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ANTIMICROBIAL EFFICACY OF INTERCANAL MEDICAMENTS AGAINST A MATURE MULTI-SPICIES BIOFILM MODEL: AN EX-VIVO STUDY

Nada Zakaria[1] | **Mohamed Mokhtar Nagy**[1,2] | **Soha Elhady** [3] | **Shehabeldin Saber** [1,4,5]

Introduction: The aim of this study was to compare the efficacy of different antimicrobial agents, including Ciprofloxacin, Doxycycline, Clindamycin, Metronidazole, Amoxicillin and Clavulanate, Triple antibiotic paste (TAP) and Calcium hydroxide (CH), as intra-canal medications for regenerative endodontics against an aged, multispecies *ex-vivo* biofilm model.

Methods: A total of one hundred fifty single-rooted human teeth were used in this study. Fifteen teeth were used to monitor the formation and maturation of a multispecies biofilm model consisting of Enterococcus faecalis, Staphylococcus epidermidis and Candida albicans. After 21 days of incubation, the other one-hundred and thirty-five teeth were divided into 9 equal groups; 7 experimental groups were medicated for one week with either Ciprofloxacin, Doxycycline, Clindamycin, Metronidazole, Amoxicillin and Clavulanate, Triple antibiotic paste (TAP) or Calcium hydroxide (CH), a positive control group was infected and not medicated, while the negative control group was neither infected nor medicated. After medication removal, bacterial samples were collected in sterile tubes to assess the colony forming unit (CFU) counts, and the effect of the tested drugs on biofilm dissolution was assessed with scanning electron microscopy (SEM). The scoring was carried out by three blinded calibrated examiners.

Results: The most effective medications were Metronidazole and TAP, with no significant difference between them ($p \geq 0.05$), followed by Clindamycin and Ciprofloxacin, with no significant difference between them ($p \geq 0.05$), and the least effective were Calcium hydroxide, Doxycycline and Augmentin, with no significant differences between them ($p \geq 0.05$). SEM results showed that there was a significant difference between different groups. Metronidazole, TAP, Clindamycin and Ciprofloxacin recorded the lowest scores, followed by CH, Doxycycline and Augmentin.

Conclusions: Within the limitations of this study, it can be concluded that metronidazole and TAP showed better antimicrobial efficacy than other drugs against the studied biofilm model. The multispecies biofilm model is a better indicator of the nature of complex root canal infections than mono-species models.

Keywords: Amoxicillin and Clavulanate, Candida albicans, Clindamycin, Doxycycline, Enterococcus faecalis, Metronidazole, Endodontic Biofilm, Regenerative endodontics, Staphylococcus epidermidis, Triple antibiotic paste.

Corresponding author:
Prof. Shehabeldin Mohamed Saber, Email: shehabeldin.saber@bue.edu.eg

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1. Department of Endodontics. Faculty of Dentistry. Ain Shams University. Cairo. Egypt.
2. Department of Endodontics. Faculty of Dentistry. Galala University. Suez. Egypt.
3. Microbiology and Immunology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.
4. Department of Endodontics, Faculty of Dentistry, The British University in Egypt (BUE), Egypt.
5. Dental Science Research Group Lead. The Health Research Center of Excellence, The British University in Egypt.
Email: nodos85@hotmail.com
E-mail: mohamed.nagy@dent.asu.edu.eg
Email: sohaelhady@yahoo.com
Email: shehabeldin.saber@bue.edu.eg

EFFICACITÉ ANTIMICROBIENNE DES MÉDICAMENTS INTRA CANALAIRES CONTRE UN MODÈLE DE BIOFILM MULTI-ÉSPECES MATURE: UNE ÉTUDE EX-VIVO

Introduction: Le but de cette étude était de comparer l'efficacité de différents agents antimicrobiens, notamment la ciprofloxacine, la doxycycline, la clindamycine, le métronidazole, l'amoxicilline et le clavulanate, la pâte antibiotique triple (TAP) et l'hydroxyde de calcium (CH), en tant que médicaments intra-canalaires pour la régénération. Endodontie contre un modèle de biofilm ex-vivo multi-espèces vieilli.

