an existing functional proton pump in their cell membrane that is capable of maintaining the homeostasis of the cytoplasm, even in a pH around 11.5. Proton pump inhibitors (PPIs) are used widely in the treatment of peptic ulcers of microbial origin along with antibiotics. The effect of incorporating PPIs with CH has been widely studied.

Chlorhexidine (CHX) gluconate (2%) has been recommended as a potential alternative to CH. In the past, many studies have been done regarding the efficacy of CH and CHX mixture and its antibacterial property and have emphasized the concept that their antimicrobial properties interact in a synergistic fashion which enhances their efficacy.^[11] The tissue reactions to the mixture of CH/CHX have also been studied, and it is found that the combination exerts good antimicrobial properties and improves healing of the periapical tissues.^[12]

The majority of these studies have been carried out using culturing techniques; however, polymerase chain reaction is currently a more accurate method for the detection of *E. faecalis*.^[13] An optical spectroscopy-based chairside method has also been studied to rapidly monitor the presence or absence of *E. faecalis* in the root canal system.^[14]

The aim of this study was to evaluate the efficacy of the association of different concentrations of PPI (Lansoprazole) with CH and CHX against *E. faecalis* using a broth dilution method. The null hypothesis states that there is no concentration-dependent effect of the PPI lansoprazole in association with CH and CHX on the inhibition of *E. faecalis*.

MATERIALS AND METHODS

Preparation of the test organism

Pure culture of *E. faecalis* (ATCC 29212) was used as the test organism and was incubated in brain–heart infusion (BHI) broth. Bacterial growth of this master broth (MB) was confirmed by the presence of turbidity. Ten milliliters of the sterilized BHI broth were inoculated with 500 μ l of *E. faecalis* from the MB in test tubes and incubated at 37°C for 5 h. The growth of bacteria was confirmed by the turbidity of the broth.

Preparation of the stock solution

CH powder (Prevest Denpro Ltd., Jammu, India) was mixed with deionized water to attain a concentration of 16 mg/ml.^[15] pH of the prepared CH was determined using a pH meter as 12.5 (Oakton Pvt. Ltd., Hyderabad, Telangana, India). PPI lansoprazole powder (Prevacid, Takeda Pharmaceuticals U. S. A.) was mixed with deionized water at a concentration of 1 mg/ml and dilutions made at 6.25 and 25 μ g/ml.^[16,17] CHX 2% solution (Anabond Asep-RC) was used.

Antibacterial assessment

Tubes were divided into five main groups. Groups 3, 4, and 5 were further subgrouped into “A” and “B” based on the concentration of lansoprazole at 6.25 μ g/ml and 25 μ g/ml, respectively, as listed below.

- Group 1: CH alone
- Group 2: CH + 2% CHX
- Group 3A: CH + Lansoprazole 6.25 μ g/ml
- Group 3B: CH + Lansoprazole 25 μ g/ml
- Group 4A: 2% CHX + Lansoprazole 6.25 μ g/ml
- Group 4B: 2% CHX + Lansoprazole 25 μ g/ml
- Group 5A: CH + 2% CHX + Lansoprazole 6.25 μ g/ml
- Group 5B: CH + 2% CHX + Lansoprazole 25 μ g/ml.

200 μ L of CH, 200 μ L of CH with 200 μ L of CHX and 200 μ L CH with lansoprazole in the two dilutions as

described were added to experimental groups yielding a total volume of 10 ml. Furthermore, reference blanks^[18] for each group were prepared. The schematic diagram of the preparation of the experimental groups is depicted in Figure 1.

Entire procedure was carried out in a biosafety hood (Class II Biohazard safety cabinet, ESCO, Singapore) maintaining sterilization and disinfection. 500 µl of *E. faecalis* was again incubated in 10 ml of sterile BHI broth to obtain MB and this served as the negative control. The MB along with the tubes (experimental groups) was incubated at 37°C for 24 h. The tubes (experimental groups, reference blanks of each groups and MB) were then evaluated for optical density (OD) at 630 nm after 24 h using ultraviolet visible Spectrophotometer (Specord 50 Plus, Analytikjena, Jena, Germany). Three readings of the experimental groups were taken corresponding to the reference blanks. Three readings of the MB were also taken to obtain its OD.

