Original Article

# In Vitro Evaluation of a New Combination of Three Antibiotic Paste Against Common Endodontic Pathogens

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**Abstract**

**Background and Aim:** Removing the pathogenic microorganisms from the root canal system is the key to a successful endodontic therapy. This study aimed to evaluate the antibacterial efficiency of three antibacterial agents and a new combination against selected endodontic pathogens.

**Materials and Methods:** In this in vitro study, the efficacy of three different antibacterial agents namely clindamycin, metronidazole, doxycycline, and their combination (CMD) was evaluated against seven bacterial strains associated with endodontic infections to determine their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). selective media were used to culture Candida albicans (C. albicans), Pseudomonas aeruginosa (P. aeruginosa), Escherichia coli (E. coli), Enterococcus faecalis (E. faecalis), Streptococcus mutans (S. mutans), Bacillus subtilis (B. subtilis) subsp. spizizenii, and Actinomyces actinomycetemcomitans (A. actinomycetemcomitans). All the tests were repeated in triplicate. The MIC and MBC values were reported as mean ± standard deviation. Data were analyzed by the Shapiro-Wilk test, the Kruskal-Wallis test, and Wilcoxon signed-rank test ($P<0.05$).

**Results:** The intergroup comparisons of MIC for clindamycin versus CMD ($P=0.036$), metronidazole versus CMD ($P=0.016$), and doxycycline versus CMD ($P=0.016$) demonstrated significant differences. No other significant difference was noted ($P>0.05$). Intergroup comparisons of MBC for clindamycin versus CMD ($p=0.036$), metronidazole versus CMD ($P=0.022$), and doxycycline versus CMD ($p=0.016$) demonstrated significant differences. No other significant difference was noted ($P>0.05$).

**Conclusion:** CMD showed superior antibacterial efficacy than each individual antibiotic, and can be used effectively against the abovementioned endodontic pathogens for their predictable elimination during endodontic therapy.

**Key Words:** Anti-Bacterial Agents; Clindamycin; Doxycycline; Metronidazole; Root Canal Therapy

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## Introduction

Bacteria residing as commensals in the oral cavity are harmless in normal conditions. But they may become harmful in compromised health, due to the expression of few virulence traits which enhance their pathogenicity [1].

One very peculiar mode of expressing pathogenicity by such microorganisms is biofilm formation [2]. Biofilms created by one species of bacteria can serve as habitat for another [3]. Such symbiotic associations among bacterial cells comprise "plaque" which is responsible for dental caries, and endodontic, periodontal, and other oral diseases. Biofilm-associated diseases are practically challenging for all professionals in the medical and dental fields [4].

During endodontic therapy, degenerated/ necrotic pulp is debrided through biomechanical preparation of the root canal system [5]. However, bacteria lodged in deeper layers of dentinal tubules are tenacious to remove, irrespective of what endodontic irrigation system is used, making endodontic treatment questionable [6]. Eliminating such microbial populations during therapeutic procedures from the root canal system and preventing biofilm formation comprise the first line of therapy [3]. Many chemical agents have been used as endodontic medicaments over few decades including phenols, aldehydes, corticosteroids, calcium hydroxide, chlorhexidine, and antibiotics [7]. But when used alone, such agents fall prey to multidrug-resistant microorganisms and lose their antimicrobial activity in-between appointments, questioning their effectiveness to kill the bacteria in complex anatomical regions [8]. The evolution of multi-drug resistant microbial strains made the researchers across the world to develop reliable and biologically safe combinations of antimicrobial agents to target such microorganisms [9].

Considering this notion, the present experimental study was planned to evaluate the antimicrobial efficacy of three antimicrobial agents namely clindamycin, metronidazole, and doxycycline, and their combination (CMD) against seven endodontic microbial strains associated with endodontic infections. The tested hypothesis was that CMD would exhibit better antibacterial efficacy than each individual antibiotic to eliminate endodontic pathogens.