Méthodes: Au total, cent cinquante dents humaines monoradiculées ont été utilisées dans cette étude. Quinze dents ont été utilisées pour surveiller la formation et la maturation d'un modèle de biofilm multi-espèces composé d'Enterococcus faecalis, Staphylococcus epidermidis et Candida albicans. Après 21 jours d'incubation, les cent trente-cinq autres dents ont été divisées en 9 groupes égaux ; 7 groupes expérimentaux ont été traités pendant une semaine avec soit de la ciprofloxacine, de la doxycycline, de la clindamycine, du métronidazole, de l'amoxicilline et du clavulanate, de la pâte antibiotique triple (TAP) ou de l'hydroxyde de calcium (CH), un groupe témoin positif a été infecté et non médicamenté, tandis que le groupe témoin négatif n'était ni infecté ni médicamenté. Après le retrait du médicament, des échantillons bactériens ont été collectés dans des tubes stériles pour évaluer le nombre d'unités formant colonies (UFC), et l'effet des médicaments testés sur la dissolution du biofilm a été évalué par microscopie électronique à balayage (MEB). La notation a été réalisée par trois examinateurs calibrés en aveugle.

Résultats: Les médicaments les plus efficaces étaient le métronidazole et le TAP, sans différence significative entre eux ($p \geq 0,05$), suivis de la clindamycine et de la ciprofloxacine, sans différence significative entre eux ($p \geq 0,05$), et les moins efficaces étaient l'hydroxyde de calcium et la doxycycline. Et Augmentin, sans différence significative entre eux ($p \geq 0,05$). Les résultats du SEM ont montré qu'il y avait une différence significative entre les différents groupes. Le métronidazole, le TAP, la clindamycine et la ciprofloxacine ont enregistré les scores les plus bas, suivis du CH, de la Doxycycline et de l'Augmentin.

Conclusions: Dans les limites de cette étude, on peut conclure que le métronidazole et le TAP ont montré une meilleure efficacité antimicrobienne que les autres médicaments contre le modèle de biofilm étudié. Le modèle de biofilm multi-espèces est un meilleur indicateur de la nature des infections canalaires complexes que les modèles mono-espèces.

Mots clés: Amoxicilline et Clavulanate, Candida albicans, Clindamycine, Doxycycline, Enterococcus faecalis, Métronidazole, Biofilm endodontique, Endodontie régénérative, Staphylococcus epidermidis, Pâte triple antibiotique.

Introduction

In immature permanent teeth with open apices, regenerative endodontic procedures (REPs) are biologically based treatments designed to restore damaged tissues, including dentine and root structures, and pulp-dentine complex cells [1]. To achieve disinfection, most REPs use irrigation and chemical disinfection using intracanal medicaments (ICM), such as local antibiotics, with little to no mechanical debridement [2]. Systemic antibiotics cannot reach the necrotic pulp area in sufficient doses; hence, this can be an effective way to disinfect root canals [3]. In addition, it limits bacterial growth, produces a barrier against bacterial recolonization, and continuously supplies antimicrobial agents [4].

In the first documented clinical technique utilizing antibiotics as ICM in REPs [5], Metronidazole and Ciprofloxacin were combined to create a Double antibiotic paste (DAP). A triple antibiotic paste (TAP) comprising minocycline, metronidazole, and Ciprofloxacin was later suggested [6]. Afterwards, several antibiotics, such as Clindamycin and various Amoxicillin combinations, were proposed to avoid the tendency of Minocycline to discolour teeth [7, 8].

However, the bacterial burden residing in the root canals is challenging due to its various species and virulence factors [9]. The bacterial capacity to colonize and arrange itself in a biofilm structure is one of the tactics that adds to its pathogenicity. Sessile multicellular microbial communities known as biofilms are defined as cells tightly bound to a substrate within a polysaccharide matrix that they have created on their own [10].

