



## Bactericidal Efficacy Of Antibiotic Combinations Against Enterococcus Faecalis – An In Vitro Study

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### INTRODUCTION

Endodontics plays a major role in the eradication of pulpal inflammation, the primary goal includes the treatment of apical periodontitis and maintaining the pulpal health. An immature necrotic tooth poses various challenges in the management of pulpal necrosis which traditionally aims at the formation of calcific barrier that aid in the obturation. The prolonged duration of the procedure with multiple review appointments, and the risk of cervical fracture have posed a challenge in the conventional apexification procedures<sup>1</sup>. Regenerative endodontics is a biological based procedure designed to replace damaged structures such as dentin, root structures, and cells of the pulp-dentin complex. The evolution of the method of biologically replacing dental tissues by regeneration has overcome the challenges by increasing the root length and thickness of the root canal walls, inducing apexogenesis<sup>2</sup>.

The outcomes of regenerative procedures rely on the complete elimination of bacteria, facilitation of root development, and closure of sinus tracts<sup>3</sup>. The infection of the root canal system is polymicrobial, consisting of predominantly facultative anaerobes and anaerobic bacteria. The existence of bacterial species results in the persistence of apical periodontitis and inflammation<sup>4</sup>. The virulence of the bacteria on causing disease and tissue damage is proportionate to the type and the numbers of bacteria present<sup>5</sup>. The microbial flora in failed endodontic teeth normally include varied microorganisms of which Enterococcus faecalis is the most prevalent and highly resistant to intracanal medicaments<sup>6</sup>.

It is an established fact that regeneration of the pulpal tissue of an infected immature tooth with open apex and apical periodontitis was considered to be impossible due to the presence of various microorganisms. Local antibiotics application has been widely used in the eradication of intracanal infection, hence the systemic routes of administration have been greatly reduced<sup>7</sup>.

To encounter the diverse flora in the root canal system, combinations of antibiotics are used known as the TRIPLE ANTIBIOTIC PASTE which comprises of combination of Ciprofloxacin, Metronidazole and Minocycline<sup>8</sup>. The synergy of antibiotics used in the triple antibiotic paste is used to efficiently combat the polymicrobial environment in the infected root dentin<sup>9</sup>. Minocycline is known for its broad-spectrum activity

but has the disadvantage of discoloration of teeth which has led to the necessity of finding an alternative antibiotic. In this study in the triple antibiotic combination minocycline is replaced with clindamycin.

This study aimed at comparing and evaluating the antibacterial efficacy of double or triple antibiotic combinations used as intracanal medicament. A standard strain of *Enterococcus faecalis* is used as a indicator organism to test the efficacy. The primary objective was to compare the efficacy of clindamycin over minocycline in combination with other antibiotics.

## MATERIALS AND METHODS

### 1. Bacterial suspension

*Enterococcus faecalis* was used as the indicator organism in the present study. A standard strain of *Enterococcus faecalis* (ATCC 29243) was procured from the Department of Microbiology, Priyadarshini Dental College & Hospital, Thiruvallur. The isolate was identified using PCR following the standard protocol<sup>10</sup>. The isolate was confirmed with the specific primer sets obtained. Further confirmation was done by heat tolerance test, biochemical reactions and with colony character on nutrient agar, MacConkey agar and blood agar. The organism is grown on triptose soya agar and the colonies were picked up and the suspension was made with nutrient broth and turbidity was compared with 0.5 McFarland standards which correspond to  $1 \times 10^8$  CFU.

### 2. Antimicrobial agents

The antibiotics used in this study included Ciprofloxacin, Metronidazole, Minocycline and Clindamycin respectively. The efficacy of these antibiotics was tested as double antibiotic combinations and triple antibiotic combinations. The ciprofloxacin and metronidazole were used in the pure form, clindamycin was available in powdered form from the capsule and minocycline was powdered finely using a porcelain mortar and pestle after removing the coatings<sup>10</sup>.

### 3. Experimental procedures

The antibacterial susceptibility of the organism was evaluated by the tube dilution method<sup>11</sup>. With the drugs', doubling dilution is done to achieve the required four concentrations as 100µg/ml, 50µg/ml, 25µg/ml, and 12.5µg/ml. With each concentration 5 tubes were taken and labeled as S1, S2, S3, S4, S5. The antibiotics were categorized into two groups. The first group comprised of different double antibiotic combinations of the four drugs chosen and the second group comprised of triple antibiotic combinations.

