Comparative Evaluation of Antimicrobial Efficacy of Calcium Hydroxide, Chitosan, Chlorhexidine and Their Combinations Against *Enterococcus Fecalis* and *Candida Albicans* Using Quantitative Real Time Polymerase Chain Reaction – An In Vitro Study

Archana Srinivasan*, Srirekha A, Kusum Bashetty, Teja Deepthi C, Ashis Mohanty and Ashwija Shetty

Department of Conservative dentistry & Endodontics, The Oxford Dental College and Hospital, Karnataka, India

Submission: September 27, 2019; Published: October 31, 2019

*Corresponding author: Archana Srinivasan, Department of Conservative dentistry & Endodontics, The Oxford Dental College and Hospital, Karnataka, India

Abstract

Objectives: To comparatively evaluate the antimicrobial efficacy and synergistic effect of calcium hydroxide, chlorhexidine gluconate, chitosan and their combinations against *E. fecalis* and using Quantitative real time PCR.

Materials and Method: 120 human extracted single rooted maxillary premolar teeth were selected. Access cavity preparation was done followed by cleaning and shaping. Middle third of the root was cut using a rotary diamond disc. The specimens were inoculated with *E. faecalis* and *Candida Albicans* for 14 days and divided into two groups with sixty teeth each

- Group 1: *E. Fecalis*.
- Group 2: *Candida Albicans*.

This was followed by placement of Intracanal medicament and the two groups were further subdivided into six subgroups with ten teeth each.

- Group 1 & 2 A: 2%Chlorhexidine.
- Group 1 & 2 B: Calcium Hydroxide and 2% Chitosan.
- Group 1 & 2 C: 2%Chitosan and 2%Chlorhexidine.
- Group 1 & 2 D: 2%Chitosan.
- Group 1 & 2 E: Calcium Hydroxide.
- Group 1 & 2 F: Control.

The dentinal chips were collected, following which DNA isolation was done. The specimens were analyzed using real-time PCR.

Result: In the *E. Fecalis* group, ANOVA test statistical analysis showed significant difference in the quantity between subgroup 1C and subgroup 1F (P>0.05), whereas, in the *Candida Albicans* group, Kruskal Alli’s test showed a statistically significant difference between subgroup 2C and subgroup 2F (P<0.02).

Conclusion: Combination of 2% Chlorhexidine and 2% Chitosan are the most effective intracanal medicament against Enterococcus Fecalis and Candida Albicans.

Clinical significance: 2% Chlorhexidine and 2% Chitosan, as intracanal medicament, due to their probable synergistic antimicrobial effect against *E. Faecalis* and *C. Albicans*, can show promising results in reducing the endodontic treatment failure cases.

Keywords: *E. Faecalis, C. Albicans*; Polymerase Chain Reaction; Chlorhexidine; Chitosan; Calcium Hydroxide
Introduction

Bacterial invasion of root canal system is crucial for the onset and maintenance of periapical diseases. Hence, one of the goals of the endodontic treatment is to kill micro-organisms in the root canal system. Although, biomechanical preparation and root canal shaping effectively reduces the microbiota, these procedures do not completely eliminate bacteria in the lateral and accessory root canals, isthmus, and apical deltas. Thus, intracanal medication between appointments is recommended to further reduce bacteria in the root canal system. *Candida albicans* is the most common species of fungi cultured from root canals of teeth with failed endodontic treatment [1]. Because of collagenolytic activity, it may be possible for the yeast to use dentin as a nutrient source and promote colonization in the root canal. Another organism commonly found in cases of failed endodontic infections and endodontic flare-ups is Enterococcus faecalis. It has the ability to survive in root canal system as a single organism without the support of other bacteria and is small enough to proficiently invade and live within the dentinal tubules [1]. Calcium hydroxide is one of the most commonly used substances in Endodontics and its antibacterial property stems from its ability to increase a solution’s pH. Chlorhexidine has emerged as an intracanal medicament because of its antimicrobial spectrum, its ability to maintain its antibacterial property stems from its ability to increase a solution’s pH. Chlorhexidine has emerged as an intracanal medicament because of its antimicrobial spectrum, its ability to maintain its antibacterial action for a prolonged duration when adhered to anionic substrates and its slow release as its concentration decreases. Chitosan is a natural polysaccharide comprising of copolymers of glucosamine and N-acetyl glucosamine.