The chronicity of diseases has been attributed to biofilms, which facilitate the evasion of the host immune system and the exchange of genetic material amongst biofilm bacteria, leading to antibiotic resistance [11]. According to Ricucci and Siqueira [12], biofilms were discovered in most canals connected to apical periodontitis, especially those with sizable apical lesions. They contribute to resistant root canal infections by hiding in anatomical intricacies and tolerating chemo-mechanical disinfection techniques [13]. The fact that biofilm bacteria have a modified phenotype that enables them to display distinct traits, such as increased pathogenicity and survival capacities, sets them apart from their planktonic counterparts [14].

It has been demonstrated that an *Enterococcus faecalis* infection would tolerate attempts to disinfect root canals and result in resistant infections [15,16]. The Gram-positive facultative anaerobes, *Enterococcus faecalis*, can invade dentinal tubules and survive deprivation of nutrients with the possibility of recovery [17]. *Candida albicans* is a naturally occurring commensal organism that colonizes mucosal surfaces. However, they can become pathogenic and create resistant biofilms in immune-compromised environments [18]. Another commensal organism of the human skin microflora, *Staphylococcus epidermidis*, is also known to be an opportunistic pathogen in immune dysfunction situations [19, 20]. Biofilm models that closely mimic the clinical setting should be used for *in-vitro* investigations of any ICM's efficacy [21].

Thus, the purpose of this work was to assess and rank the antibacterial efficacy of various local antimicrobial agents (ICM) employed during REPs against an established mixed-species biofilm model. Ranking of these medications in order of local efficacy is important to know, if allergy exists and the use of any of them is contra-indicated. The null hypothesis tested is that there is no difference in the antimicrobial effectiveness among the tested antimicrobial agents.

Materials et Methods

Sample size calculation

To have sufficient power to execute a two-sided statistical test of the null hypotheses, a power analysis was created. Based on the findings of Saber & ElHady [3], with a power of 80%, an alpha (α) level of 0.05 (5%) and a beta (β) level of 0.20 (20%), the anticipated sample size (n) was 135 teeth in total. To calculate the sample size, G*Power 3.1.9.2 was used.

Selection and preparation of the teeth

Ethical approval was obtained from the Faculty of Dentistry, Ain Shams University (ethical committee number: FDASU-RecM071206). One hundred fifty extracted human single-rooted mature teeth were used in this study; one hundred thirty-five teeth were used for the experimental groups, and fifteen were used to monitor biofilm maturation. They were collected from the outpatient clinic at the Oral Surgery department of the Faculty of Dentistry, Ain Shams University, Cairo, Egypt. Patients were informed that their teeth would be used for scientific purposes. An ultrasonic scaler cleaned all hard and soft tissue deposits. Periapical radiographs confirmed the presence of a single patent root canal. De-coronation with a diamond disc was performed to standardize the length of the specimens to 16 mm. Longitudinal grooves were cut along proximal surfaces of all the samples before root canal preparation. Instrumentation was performed using the large Reciproc Blue files R25, R40 and R50 in the presence of 2.5% NaOCl [22]. Following preparation, all samples received a final irrigation sequence of 5 ml of 17% EDTA, followed by 5 ml of 2.5% NaOCL and 5 ml of distilled water to remove the smear layer. Teeth were air-dried and steam autoclaved at 121°C for 30 minutes

after coating the entire root surface, including the apices, with two layers of nail polish (Max factor, cosmetics, and fragrances, London, UK) to seal the roots.

Classification of the teeth

Teeth were classified into nine groups (n=15) according to the antimicrobial agents used. Group 1: medicated with Ciprofloxacin (Amriya Pharm Ind. Alexandria, Egypt), Group 2: medicated with Doxycycline (The Nile, Al Sawah, Cairo, Egypt), Group 3: medicated with Clindamycin (Pfizer Middle East. Cairo, Egypt), Group 4: medicated with Metronidazole (Amriya Pharm Ind. Alexandria, Egypt), Group 5: medicated with Amoxicillin and Clavulanate (Augmentin, Glaxosmith Kline, Cairo, Egypt), Group 6: medicated with a triple antibiotic paste (equal powder ratios of Doxycycline, Ciprofloxacin, and Metronidazole mixed with propylene glycol as an inert vehicle), Group 7 (positive control): medicated with Calcium hydroxide (CH) paste (Urbical, Promedica, Germany), Group 8 (positive control): infected and not medicated samples, and Group 9 (negative control): non-infected, non-medicated samples to check for the sterility of the procedures.