The percentage inhibition of growth was calculated as per Wang et al.^[19]

Percentage (%) inhibition

$$= \frac{(OD)_{MB} - (OD)_{Test}}{(OD)_{MB}} \times 100\%$$

Where, (OD)_{MB} was an average of three replicates of light absorption values at wavelength 630 nm of the control, and (OD)_{Test} was an average of three replicates of light absorption values at wavelength 630 nm of the experimental groups.

Statistical analysis

Comparison between the groups was done by the one-way analysis of variance and Kruskal–Wallis test for multiple comparisons. The level of significance (P) was set at 0.05.

RESULTS

The null hypothesis was rejected as there was a definite concentration-dependent effect of the PPIs on the growth inhibition of *E. faecalis* when associated with CH and CHX.

The mean percentage inhibition of Group 5A (CH + 2% CHX + lansoprazole 6.25 µg/ml) is observed to be 96.03% ± 0.60% which indicates the highest efficiency compared to all other groups, followed by Group 2 (CH + 2% CHX) with a mean of 88.44% ± 0.24% and Group 4A (2% CHX + lansoprazole 6.25 µg/ml) with a mean of 80.61% ± 0.49%. The lowest mean value of 14.74% ± 0.24% is observed in Group 3A (CH + lansoprazole 6.25 µg/ml) indicating

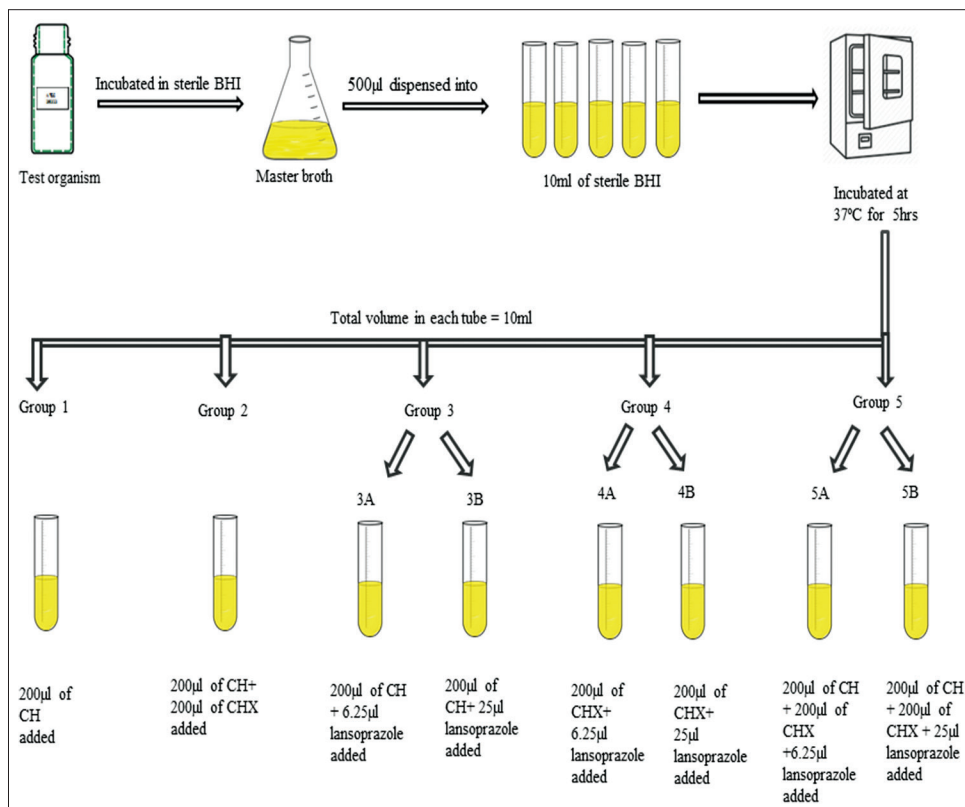


Figure 1: (Original): Schematic diagram showing the preparation of the experimental groups

least efficiency. Kruskal–Wallis test at 0.05 significance level shows that the difference in the values is statistically significant with $P = 0.002$ [Table 1].