In endodontics its role as antifungal and antibacterial agent has not been subjected to adequate scrutiny. There is increasing scientific evidence regarding the role of *C. albicans* and *E. faecalis* particularly as members of secondary or persistent infections associated with failed endodontic therapy and their resistance against commonly used intracanal medicaments [2]. PCR assays are very sensitive and enable rapid identification of microbial species and strains that are difficult or even impossible to culture. Also, in cases with small number of bacteria, negative culture may result, even though viable bacteria may be present because of low sensitivity of culturing technique [3]. Thus, a more sophisticated and sensitive molecular technique like PCR was used to assess the effect of antimicrobial agents against *E. faecalis* [4].

Materials and Methods

Preparation of Dentine Specimens

The model proposed by Haapasalo & Ørstavik was modified [5]. One hundred and twenty single-rooted human maxillary premolar teeth freshly extracted for orthodontic reasons were selected. A rotary diamond disc was used to decorate the teeth below the cementoenamel junction and the apical part of the root to obtain 6 mm of the middle third of the root. Gates Glidden drills no. 3 (Mani Inc, Tachigi-ken, Japan) in a slow-speed handpiece was used to remove 6 mm of the middle third of the root. Gates Glidden drills no. 6 were used to locate the cementoenamel junction and the apical part of the root to obtain 6 mm. A rotary diamond disc was used to decoronate the teeth below the root. Molar teeth freshly extracted for orthodontic reasons were selected [5]. One hundred and twenty single-rooted human maxillary premolar teeth were divided into 60 teeth each for Group 1 & 2 A: 2% Chlorhexidine.
b. Group 1 & 2 B: Calcium Hydroxide and 2% Chitosan.
c. Group 1 & 2 C: 2% Chitosan and 2% Chlorhexidine.
d. Group 1 & 2 D: 2% Chitosan.
e. Group 1 & 2 E: Calcium Hydroxide.
f. Group 1 & 2 F: Control.

Teeth after placement of intracanal medicament were sealed with a temporary restorative material and incubated in an aerobic environment at 37 degrees celsius. An antimicrobial assessment was performed at the end of 14 days after placing intracanal medicament using quantitative real time PCR.

Antimicrobial Assesment

At the end of 14th day, the blocks were irrigated with 5 mL of sterile water to remove the inoculated broth. 120 sample teeth were divided into 60 teeth each for *E. Faecalis* and *Candida Albi- cans*. This was followed by placement of Intracanal medicament and the two groups were further subdivided into six subgroups with ten teeth each.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Materials and Medicaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>Group 1 &amp; 2 A: 2% Chlorhexidine</td>
</tr>
<tr>
<td>b.</td>
<td>Group 1 &amp; 2 B: Calcium Hydroxide and 2% Chitosan</td>
</tr>
<tr>
<td>c.</td>
<td>Group 1 &amp; 2 C: 2% Chitosan and 2% Chlorhexidine</td>
</tr>
<tr>
<td>d.</td>
<td>Group 1 &amp; 2 D: 2% Chitosan</td>
</tr>
<tr>
<td>e.</td>
<td>Group 1 &amp; 2 E: Calcium Hydroxide</td>
</tr>
<tr>
<td>f.</td>
<td>Group 1 &amp; 2 F: Control</td>
</tr>
</tbody>
</table>