Development of polymicrobial intracanal biofilm

Three clinical isolates of *Enterococcus faecalis*, *Staphylococcus epidermidis*, and *Candida albicans* from the microbiology laboratory (Central Laboratories, Ministry of Health, Egypt) were used for biofilm formation. A 24-hour pure culture suspension of the selected organisms grown in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) was used to infect samples of the experimental groups (105 teeth) and the positive control group (15 teeth). The suspension was then adjusted to the No. 1/2 MacFarland turbidity standard. The culture was replenished 72 hours for 21 days. To verify the sterility of the processes, the negative control samples (15 teeth) were submerged in sterile BHI broth that was replaced every 72 hours with sterile saline. For 21 days, the teeth were kept at 37°C in a humid atmosphere.

Monitoring biofilm formation and maturation by SEM

The development of bacterial biofilms on the root canal dentin was assessed by SEM examination at three-time intervals (days 7, 14, 21) using an SEM Model Quanta 250 FEG (field emission gun) connected to an EDX unit (energy dispersive X-ray analyses) with an accelerating voltage of 30 kV. Fifteen more teeth were randomly chosen at each time interval (n = 5) and split into two halves using a hammer and chisel. Each half was then immersed in 2.5% glutaraldehyde (pH 7) for two hours at 4°C for fixation, followed by phosphate buffer saline (PBS) for thirty minutes and dehydrated in an ascending alcohol series (50%, 70%, 80%, 90%, and 100%) for thirty minutes each, and finally, acetone for thirty minutes. Each sample was mounted and sputter-coated with a 200°A layer of gold-palladium, and the whole canal was observed by SEM at 30 kV (FEI Company, Netherlands) for thickness, homogeneity, and presence of an extracellular polymeric substance, as shown in Figure 1.

Determination of minimum inhibitory concentration (MIC)

Before use, the minimum inhibitory concentration (MIC) for each antimicrobial agent was ascertained independently for each pathogen. The greatest value among the three organisms was chosen for each medication and used on the polymicrobial biofilm. Simultaneously, the TAP's MIC value (0.003 mg/mL) was determined according to Sabrah et al. [23]. The least quantity of antibiotic needed to stop the microorganism's growth *in-vitro* was found using the tube dilution approach. Each antibiotic was serially diluted in tubes, and each tube was then filled with an organism under test

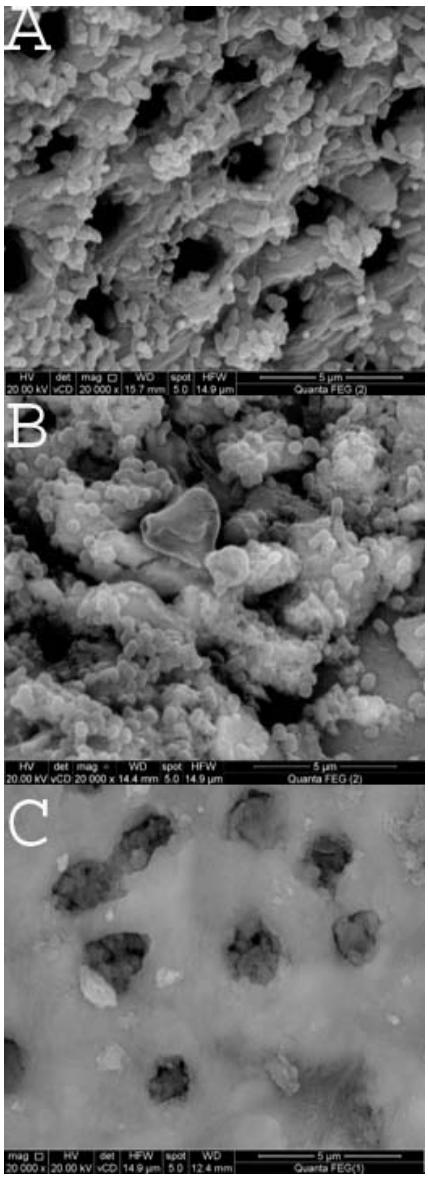

Figure 1. Scanning electron micrograph showing that microorganisms consistently adhered to collagen structures after 21 days.