Group 1 (Double Antibiotic Combinations):

- ▶ A – Ciprofloxacin + Metronidazole
- ▶ B – Metronidazole + Minocycline
- ▶ C – Ciprofloxacin + Minocycline
- ▶ D – Ciprofloxacin + Clindamycin
- ▶ E – Metronidazole + Clindamycin

Group 2 (Triple Antibiotic Combinations):

- ▶ F – Ciprofloxacin + Metronidazole + Minocycline
- ▶ G – Ciprofloxacin + Metronidazole + Clindamycin

From the broth suspension 10µl was pipette and added to all the tubes. The tubes were incubated at 37°C aerobically for 24hours. The Minimal Inhibitory Concentration (MIC) of various drug combinations was evaluated with the presence or absence of turbidity by comparing with the positive and negative control groups. Further the Minimal Bactericidal Concentration (MBC) was determined by subculturing on Triptose soya agar which was incubated at 37°C aerobically for 24 hours to check the viability of the cells. The dilution which shows no colony forming units is the end point and taken as minimum bactericidal concentration of the preparation.

## RESULTS:

The drug in double and triple antibiotic combination is taken in increasing dilution to assess the MIC and MBC value. The minimal inhibitory concentration of the two-drug combination was observed effective in the combination of minocycline, with ciprofloxacin and metronidazole at 25µg/ml. At 25µg/ml, the combinations

with ciprofloxacin and metronidazole showed turbidity but no turbidity seen at 50µg/ml. The MIC of triple antibiotic combination including clindamycin was free of turbidity at a lesser concentration of 12.5µg/ml whereas the combination including minocycline was effective at 25µg/ml.

The minimal bactericidal concentration was lower for the triple antibiotic group that contained ciprofloxacin, clindamycin and metronidazole at 25µg/ml than the minocycline combination with ciprofloxacin and metronidazole at 50µg/ml (Table – 1).

**TABLE – 1**

S.No	Antibiotics	MIC (µg/ml)	MBC (µg/ml)
A	Ciprofloxacin + Metronidazole	25	100
B	Ciprofloxacin + Minocycline	25	100
C	Ciprofloxacin + Clindamycin	25	100
D	Metronidazole + Minocycline	25	100
E	Metronidazole + Clindamycin	50	100
F	Ciprofloxacin + Metronidazole + Minocycline	25	50
G	Ciprofloxacin + Metronidazole + Clindamycin	12.5	25

## DISCUSSION:

The presence of microorganisms in the root dentin varies widely. The resistance of microorganisms to antibacterial drugs increases greatly in root canal treatment failure teeth in systemic administration. The bacteria remaining in the canals or the periapical tissues need to be eliminated to improve the condition of the tooth. It is stated that obligate anaerobes and microaerophilic bacteria are most commonly associated in carious lesions, infected root dentine and unexposed pulp tissues<sup>9</sup>. With the advent of the triple antibiotic paste it became possible to eliminate the polymicrobial infection and facilitate healing of periapical tissue.

*Enterococcus faecalis* is a gram-positive diplococcus and it is a facultative anaerobe. The organism has various survival factors which include the encoded surface proteins, collagen binding proteins, gelatinase serine protease<sup>12</sup>. *E. faecalis* has high rate of resistance to various antibiotics but most strains are sensitive to ampicillin and vancomycin. It may become resistant even to these drugs due to widespread use of these antibiotics in treating systemic complications<sup>13</sup>. The prevalence of *Enterococcus faecalis* is higher in persistent infections that are asymptomatic than in symptomatic primary endodontic infections. It has the ability to invade dentinal tubules, and resist nutritional deprivation; it also requires good technique of asepsis with increased apical preparation sizes with effective irrigation modalities<sup>14</sup>. It has been documented that *E. faecalis* isolates from the endodontic lesions shows multi-resistance to antibiotics. According to one study the conventional triple antibiotic combination is effective against eight different isolates of *Enterococcus faecalis*<sup>10</sup>.

The antibiotic susceptibility testing is more accurate when quantitative analysis like agar dilution or broth dilution is used when compared to qualitative analysis such as disc diffusion methods<sup>15</sup>. The macro dilution method is one of the earliest antibacterial susceptibility testing methods. In this study the drugs are diluted by doubling dilution method starting from 100µg/ml to 12.5µg/ml<sup>16</sup>.

Ciprofloxacin, a fluoroquinolone has high activity against gram positive and gram-negative pathogens. It has good tissue penetration and is structurally related to nalidixic acid. It functions primarily by inhibiting the bacterial DNA gyrase. Ciprofloxacin is effective against isolates of *Enterococcus faecalis* at a concentration as low as 0.32µg/ml in macro broth dilution<sup>17</sup>. Metronidazole is highly active against anaerobic pathogens. It is active against most of the obligate anaerobes in vitro but shows no clinical activity against facultative anaerobes or obligate aerobes. Minocycline has a spectrum of activity similar to tetracycline. It has high tissue concentrations. In an in vivo study it has been proved that minocycline alone at 75µg/ml is effective in completely eradicating the polymicrobial flora whereas in combination with ciprofloxacin and metronidazole is effective at 25µg/ml<sup>9</sup>.

