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COMPARATIVE ANTIBACTERIAL ASSESSMENT OF ANTIBIOTIC BASED IRRIGANTS AGAINST ENTEROCOCCUS FAECALIS – AN IN VITRO STUDY

Dental Science

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ABSTRACT

AIM: The aim of the study is to compare the efficacy of 4 different generations of cephalosporins as an irrigant in eliminating E.faecalis by various antibacterial assays such as disc diffusion assay, tube dilution assay and finally colony forming units

METHODS: Sixty intact lower premolar teeth were selected for this study. The tooth was decoronated below cementoenamel junction. Each specimen was individually packed in vials containing 5ml of BHI broth. The specimens were autoclaved at 121˚c for 15 lbs for 15 minutes

A total of 60 specimens were divided into 6 groups. The specimens were placed in 5 ml of the sterile BHI broth belonging to groups were chosen for contamination with Enterococcus faecalis. From each vial 2ml of sterile BHI broth was discarded and replaced with 2ml of Enterococcus faecalis suspension prepared as stated previously under sterile conditions. The inoculated vials were incubated at 37˚C for 14 days, each of the tooth specimens was taken from each vial under sterile conditions and was rinsed with 5ml of sterile saline solution. 0.5% of the irrigating solutions were inserted into the canal with sterile syringes and 27 gauge needle. The irrigation solution was allowed to remain for 10 minutes after with they were removed using sterile paper points. The samples were diluted using sterile saline. 100µl of sample from each dilutions was spread out on to the surface of BHI agar plates. The plates were incubated at 37˚C for an overnight period. After incubation the colonies were counted and expressed as CFU/ML.

RESULTS: The results of the present study show group 2 (cefuroxime) with least antimicrobial activity against Enterococcus faecalis. Whereas, group 3 (cefoperazone) had better antibacterial activity compared to group 2 (cefuroxime) but had less antimictobial property when compared to group 1 (cephazolin) and group 4 (cefipime). Group 1 (cephzolin) had better antimicrobial property when compared with group 2 (cefuroxime) and group 3 (cefoperazone). Group 4 (cefipime) had better inhibition of bacterial growth of all other groups used in this experiment.

KEYWORDS

INTRODUCTION

For many years, the intracanal irrigants have been used as an adjunct to enhance the antimicrobial effect of cleaning and shaping in endodontics. Nevertheless, because of the anatomical complexities of many root canals, organic residues and bacteria located in the dentinal tubules cannot be sufficiently cleaned, even after meticulous mechanical procedures; therefore various substances have been used during and immediately after root canal preparation to remove debris, necrotic pulp tissue and to eliminate microorganisms from the root canal.

E.faecalis is facultative anaerobic gram positive cocci, they are most common and occasionally the only single isolated bacteria from the root canal of the teeth (59). Root canal treated teeth are about 9 times more likely to harbour E.faecalis than cases of primary infections. They resist bile salts, detergents, heavy metals, ethanol, azide and desiccation. E.faecalis can bind to dentin and invade the dentinal tubules and may survive chemo-mechanical instrumentation and intracanal medication. (3).

Several irrigating solutions are used during endodontic treatment to achieve antimicrobial activity. Sodium hypochlorite remains the most widely used irrigant solution because of its pronounced antimicrobial activity. Chlorexidine has been proposed as a potential substitute for hypochlorite given its optimum effects against endodontic bacteria.

Some experimental antimicrobial root canal irrigants are found to be more effective on bacterial bio-films(5). Antibiotics can be used as an adjunct to endodontic treatment. Its ineffectiveness in systemic route of administration has led to intracanal application to increase its efficacy (27).

Cephalosporins are beta lactam antibiotics which are bactericidal and have the same mode of action as other beta-lactam antibiotics. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of mucopeptides (peptidoglycan precursors) to crosslink the peptidoglycan. Beta-lactam antibiotics mimic the D-Ala-D-Ala site, thereby competitively inhibiting PBP crosslinking of peptidoglycan.

Cephalosporins are indicated for the prophylaxis and treatment of infections caused by bacteria susceptible to this particular form of antibiotic. First generation cephalosporins are active predominantly against gram positive bacteria and successive generations have increased activity against gram negative bacteria. Resistance to cephalosporin antibiotics can involve either reduced affinity of existing penicillin binding protein components or the acquisition of a supplementary beta-lactam insensitive penicillin-binding-protein (67).

The cephalosporin nucleus can be modified to gain different properties. Cephalosporins are grouped in to generations by their antimicrobial properties. The first developed cephalosporins were designated as first generation cephalosporins, whereas more extended spectrum cephalosporins were classified as second generation cephalosporin. Each newer generation has significantly greater gram negative antimicrobial properties than the preceding generation, in most cases with decreased activity against gram positive organisms. Fourth generation cephalosporins, have true broad spectrum activity. Fourth generation cephalosporins are considered to be a class of highly potent antibiotics that are medicines last defences against several serious human infection (72).