at a known concentration. Two control tubes were set up: one for the culture control contained a standardized fluid culture of the organism without antibiotics, and the other for the antibiotic control contained antibiotics without organisms. The inspection was done after the incubation period of one night. Examining the control tubes to ensure test validity revealed that they were clear, signifying a functional antibiotic solution free of contamination, and the culture control tube was turbid, signifying a viable tested organism.

The test tubes were also examined to see which was the final one to exhibit no turbidity, which was the MIC.

Application of the Intracanal medicaments

Following a 21-day incubation period, intracanal medications were applied Using a disposable plastic syringe, all antimicrobial agents, apart from Calcium hydroxide, were transferred to an inert cream base compound consisting of glycerol, purified water, methyl para hydroxide benzoate, and propyl para hydroxide benzoate. Group 1 received 0.5 ml of 0.04 mg Ciprofloxacin, Group 2 received 0.5 ml of 10 mg Doxycycline, Group 3 received 0.5 ml of 30 mg Clindamycin, Group 4 received 0.5 ml of 0.1 mg Metronidazole, Group 5 received 0.5 ml of 24 mg Augmentin, Group 6 received 0.5 ml of 300 mg Triple antibiotic paste, consisting of equal ratios of Doxycycline, Ciprofloxacin, and Metronidazole, and Group 7 received 0.5 ml of Calcium hydroxide.

Wax was used to seal the apex and the coronal access cavity, and aluminium foil was used to envelop the samples. All samples were incubated for one week at 37°C under humid conditions in saline.

Bacterial sampling

Following a week, all samples were irrigated with 20 ml of sterile saline solution to remove the contents of the root canals, and bacterial samples were collected using a conventional technique. Root canals were filled with sterile saline solution, then a k-file size 15 was inserted 1 mm of the working length and used in a circular filling motion for 10 seconds. To determine the number of colony-forming units (CFU), three sterile paper points of size 35 were passively placed in each canal and collected in sterile tubes filled with saline. All samples were vortexed for twenty seconds, and tenfold dilutions (1:10, 1:100 and 1:1000) were prepared in saline. Aliquots of 0.1 ml were spread onto media plates (tryptone soy agar) and incubated at 37°C for 48 hours. Colony-forming units per 1 ml were counted.

Scanning electron microscope (SEM) evaluation

The remaining bacterial biofilm adherent to the root canal dentin was assessed by SEM examination using an SEM Model Quanta 250 FEG (field emission gun) attached to an EDX unit (energy dispersive X-ray analyses) with an accelerating voltage of 30 kV. All samples were processed as mentioned before.

Scoring for bacteria was performed under 10000X magnification according to the following criteria:

Score 1: No bacteria on the surface of the root canal.

Score 2: Isolated bacteria over the surface with no signs of viability/organization.

Score 3: Agglomeration of bacteria with signs of viability/organization.

Score 4: Over 50% of the root canal walls were covered with viable bacteria.

Score 5: Complete or nearly complete root canal wall coverage with viable bacteria.

Statistical analysis

The SAS software (SAS, Statistical Analysis Systems, STAT/User's Guide 6.03 ed., SAS Institute, Cary NC, USA) was used for statistical analysis. Using the Shapiro-Wilk and Kolmogorov-Smirnov tests, data were examined for normality and revealed a parametric distribution. Duncan's multiple range test was performed to examine the impact of treatment on CFU after one-way ANOVA was utilized to test for differences between more than two groups. The student's t-test was used to analyse the differences between the SEM scores. All analyses were conducted with a significance level of $P < .05$.