## Materials and Methods

The present in vitro study was carried out at the Department of Pedodontics and Department of Microbiology after gaining clearance from the Institutional Ethical Committee, letter no. DMIMS(DU)/IEC/2015-16/1744, dated: 31/12/2015. The antimicrobial activity of different antimicrobial agents was tested against standard strains of microorganisms.

*Materials used for the microbiological experiment:*

The test agents used in the present study were commercial analytical-grade antibacterial agents namely clindamycin HCL (Himedia Labs Pvt Ltd., Mumbai, India), doxycycline HCL (Himedia Labs Pvt Ltd., Mumbai, India), and metronidazole (MP Biomedicals, LLC, France).

*Bacterial strains tested:*

The ATCC bacterial strains (Microbiologics, USA) were purchased from HiMedia Labs Pvt Ltd., Mumbai, India. Candida albicans (ATCC 10231) (C. albicans), Pseudomonas aeruginosa (ATCC 27853) (P. aeruginosa), Escherichia coli (ATCC 25922) (E. coli), Enterococcus faecalis (ATCC 35550) (E. faecalis), Streptococcus mutans (ATCC 25175) (S. mutans), Bacillus subtilis subsp. spizizenii (ATCC 6633) (B. subtilis), and Aggregatibacter actinomycetemcomitans (ATCC 29523) (A. actinomycetemcomitans) were used in this study based on their correlation with clinical symptoms of endodontic infections.

*Preparation of microbial inocula:*

All the lyophilized bacterial cells were revived to prepare a primary bacterial suspension by using 0.5 mL of sterile brain heart infusion (BHI) broth aseptically under a laminar flow biological safety cabinet (Bio-Clean Air Devices, Chennai, TN, India) at room temperature to prevent cross-contamination. This suspension was further expanded by adding sterile BHI broth to prepare secondary bacterial suspensions. All the aliquots were finally calibrated to 0.5 McFarland standards comprising of $10^7$ colony forming units (CFU)/mL of bacterial cells [10].

*Preparing the antibacterial agent stock solutions:*

All the antibiotics were converted into stock solutions as per the procedures mentioned by Panpaliya et al [10]. While preparing the stock

solution, 2 mg of the antibacterial agent was dissolved in 2 mL of sterile distilled water homogeneously to obtain the stock solution at a concentration of 1000 µg/mL [10,11]. All the stock solutions were preserved at 4 to 8°C in a non-transparent screw-capped container to prevent desiccation and oxidation of the active ingredients [11].

*Determining the minimum inhibitory concentration (MIC) of antibacterial agents:*

In this study, the MIC of all the antibacterial agents was determined through serial dilution method utilizing BHI broth. For this purpose, 1 mL of each stock solution was dispensed into a sterile test tube and diluted further serially from 1000 µg/mL to 0.2 µg/mL making 13 MIC tubes, respectively, and the last MIC tube with sterile BHI broth without any test agent was kept as the negative control (making a total of 14 MIC tubes per test agent). The guidelines given by the CLSI were followed to determine the MIC of each agent [12]. Five microliters of each bacterial aliquot were added to all MIC tubes and mixed on a vortex mixing device (SPINWIN Centrifuge, Korea) to obtain a homogenous suspension. All the test tubes were then incubated in a phase change microbiological incubator (Adarsh International, Haryana, India) in aerobic and anaerobic modes at 37°C for 24-48 hours to achieve bacterial growth [12]. The MIC values of all test agents were determined by visual inspection and confirmed by using a spectrophotometer (Orion™, Aqua-Mate 8000 UV-Vis, Thermo Fisher Scientific, US) at an optical density ($OD_{600}$) of 0.6-0.7 [11]. The concentration of antibacterial agents demonstrating no appearance of turbidity (no evidence of bacterial growth) was considered as the MIC of the respective agent for that particular microorganism [10].

*Determining the minimum bactericidal concentration (MBC) of the antibacterial agents:*

To determine the MBC of the antimicrobial agents, 5 µL of incubated MIC broth from each tube was streaked on nutrient agar plates and incubated at 37°C aerobically and anaerobically for 48 hours.