Extraction of RNA

All the plastic wares and glass wares used for this experiment were made RNase free by treating with DEPC for overnight and autoclaving twice at 121 °C until the traces of DEPC removed. These were then dried in Hot Air Oven at 90 °C before use. The samples were vortexed, the dentine samples were taken out, the broth was centrifuged, and the pellet was collected for the study. The pellet was suspended in 0.5 ml of Trizol Reagent (Takara Bio Inc, Japan),...
mixed, transferred to a 2ml microfuge tube and incubated at room temperature for 15 – 20 mins. It was then centrifuged at 10,000 rpm (Sigma) for 5 min and the supernatant was transferred to a fresh sterile microfuge tube. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed gently and incubated in ice for 2 minutes. After centrifuging the above at 12,000 rpm for 5 minutes the supernatant was transferred to a fresh vial and then incubated in ice for 2 minutes after adding 500 μl of isopropanol. The RNA was pelleted down by centrifuging the above at 10,000 rpm for 10 minutes and the pellet was vortexed gently with 300 μl of 70% of ethanol. Centrifugation at 10,000 rpm was repeated for 10 minutes and the pellet was air dried. 20μl of RNase free water was added to the pellet and heated gently (60 °C) to dissolve the pellet in water.

**DNase Treatment**

The extracted RNA was treated with DNase enzyme to remove any traces of DNA contamination. One micro liter of DNase was added to a above isolated RNA and incubated for 1hr at 37 °C and after the temperature was raised to 70 °C for 5 minutes to inactivate the enzyme. The RNA was stored at -20 °C for later use.

**RNA Quantification**

The concentration and purity of RNA was assessed using a spectrophotometer (Sartorius). A 1μl aliquot of RNA was pipetted onto the apparatus pedestal. RNA with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2 was deemed indicative of pure RNA.

**cDNA Synthesis**

After quantification, RNA was reverse transcribed using oligo dT (Sigma Aldrich). Hundred nano gram of RNA was aliquoted to a fresh sterile microfuge tube and 2μl of oligo dT was added and incubated at 70 °C for 5 minutes and immediately transferred to ice. To this 2μl of dNTPs, 1μl of Reverse Transcriptase enzyme (Bioline, New England) and 2 μl of 10x Reverse transcriptase buffer were added and made up the volume to 25μl using RNase free water. This mixture was incubated at 42 °C for 90 minutes and reaction was terminated by incubating at 70 °C for 15 minutes.

**Real-time PCR (qPCR)**

Relative Quantification using Real Time PCR. The quantification was carried out in Applied Biosystems Step One Real Time PCR using the SYBR Green Chemistry.

**Statistical Analysis**

Since the the *E. Faecalis* group sample was normally distributed, parametric test ANOVA was applied for the comparison between the groups, followed by Tukey multiple comparisons means to check the difference in bacterial inhibition between groups (P < 0.05). For the *Candida Albicans* group since the sample was not normally distributed Kruskal Walli’s test was applied followed by post hoc comparison using Dunn test to check the difference in microbial inhibition between groups (P < 0.02).

**Results**

**E. Faecalis Group**

Mean and Median for the samples were calculated and since there was a statistically significant difference among the groups, Tukey Post-Hoc was applied for multiple comparisons. There was a statistically significant difference between Group C (Chlorhexidine +Chitosan) and Group F (Control). In the *E. Faecalis* group, statistical analysis showed significant difference in the quantity between subgroup 1C (chlorhexidine and chitosan combination) and subgroup 1F(control) (P>0.05) (Table 1). Maximum reduction of the bacteria was seen in the third group, i.e, chlorhexidine and chitosan combination (Table 2), whereas the least reduction was seen in the control group, i.e, without any intracanal medicament (Table 2). Following the third group, the next group to show reduction in the bacteria was the chlorhexidine group, followed by chitosan group. Least reduction was seen with the Calcium hydroxide, calcium hydroxide and chitosan combination and the Control group (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>1863.618</td>
<td>504.3971</td>
<td>159.5044</td>
<td>1217.55</td>
<td>2569.5</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>1962.324</td>
<td>484.8789</td>
<td>153.3322</td>
<td>1181.94</td>
<td>2727.95</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>1393.291</td>
<td>413.5574</td>
<td>130.7783</td>
<td>800.28</td>
<td>1951.45</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>1925.836</td>
<td>803.0171</td>
<td>253.9363</td>
<td>386.03</td>
<td>2910.37</td>
</tr>
</tbody>
</table>