Results

Bacterial reduction (CFU) after the application of antimicrobial agents is presented in (Table 1). Statistical analysis showed that all the experimental groups demonstrated significantly fewer CFU counts than the positive control group ($p < 0.0001$). Regarding the antimicrobial effectiveness of the tested drugs, it was found that the most effective were Metronidazole and TAP, with no significant difference between them

Table 1: Mean and standard deviation values of CFUs of the experimental and control groups.

Variables	Bacterial count
	Mean ± SD
Doxycycline	11.50 x10x10 ± 2.50 x10x10^{a}
Ciprofloxacin	7.50 x10x10 ± 1.92 x10x10^{b}
Augmentin	13.37 x10x10 ± 2.82 x10x10^{a}
Clindamycin	6.87 x10x10 ± 2.29 x10x10^{b}
Metronidazole	0.87 x10x10 ± 0.83 x10x10^{c}
Triple antibiotic paste	3.12 x10x10 ± 1.12 x10x10^{cb}
Calcium hydroxide	11.25 x10x10 ± 3.01 x10x10^{ab}
Positive Control	24.87 x10x10^{3} ± 4.12 x10x10^{3} ^{d}
Negative Control	0.00 x10x10 ± 0.00 x10x10^{c}
P-value	**0.0001***

* Significant ($p<0.05$). Different superscript letters indicate statistically significant difference.
Similar superscript letters indicate a non-significant difference.

Table 2: Mean SEM biofilm scores of different groups.

	Doxycycline	Ciprofloxacin	Augmentin	Clindamycin	Metronidazole	TAP	CaOH	Positive	P-Value
mean score	$2.5^{cdi}\pm0.53$	$1.625^{eikm}\pm0.74$	$2.75^{bd}\pm1.03$	$1.5^{fjlm}\pm0.76$	$1^{hm}\pm0$	$1.125^{gklm}\pm0.35$	$2.37^{dij}\pm0.52$	$4.75^{a}\pm0.46$	$P<0.001$

* Significant ($p<0.05$). Different superscript letters indicate statistically significant difference. Similar superscript letters indicate a non-significant difference.

($p\geq0.05$). This was followed by Clindamycin and Ciprofloxacin, with no significant difference between them ($p\geq0.05$), and the least effective were Calcium hydroxide, Doxycycline, and Augmentin, with no significant differences between them ($p\geq0.05$).

SEM results showed that there was a significant difference between different groups. Metronidazole, TAP, Clindamycin & Ciprofloxacin recorded the lowest scores, followed by Calcium hydroxide, Doxycycline & Augmentin (Table 2) & (Figure 2).

Discussion

The biofilm structure of microorganisms is a strategy that allows them to adapt to almost any environmental circumstance. Colonizing bacteria can benefit greatly from this complex structure in many ways, such as the creation of a wide range of habitats for growth, increased metabolic diversity and efficiency, improved opportunities for genetic exchanges and bacterial intercommunications (quorum-sensing systems), and defence against external threats (host defences, competing microorganisms, antimicrobial agents, and environmental stress) [24]. Bacteria persist in root canal systems that are inaccessible to irrigants and mechanical cleaning [25]. Therefore, there is a rationale for the local administration of drugs that might reach these regions.