The lowest concentration of the antibacterial agent inhibiting 99% of bacterial growth, in terms of CFU appearance, was noted as MBC of the agent against that particular microorganism, respectively [13]

**Statistical analysis:**

All the procedures were repeated in triplicate (n=3) to average out the readings and minimize the errors. The data obtained from each bacterial species and the antibacterial test were subjected to statistical analysis using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA) by descriptive statistics. The MIC and MBC of each antibacterial agent against each microorganism were calculated as mean ± standard deviation, and the range. The Shapiro-Wilk test was utilized to evaluate the normality of the obtained data during statistical analysis. The MIC and MBC were both analyzed using the Kruskal-Wallis test for intergroup comparisons, while the intragroup pairwise comparisons were done using the Wilcoxon signed-rank test. The confidence level was set at 95% ($P<0.05$)

## Results

The MIC and MBC of all the test agents are summarized in Tables 1 and 2, respectively. Among all the tested bacteria, C. albicans, and S. mutans were found sensitive against all the test agents, particularly to CMD; while P. aeruginosa and E. faecalis were found less sensitive to individual agents like clindamycin, metronidazole, and doxycycline, but had good sensitivity to CMD.

The intergroup comparisons of all the test agents in terms of MIC and MBC using the Kruskal-Wallis test revealed no significant difference ($P>0.05$) (Tables 3 and 4, respectively). The Shapiro-Wilk test demonstrated the normal distribution of data ($P>0.05$) for both MIC and MBC, and no significant difference ($P>0.05$), except for intra-group comparison of clindamycin versus intra-group comparison of clindamycin versus doxycycline ($P<0.05$; Table 3).

The analysis of MIC through pairwise intra-group comparisons of clindamycin versus

**Table 1.** MIC of all the test agents against selected bacteria (range in ug/mL)

| Bacteria, (n =3) | Clindamycin | | Metronidazole | | Doxycycline | | CMD | |
|---|---|---|---|---|---|---|---|---|
| | Mean±SD | Range | Mean±SD | Range | Mean±SD | Range | Mean±SD | Range |
| C. albicans | 0.53±0.23 | 0.4-0.8 | 5.22±1.79 | 3.15-6.25 | 2.12±0.90 | 1.6-3.15 | 0.53±0.23 | 0.4-0.8 |
| P. aeruginosa | 33.33±14.4 | 25-50 | 20.7±7.49 | 12.5-25 | 16.67±7.22 | 12.5-25 | 10.42±3.61 | 6.25-12.5 |
| E. coli | 16.67±7.22 | 12.5-25 | 10.42±3.61 | 6.25-12.5 | 16.67±7.22 | 12.5-25 | 5.22±1.79 | 3.15-6.25 |
| E. faecalis | 20.83±7.22 | 12.5-25 | 41.67±14.43 | 25-50 | 25±0.00 | 12.5-25 | 16.67±7.22 | 12.5-25 |
| S. mutans | 1.07±0.46 | 0.8-1.6 | 8.33±3.61 | 6.25-12.5 | 4.18±1.79 | 3.15-6.25 | 0.53±0.23 | 0.4-0.8 |
| B. subtilis s. spiz | 16.67±7.22 | 12.5-25 | 20.83±7.22 | 12.5-25 | 20.83±7.22 | 12.5-25 | 8.33±3.61 | 6.25-12.5 |
| A. actinomycetemcomitans | 5.22±1.79 | 3.15-6.25 | 16.67±7.22 | 12.5-25 | 5.23±1.76 | 3.15-6.25 | 2.12±0.90 | 1.6-3.15 |