The pairwise comparison of the groups is depicted in Table 2. It is observed that the significant difference in terms of mean percentage zone of inhibition exists between Group 3A and Group 5A ($P < 0.001$). Group 5A is also found to be significantly different from Group 4B ($P = 0.002$), Group 1 ($P = 0.009$) and Group 5B ($P = 0.038$). Group 3A is also found to be significantly different from Group 2 ($P = 0.002$), Group 4A ($P = 0.009$) and Group 3B ($P = 0.038$).

DISCUSSION

The oral cavity may serve as a reservoir for the bacterial pathogens of medical importance such as *Enterococci* in systemically healthy or diseased participants. Oral *E. faecalis* possess virulence factors that may contribute to the pathogenesis of apical periodontitis and failed root canal treatment. The success of the root canal treatment entirely relies on the complete elimination of microorganisms from the root canal anatomy. Intracanal medicaments by virtue of their longer contact time with the microorganisms can effectively eliminate these pathogens from the root canal system. CH is the most commonly used intracanal medicament. The antimicrobial effect of CH is due to the release of hydroxyl ions, a highly reactive oxidative species in an aqueous environment which diffuse into the dentinal tubules.^[20] However,

inside the root canal pH of CH is never maintained. It is because of the buffering capacity of dentin due to the presence of proton donors such as $H_2PO_4^-$, H_2CO_3 , and HCO_3^- from hydroxyapatite.^[21]

CHX (2%) have been found to have increased antimicrobial activity when compared with CH.^[22] CHX exhibits its antimicrobial activity by the interaction of the positive charge of the molecule and the negatively charged phosphate groups on the microbial cell walls,^[23] thereby altering the cells osmotic equilibrium. This increases the permeability of the cell wall, which allows the CHX molecule to penetrate into the bacterial cell. Combined use of 2% CHX with CH was found to be more effective in the elimination of *E. faecalis* than CH in water.^[24,25]

Various reasons have been proposed for the resistance of *E. faecalis* with CH, out of which the most relevant seems to be the presence of a proton pump, which lowers the cytoplasmic pH by pumping protons into the cell.^[10] PPIs are commonly used in association with antibiotics to treat peptic ulcer disease of microbial origin (in the presence of *Helicobacter pylori*). These cause an irreversible inhibition of the H^+/K^+ ATPase in the parietal cells of the stomach leading to reduced gastric acid secretion, and thus are a drug of choice for gastric/duodenal ulcers and gastroesophageal reflux diseases. They increase the efficacy of antibiotics by decreasing the intragastric acidity.^[26] Lansoprazole represents the new generation of PPIs used extensively in medicine for gastric disorders.

Table 1: Comparison of the mean percentage inhibition among the groups

	n	Percentage inhibition				Kruskal-Wallis test, P
		Mean percentage inhibition (%)	SD (%)	Minimum (%)	Maximum (%)	
Group 1	3	71.39	0.26	71.20	71.69	0.002*
Group 2	3	88.44	0.24	88.17	88.58	
Group 3A	3	14.74	1.43	13.27	16.13	
Group 3B	3	76.58	0.86	75.93	77.56	
Group 4A	3	80.61	0.49	80.09	81.07	
Group 4B	3	50.86	0.65	50.23	51.54	
Group 5A	3	96.03	0.60	95	96	
Group 5B	3	72.94	0.95	72.10	73.97	

*Significant. SD: Standard deviation

Table 2: Kruskal-Wallis pairwise comparisons of the groups with statistically significant differences in mean percent zone of inhibition at 0.05 significance level