According to Esterala et al. [26], the bacterial colonization structure,

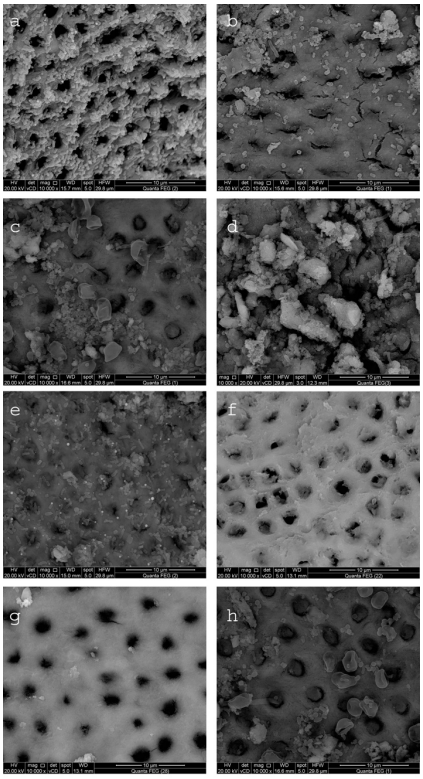

Figure (2): Representative scanning electron micrographs of different groups. a: positive control, b: Calcium hydroxide, c: Doxycycline, d: Augmentin, e: Metronidazole, f: Clindamycin, g: TAP. h: Ciprofloxacin.

the biological indicator, and the amount of time required for biofilm formation are the three factors that need to be considered to verify biofilm models. Our investigation succeeded in achieving each of these goals. The human root canal, with its microenvironment, served as the structure for bacterial colonization. Three endodontic pathogens exhibiting multiple virulence factors made up the biological marker. Lastly, SEM analysis demonstrated that the infection duration was adequate for the bacterial biofilm's growth and maturity. Therefore, researchers looking into the antibacterial potential of endodontic materials can profit from our approach.

E. faecalis is an extensively evaluated biological indicator. Rather than being a persister from original infections that were not successfully treated, it has been suggested that this species is a subsequent opportunistic colonizer in treated root canals [27]. The data from next-generation sequencing (NGS) have cast doubt on the role of *E. faecalis* in secondary or persistent infections. In contrast to previous research, which found that *E. faecalis* prevalence values in treated root canal patients could exceed 90%, 16S sequencing of persistent/secondary infections revealed the species at much lower rates (~30%) [28, 29].

Oral infectious disorders such as jaw osteomyelitis, severe marginal periodontitis, refractory marginal periodontitis, root canal infections, and root surface caries have all been linked to *staphylococci* [19].

The most common fungus found isolated from infected root canals and linked to cases of chronic root canal infections is *Candida albicans* [30]. This species is allegedly not a persister from unsealed root canals, but rather a secondary opportunistic colonizer.

This study employed the culture method to assess variations in the

total bacterial count. As opposed to other molecular techniques, which can detect uncultivable or difficult-to-grow bacteria or examine more specific effects [31], this method is a useful primary investigation method for quickly quantifying cultivable microorganisms in samples or correlating some bacteria to certain clinical findings.

Ciprofloxacin is a bactericidal fluoroquinolone, with strong effects on gram-negative bacteria but little effect on gram-positive bacteria [32]. Because it binds to the microorganism's 30S ribosomes and prevents protein synthesis, tetracycline is bacteriostatic. They have a wide range of antimicrobial action against gram-positive and gram-negative bacteria [33].

Clindamycin and Metronidazole exhibit exceptional antibacterial effectiveness, especially on endodontic microbiota, including anaerobic bacteria. They have been utilized as intracanal therapy for persistent infections [34].

A combination of Amoxicillin with Clavulanate (AugmentinTM) exhibit a broad-spectrum antibacterial action against anaerobic bacteria, while Clavulanate is a competitive inhibitor of the beta-lactamase enzyme produced by bacteria to inactivate penicillin. Another combination of antibiotics commonly used is triple antibiotic paste, which consists of a mixture of Metronidazole, Ciprofloxacin, and Minocycline. This drug combination was sufficiently potent to eradicate bacteria from infected root canal dentin [35].

The medication that is applied most frequently is the Calcium hydroxide (CH). Because of its high pH, which causes bacterial membrane enzymes to become inactive, it has antibacterial properties [36]. The hydroxyl ions released by CH are thought to cause the bacterial cell wall and DNA to break down, thereby having an antibacterial action [37]. Numerous investigations have revealed that following the insertion of CH into the root canal system, the hydroxyl ions diffuse to the root's outer surface via the dentinal tubules [38]. These studies suggest that CH may have an immunomodulatory effect through localized inflammatory mediator denaturation, either through proinflammatory mediator denaturation [39] or amide bond alkaline hydrolysis [40]. Nevertheless, the effectiveness of CH has also been questioned, especially when it comes to bacteria like Candida albicans and E. faecalis that are linked to refractory apical periodontitis [41]. Additionally, it has a limited capacity to disinfect dentinal tubules sufficiently [42] and, if applied repeatedly, may impair the root structure [43].