**Table 2.** MBC of all the test agents against selected bacteria (range in ug/mL)

| Bacteria, (n =3) | Clindamycin | | Metronidazole | | Doxycycline | | CMD | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range |
| **C. albicans** | 5.27 ± 1.79 | 3.2 - 6.3 | 12.50 ± 0.00 | 12.5 | 20.83 ± 7.22 | 12.5 - 25 | 5.27 ± 1.79 | 3.2 - 6.3 |
| **P. aeruginosa** | 50.00 ± 0.00 | 50 | 50.00 ± 0.00 | 50 | 41.67± 14.43 | 25 - 50 | 25.00 ± 0.00 | 25 |
| **E. coli** | 25.00 ± 0.00 | 25 | 41.67 ± 14.43 | 25-50 | 50.00 ± 0.00 | 50 | 12.50 ± 0.00 | 12.5 |
| **E. faecalis** | 33.33 ± 14.43 | 25-50 | 33.33 ± 14.43 | 25 - 50 | 50.00 ± 0.00 | 50 | 25.00 ± 0.00 | 25 |
| **S. mutans** | 8.37 ± 3.58 | 6.3 - 12.5 | 25.00 ± 0.00 | 25 | 12.50 ± 0.00 | 12.5 | 5.27 ± 1.79 | 3.2 - 6.3 |
| **B. subtilis s. spiz** | 41.67 ± 14.43 | 25 - 50 | 41.67 ± 14.43 | 25 - 50 | 25.00 ± 0.00 | 25 | 12.50 ± 0.00 | 12.5 |
| **A. actinomycetemcomitans** | 33.33 ± 14.43 | 25 - 50 | 25.00 ± 0.00 | 25 | 12.50 ± 0.00 | 12.5 | 8.37 ± 3.58 | 6.3 - 12.5 |

**Table 3.** Inter-group and intra-group comparisons of MIC

| **Kruskal-Wallis test** | $X^2$ | Df | p |
|---|---|---|---|
| **Clindamycin** | 6.00 | 6 | 0.423 |
| **Metronidazole** | 6.00 | 6 | 0.423 |
| **Doxycycline** | 6.00 | 6 | 0.423 |
| **CMD** | 6.00 | 6 | 0.423 |

| **Pairwise Comparisons** | **Normality (S-W) test** | | **Wilcoxon signed-rank test** | | | |
|---|---|---|---|---|---|---|
| | **W** | **p** | **Statistic** | **P value** | **Mean difference** | **SE difference** |
| **Clindamycin vs Metronidazole** | 0.97 | 0.908 (NS) | 9.00 | 0.469 (NS) | -4.56 | 4.17 |
| **Clindamycin vs Doxycycline** | 0.67 | 0.002 (S) | 6.00[a] | 0.402 (NS) | -2.09 | 2.77 |
| **Clindamycin vs CMD** | 0.86 | 0.165 (NS) | 21.00[a] | 0.036 (S) | 7.28 | 3.04 |
| **Metronidazole vs Doxycycline** | 0.96 | 0.809 (NS) | 17.00[a] | 0.208 (NS) | 4.15 | 2.82 |
| **Metronidazole vs CMD** | 0.89 | 0.265 (NS) | 28.00[a] | 0.016 (S) | 10.21 | 2.64 |
| **Doxycycline vs CMD** | 0.93 | 0.540 (NS) | 28.00[a] | 0.016 (S) | 6.78 | 1.60 |

[a] - 1 pair(s) of values were tied; A low 'p-value' suggests a violation of the assumption of normality

SD – Standard Deviation; SE – Standard Error; df – degree of freedom; S-W - Shapiro-Wilk test (W=0.75);
HS - Highly significant ($p<0.001$), S - Significant ($p<0.05$), NS - Not significant ($p>0.05$).

**Table 4**. Inter-group and intra-group comparisons of MBC of antimicrobial agents

| **Kruskal-Wallis test** | **$X^2$** | | **df** | | **p** | |
|---|---|---|---|---|---|---|
| Clindamycin | 6.00 | | 6 | | 0.423 | |
| Metronidazole | 6.00 | | 6 | | 0.423 | |
| Doxycycline | 6.00 | | 6 | | 0.423 | |
| CMD | 6.00 | | 6 | | 0.423 | |
| **Pair-wise Comparisons** | **Normality (S-W) test** | | **Wilcoxon signed-rank test** | | | |
| | **W** | **p** | **Statistic** | **p value** | **Mean difference** | **SE difference** |
| Clindamycin vs Metronidazole | 0.89 | 0.258 (NS) | 2.00[a] | 0.361 (NS) | -9.06 | 3.55 |
| Clindamycin vs Doxycycline | 0.93 | 0.514 (NS) | 12.50 | 0.866 (NS) | -2.97 | 6.74 |
| Clindamycin vs CMD | 0.90 | 0.350 (NS) | 21.00[b] | 0.036 (S) | 16.66 | 4.41 |
| Metronidazole vs Doxycycline | 0.87 | 0.184 (NS) | 17.50 | 0.608 (NS) | 2.08 | 4.97 |
| Metronidazole vs CMD | 0.89 | 0.301 (NS) | 28.00 | 0.022 (S) | 18.75 | 3.45 |
| Doxycycline vs CMD | 0.93 | 0.578 (NS) | 28.00 | 0.016 (S) | 15.84 | 4.28 |