Pairwise groups	1	2	3A	3B	4A	4B	5A	5B
2	0.038, S	-	-	-	-	-	-	-
3A	0.299, NS	0.002, S	-	-	-	-	-	-
3B	0.299, NS	0.299, NS	0.038, S	-	-	-	-	-
4A	0.119, NS	0.603, NS	0.603, NS	0.603, NS	-	-	-	-
4B	0.603, NS	0.009, S	0.603, NS	0.119, NS	0.038, S	-	-	-
5A	0.009, S	0.603, NS	$P < 0.001$, S	0.119, NS	0.299, NS	0.002, S	-	-
5B	0.603, NS	0.119, NS	0.119, NS	0.603, NS	0.299, NS	0.299, NS	0.038, S	0.038, S

S: Significant, NS: Nonsignificant

In dental literature, Wagner *et al.*^[27] were the first to attempt eradication of *E. faecalis* using CH supplemented with PPI omeprazole, who used a rat model of periapical lesion and compared it to conventional CH intracanal dressing. They obtained superior healing rates with CH supplemented with omeprazole than conventional CH dressing. Ganesh *et al.*^[16] reported concentration dependent effect of PPI (lansoprazole) with CH against *E. faecalis*. Mehta *et al.*^[28] reported that PPI enhanced the antibacterial efficacy of CH against *E. faecalis* using microdilution assay; however, variation in the concentration of omeprazole did not affect the result. Kar *et al.*^[29] also reported that the antimicrobial efficacy of omeprazole (PPI) combined with CH showed the maximum zone of inhibition against *E. faecalis*. The studies that contradicts the result of this study are by Mitthra *et al.*^[30] and Suresh *et al.*^[31] who demonstrated an *in vitro* agar diffusion assay that showed that the addition of PPI (Pantoprazole) to CH did not enhance the antimicrobial efficacy of CH when compared to CHX. The variation in results may be due to the difference in the tested drugs and methodology used.

The qualitative and quantitative applications of absorption spectrometry make use of the Beer Lambert's law and display characteristic absorption spectrum for molecular identification.^[32] The optical spectroscopic analysis of *E. faecalis* suspension have shown a conspicuous increase in absorbance intensity at spectral range from 600 to 654 nm,^[14] and hence, 630 nm is chosen to determine the OD in this broth dilution study.

In the present study, the maximum *E. faecalis* growth inhibition was shown by Group 5A (CH + 2% CHX + lansoprazole 6.25 µg/ml), followed by Group 2 (CH + 2% CHX) and Group 4A (2% CHX + lansoprazole 6.25 µg/ml). The least inhibition was observed in Group 3A (CH + lansoprazole 6.25 µg/ml). The expected mechanism of action favoring enhanced action in Group 5A is PPI blocks the proton pump in the cell wall of *E. faecalis*. This blocking makes the CH and CHX readily available for action directly over the organism, thereby resulting in enhanced elimination of *E. faecalis*. The groups containing lansoprazole at a concentration of 6.25 µg/ml (Group 4A and 5A) have exhibited extremely good results when associated with CHX. This better result can be attributed to the property of substantivity of CHX. However, when associated with CH alone, high concentration of lansoprazole (25 µg/ml) seems to be effective against *E. faecalis*.

The exact mechanism of concentration-dependent effect of PPIs on CH/CHX mixture is not clearly understood.

Further studies are needed to evaluate the chemical interaction between lansoprazole, CH and CHX and its antibacterial efficacy against *E. faecalis* at various concentrations and also *in vivo* conditions.

CONCLUSION

Within the limitations of this *in vitro* study, it can be concluded that there was a concentration dependent effect of PPI (Lansoprazole) on CH and CHX against *E. faecalis*. The maximum efficacy was found when the lower concentration of PPI was associated with CH/CHX mixture.

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Conflicts of interest

There are no conflicts of interest.

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