The concentrations employed were designed by each drug's minimal inhibitory concentration to enable the intracanal medications' antibacterial qualities to be expressed in clinical settings. To determine each organism's affinity for the antibiotic under test, the test was run on it independently, and the highest result was chosen. The study by Sabrah et al. provided the basis for the minimal inhibitory concentration of TAP (0.003 mg/mL) [44].

The results of this study indicated that the most effective medications were Metronidazole and TAP, which did not differ significantly from one another. Clindamycin and Ciprofloxacin were next in line, also not significantly different from one another, and the least effective medications were Calcium hydroxide, Doxycycline, and Augmentin. SEM scores produced comparable outcomes. The acquired results' validity was strengthened by the discovery of a strong correlation between the CFU results and SEM scores. Metronidazole exhibits a broad spectrum of activity against protozoa and anaerobic bacteria. This might be attributed to specific redox proteins, which reduce the nitro group of this compound and produce free radicals that enter the cell and induce DNA damage, resulting in rapid cell death [44]. Our findings agreed with previous studies by Roche and Yoshimori [45] and Siqueira and de Uzeda [46].

It has been well documented that TAP, as an antimicrobial dressing, exhibits a broad antibacterial range and outperforms numerous other intracanal medications. This supports several earlier research [35, 47, 48]. While most known interactions between *Candida albicans* and bacteria are inhibitory, Tsui et al. [49] noted that enhanced adherence or antibiotic resistance are possible symbiotic interactions. They demonstrated that these biofilms are much more resistant to antimicrobial treatment, which may lead to the development of novel cross-protective mechanisms. This presents an additional difficulty in the management and elimination of biofilms, since these polymicrobial biofilms typically call for a mix of treatments. This explains why the biofilm that was formed in this study responded more favourably to TAP, a combination of antibiotics than it did to any one of the individual antibiotics. It is worth mentioning that Ciprofloxacin, which is a bactericidal broad-spectrum fluoroquinolone that inhibits microbial nucleic acid synthesis, exhibits very potent activity against gram-negative bacteria but minimal activity against gram-positive bacteria [32].

On the other hand, there were no significant differences ($p \geq 0.05$) in the antibacterial efficacy of Calcium hydroxide, Doxycycline, and Augmentin. This could be because calcium hydroxide was employed in this investigation in paste form. An aqueous Calcium hydroxide suspension exhibited a greater degree of hydroxyl ion release than cementor paste-type Calcium hydroxide products, as shown by Stashle et al. [50]. This finding aligns with previous studies [18, 30, 51, 52], which reported that Calcium hydroxide exhibited reduced bacterial activity against *E. faecalis*. These findings, however, are at odds with those of Baik et al. [53], who postulated that Calcium hydroxide might detoxify lipoteichoic acid, a prominent gram-positive bacterium virulence component, hence lowering the in-

flammatory response to *E. faecalis*. This discrepancy might result from the fact that in their investigation, the bacteria were exposed to calcium hydroxide on planktonic bacteria, as opposed to our study, which involved a biofilm structure where the bacteria were able to withstand alkaline stress [52]. One of the least successful drugs at breaking down biofilms was Doxycycline. This finding aligns with numerous prior research [54-56] and suggests that *E. faecalis* resistance to Doxycycline may be the cause.

Conclusions

Metronidazole and TAP demonstrated superior antibacterial activity over other medications against the examined biofilm model, within the limitations of this investigation. It was impossible for any of the antimicrobial treatments to fully eradicate the bacteria from the root canals. Compared to monospecies models, the multispecies biofilm model more accurately represents the characteristics of complex root canal infections.

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