[a] - 3 pair(s) of values were tied; [b] - 1 pair(s) of values were tied; A low 'p-value' suggests a violation of the assumption of normality

SD – Standard Deviation; SE – Standard Error; df – degree of freedom; S-W - Shapiro-Wilk test (W=0.75);
HS - Highly significant (p<0.001), S - Significant (p<0.05), NS - Not significant (p>0.05).

CMD (P=0.036), metronidazole versus CMD (P=0.016), and doxycycline versus CMD (P=0.016) demonstrated statistically significant differences, showing higher antibacterial efficacy of CMD than individual agents (P<0.05). However, comparison of individual agents revealed no significant difference (P>0.05; Table 3). The analysis of MBC through pairwise intra-group comparisons of clindamycin versus CMD (P=0.036), metronidazole versus CMD (P=0.022), and doxycycline versus CMD (P=0.016) demonstrated statistically significant differences in terms of antibacterial efficacy (P<0.05). No other significant differences were noted (P>0.05; Table 4).

## Discussion

Due to having limited vasculature and lymphatic drainage, dental pulp may exhibit diverse responses to biological, physical, or chemical stimuli. At times, pulpal tissues of immature teeth may suffer insults for a considerable time, but the mature pulp usually does not withstand such insults for a long time and loses its vitality [5]. Degenerated/necrotic pulp and various degradation products released from such pulp tissues provide a favorable nidus for colonization and growth of multiple bacterial species within the pulp chamber. Few bacterial strains possess the potential to generate and sustain a high acidic or alkaline environment [5,14]

Bacteria causing endodontic infections are originated from the oral cavity, carious teeth, anachoresis, or pre-contaminated dentinal tubules that are insufficiently disinfected in early phases of treatment. Endodontic pathogens most commonly identified from the root canals may include aerobic bacteria like B. subtilis, Candida, and Pseudomonas, as well as anaerobic bacteria like Aggregatibacter, Enterococcus, Escherichia, and Streptococcus [15]. In endodontic infections, Gram-negative anaerobic bacteria are the predominant causative agents responsible for characteristic clinicopathologic features of few periradicular diseases. C. albicans has been isolated in cases with pulp necrosis, symptomatic as well as asymptomatic chronic apical periodontitis, and failed endodontic treatments [16]. Isolates of B. subtilis have been identified from cases of refractory endodontic and apical periodontitis [17]. These bacteria are associated with biofilm formation in accessory canals, apical ramifications, and gaps between the filling material and dentinal walls [18]. B. subtilis exhibits its virulence through the formation of spore and mesh-like tenacious exopolysaccharide biofilm facilitating its attachment and survival against antibacterial agents [19]. E. faecalis has been recovered at low numbers from untreated canals. But during endodontic procedures, due to inadequate biomechanical preparations or disinfection, it can proliferate and lead to persistent periapical infections. E. faecalis sustains a wide range of temperature and pH in presence of intracanal medicaments. It harbors capsular polysaccharide rendering it resistant to many antibacterial agents [20]. A. actinomycetem-comitans can cause endodontic-periodontal lesions by entering through lateral and apical accessory canals into the apical and periapical areas [21]. P. aeruginosa can synthesize biofilm making it resistant to antimicrobial therapies [22]. S. mutans, being deeply harbored in dentin caries, is the primary source of endodontic infections [23]. All these bacteria show different characteristics as a single entity. But when residing in mixed microbial ecology, they exhibit complex associations and diverse behaviors altogether. These bacteria enhance the propagation, growth, and survival of each other, due to the presence of extracellular proteins, glycoproteins, or mucopolysaccha-rides favoring attachment and preventing antimicrobial agents from reaching the bacterial cells [20,23]