**Table 1:** Multiple Comparisons.

**Table 2:** Mean for various intracanal medicaments at 14th day for *E. Faecalis*.

---

Candida Albicans Group

Table 3: Median, and interquartile range for various intracanal medicaments at 14th day for Candida Albicans

<table>
<thead>
<tr>
<th>Medicament</th>
<th>Median</th>
<th>First_q</th>
<th>Third_q</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX</td>
<td>1118.95</td>
<td>667.03</td>
<td>1835.69</td>
<td>443.45</td>
<td>3550.41</td>
</tr>
<tr>
<td>Ca(OH)2+CHITOSAN</td>
<td>1874.84</td>
<td>832.78</td>
<td>2395.18</td>
<td>507.72</td>
<td>3656.62</td>
</tr>
<tr>
<td>CHITOSAN+CHX</td>
<td>710.43</td>
<td>585.92</td>
<td>1264.94</td>
<td>556.65</td>
<td>1806.49</td>
</tr>
<tr>
<td>CHITOSAN</td>
<td>1540.52</td>
<td>833.64</td>
<td>1618.71</td>
<td>399.67</td>
<td>3974.73</td>
</tr>
<tr>
<td>CA(OH)2</td>
<td>2605.85</td>
<td>1221.28</td>
<td>7476.41</td>
<td>224.81</td>
<td>5067.59</td>
</tr>
<tr>
<td>CONTROL</td>
<td>3441.47</td>
<td>2691.78</td>
<td>7365.14</td>
<td>1692.06</td>
<td>7886.51</td>
</tr>
</tbody>
</table>

Chlorhexidine in gel formulation has been chosen for this study due to its low toxicity on the periapical tissues, solubility in water and viscosity that keeps the active agent in contact with the root canal walls and dentinal tubules [14-16]. In this work, the results corroborated previous study and indicate that treatment with Ca(OH)₂ alone has minor effect on C. Albicans and E. Faecalis as compared to the treatment with chlorhexidine alone [17,18] (2046±402 > 1863±504.3 - E. Faecalis) and the combination of chlorhexidine and Chitosan (2605.85±710.43 - C. Albicans and (2406±402 > 1393.2±413.5 - E. Faecalis) (Tables 2 & 4) respectively. Possible reason could be that although both calcium hydroxide and chlor-
hexidine exert their bactericidal activity by disintegrating membranes, additionally, chlorhexidine may induce reactive oxygen species production in alkaline environment, which would inhibit the E. Faecalis growth because of the destruction of cell wall and the plasma membrane mediated by nitric oxide [18]. Whereas, the combination of Calcium Hydroxide and Chitosan have shown better results for both the groups of micro-organisms as compared to Calcium hydroxide alone. (C. Albicans -1874.8-2605.85), E. Faecalis – (2219.8±441 <2406±402) (Tables 2 & 4) respectively. Possible reason could be the antimicrobial action of chitosan that could have enhanced the antimicrobial action of calcium hydroxide. Hence, this study demonstrated that combining Chlorhexidine gluconate with Chitosan had a good anti-microbial effect against C. albicans and E. faecalis. However, further investigations are required to prove the results in vivo.

Conclusion
A combination of 2% Chlorhexidine and 2% Chitosan showed better antibacterial efficacy against E. faecalis and antifungal activity against Candida Albicans by using real-time PCR.

References