There are various studies (Iwaya et al,2001; Banchs & Trope,2004; Cheuh et al,2006; Petrino et al,2007; Cotti et al,2008; Jung et al,2008; Ali Nosrat et al,2011) that have shown beneficial results by the usage of the antibiotic paste combination. The antibiotic mixture is used in the concentration of 1:1:1 and the use of dentin bonding agents are advised prior to placement of the antibiotic paste as stated in the AAE recommendations. The staining can be minimized also by replacing the minocycline in the antibiotic paste with other antibiotics or the use of double antibiotic combinations<sup>18</sup>.

Clindamycin was chosen as one of the antibiotics to replace minocycline as it is a lincosamide antibiotic that has high level activity against facultative and strict anaerobes and at variety of gram positive and gram-negative organisms. It is present in high concentrations in saliva, GCF and bone. It is the only proven antibiotic that reduces the adherence of bacteria to the epithelial cells of mucosal surfaces<sup>19</sup>. Clindamycin hydrochloride has an equivalent spectrum of antibacterial activity similar to tetracyclines but exhibits minimal staining of teeth<sup>20</sup>. Post antibiotic effect is one of the advantages of clindamycin which has a half life of two weeks<sup>19</sup>.

Clindamycin has various systemic applications and more widely used in dermatological problems. It is recommended in the prophylaxis of infective endocarditis (600mg orally 1hr before treatment). It is used locally in endodontic infections such as acute inflammatory conditions, in flare-ups and periapical abscess<sup>21</sup>. Clindamycin is being used in dentistry widely. The Clindamycin / EVA fibers have proven to reduce the number of bacteria present in extracted human teeth. The Clindamycin / EVA fibers releases active drug for two weeks<sup>22</sup>. Clindamycin has greater dentinal tubule penetrating ability when compared with tetracycline. It was found that at 1/3 and 1/9 dilutions, clindamycin had better antibacterial effect than tetracycline<sup>23</sup>. Recently Clindamycin hydrochloride has replaced tetracycline in Ledermix paste as clindamycin is said to have an equivalent spectrum of antibacterial activity and exhibits minimal staining of teeth when compared to tetracycline group of drugs<sup>20</sup>.

The use of clindamycin has been evolving widely as an alternative to minocycline in the triple antibiotic combination that is used for lesion sterilization and tissue repair of infected root canals. It has been recorded those concentrations of clindamycin as low as 1mg/mL has been effective in reduction of the microbial activity<sup>24</sup>. Clindamycin when used as a component of the triple antibiotic paste is noted to have greater efficiency of almost 99.9% in eradicating colony forming units/ mg of *E.faecalis* when evaluated in comparison with photodynamic therapy and calcium hydroxide<sup>25</sup>. The angiogenic property also plays a vital role in the use of antibiotic combinations to enhance the regenerative potential. Clindamycin has less cytotoxic potential and at concentrations of 30µg/ml and 50µg/ml is observed to have proangiogenic activity when compared to 50ng/ml of minocycline<sup>26</sup>.

The combinations of clindamycin and minocycline with ciprofloxacin and metronidazole have shown to be synergistic in the present study against *Enterococcus faecalis*. The combination of ciprofloxacin with clindamycin and metronidazole shows an occasional synergistic effect in the combinations of ciprofloxacin with clindamycin but with metronidazole they have an antagonistic action against certain bacterial species<sup>23</sup>. The combination of ciprofloxacin with metronidazole is synergistic against *actinomyces* species<sup>27</sup>. The conventional triple antibiotic mixture has proved to be effective against various isolates of *Enterococcus*<sup>10</sup>. The modified combination of triple antibiotic paste with clindamycin substituting minocycline has been equally effective in lower concentrations against *E.faecalis* and also in lesion sterilization<sup>28</sup>. This study has evolved the use of minimal concentrations of modified drug combination that is effective against *Enterococcus faecalis*.

## CONCLUSION:

Within the limits of the present study the triple antibiotic combinations of clindamycin, ciprofloxacin and metronidazole is more effective than the combination with minocycline against *Enterococcus faecalis*. The double antibiotic combinations used are less effective than the triple antibiotic combinations. Further studies can be done to evaluate the clinical usage of the clindamycin combination with respect to the disinfection of the root canal and in minimizing the discolouration of the teeth.

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