The key factor for success and predictable prognosis of endodontic therapy is the elimination of all endodontic pathogens, along with their byproducts [23,24]. For a long time, the treatment of choice to eradicate causative agents from the root canal systems has been the administration of antibacterial agents through systemic routes [13]. However, such type of modality may not prove successful to treat chronic infections in pulpless teeth, as the

amount of drug reaching the root canals in such teeth is insignificant [16]. Few endodontic infections and periradicular lesions are not affected by the use of systemic antibiotic therapy. This evolved the concept of topical drug application such as lesion sterilization and tissue repair by sustained release devices, final rinse, or pastes to eliminate endodontic lesions and heal periradicular conditions [7,25]

Microbial ecology associated with endodontic infections comprises of facultative as well as obligate aerobes and anaerobes [18,19]. In the present study, it was noted that among all the antibacterial agents tested, metronidazole exhibited the least sensitivity against all the bacteria. Metronidazole is effective against protozoa and anaerobic bacteria, but is highly susceptible to the development of microbial resistance particularly anaerobic bacteria if its dosage is altered either qualitatively or quantitatively [26]. When used along with penicillin, it has always given good results to combat endodontic and odontogenic infections. These results are in agreement with previous findings [27]. Doxycycline, a broad-spectrum antibiotic from the tetracycline group, is effective against a wide range of endodontic microorganisms when used as an intracanal topical antibiotic [13,26], and as a final root canal irrigant [28]. The results of the present study were also in agreement with previous findings [29] and demonstrated comparable effect of doxycycline in vitro against facultative and obligatory anaerobes. Clindamycin hinders bacterial protein synthesis and damages the bacterial cells beyond the level of repair [30]. It has been observed that, at sub-inhibitory concentrations, it helps decline toxin production and enhances opsonization and phagocytosis of microbial cells [31]. Clindamycin showed the highest sensitivity against almost all the bacterial isolates used in this experiment, which was in agreement with previous studies [13,28]. Considering the mechanism of action of each antibacterial agent, it was decided to combine clindamycin, metronidazole, and doxycycline, at a ratio of 5:5:1, respectively. This combination demonstrated good antibacterial efficacy, indicating high elimination potential and minimal development of antibiotic resistance. The reason for such observations for this combination can be attributed to its multi-location damage to the bacterial cell, targeting bacterial cell wall, microsomal apparatus, ribosome, mitochondria, RNA, and protein synthesis cycle [31]. Also, there are minimal chances of the bacterial cells surviving such intense damage. Application of this antimicrobial combination in root canals and periradicular tissue as an irrigant may prove to be effective in disinfecting the local endodontic system. This will help decrease the failure of endodontic therapy due to infection by such bacteria. For antimicrobial agents, concentration-dependent activity, as for CMD, may be preferred to time-dependent antimicrobial agents as thecontact time would be limited. Thus, the hypothesis of combining clindamycin, doxycycline, and metronidazole exhibiting better efficacy than individual agents to eliminate selected endodontic pathogens, was proven.

**Limitations of the study:**

1) Being an in-vitro experiment, the present study cannot reproduce the clinical scenarios.
2) Due to the financial constraints, limited bacterial strains were included in this study.
3) The test agents used in this study were not compared with standard non-antibiotic agents.

## Conclusion

In this study, CMD combination was found effective against the selected bacterial species associated with endodontic infections. Based on the results obtained, the present combination can be recommended as an effective tool for topical application (e.g. intracanal medicament) rather than an individual antibacterial agent for elimination of all the vegetative and non-vegetative bacterial forms. It can also be worked upon for the development of drug delivery systems like intracanal rinse/paste, or controlled release gels to eliminate endodontic infections. However, further studies are recommended to assess its clinical applications.

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