MATERIALS AND METHODS:

ANTIBACTERIAL TEST ASSAY:

The Cephalosporin drugs used in the study are, Cefzolin (1st generation), Cefuroxime (2nd generation), Cefoperazone (3rd generation), Cefipime (4th generation)

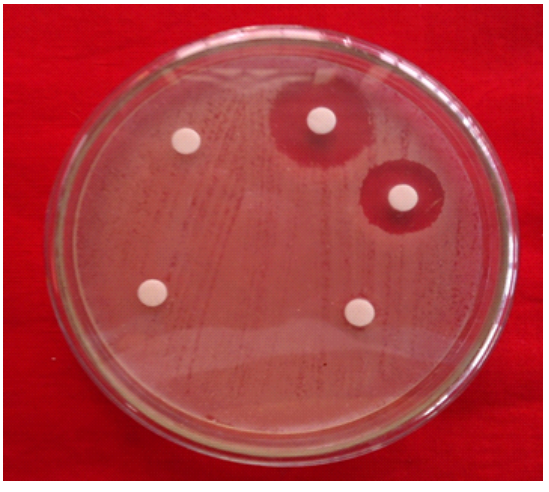
The antibacterial efficacies of the drugs have been checked by the following experiments:

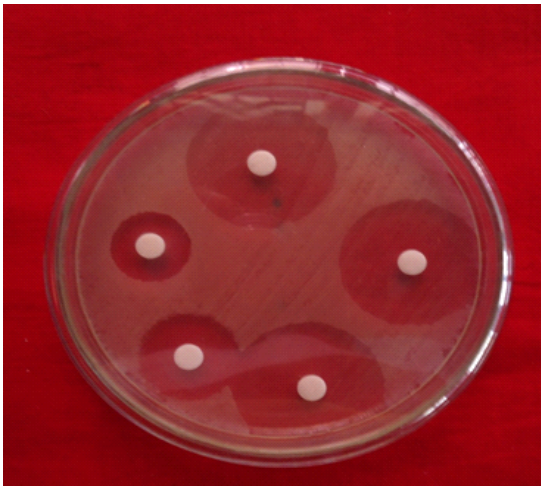
1. Disc diffusion assay
2. Serial dilution method to check minimum inhibitory concentration
3. Minimum bactericidal concentration study
4. Colony forming units

PREPARATION OF E.FAECALIS SUSPENSION:

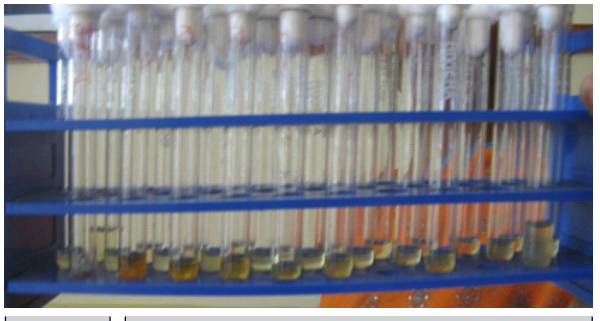
A loop full of growth from overnight incubated E.faecalis (American type culture collection) on BHI agar was used to inoculate BHI broth. The inoculated broth was incubated overnight at 37˚C.The turbidity was matched to mac farlands 0.5 standard. This suspension was used for inoculation.

Zone of inhibition

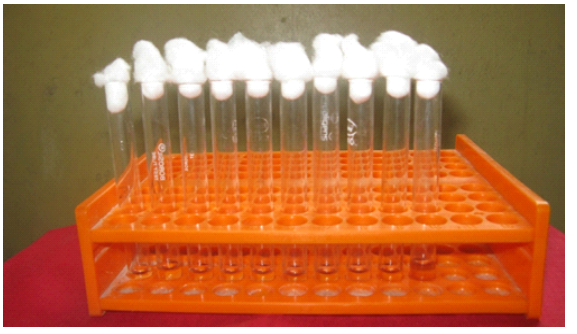




Minimum inhibitory concentration



Minimum bactericidal concentration



COLONY FORMING UNITS:

60 single rooted premolars were selected to detect the efficacy of irrigants to solution before 7 days.

SPECIMEN PREPARATION:

The teeth were decoronated below the cementoenamel junction. The cementum was removed from root surface.

The canals of the teeth were prepared using no.2 gg drill at slow speed. The canals were enlarged till F2 size to standardize internal diameter of the canal. The specimen was preserved in vials containing tap water during the procedure to avoid dehydration.

SPECIMEN STERILISATION:

Composition of brain heart infusion (BHI) broth:
Each specimen is individually packed in a glass vials containing 5ml of BHI broth.

1. Calf brain infusion -200 gm/l
2. Beef heart infusion -250 gm/l
3. Protease peptone -10 gm/l
4. Dextrose -2 gm/l
5. Sodium chloride -5 gm/l
6. Disodium phosphate -2.5 gm/l
7. Distilled water -1 liter

Enterococcal surface The specimens were autoclaved at 121degree centigrade for 15 lbs for 15mins. The autoclaved specimens were incubated at 37˚c for 24hrs for sterility check, a total of 60 specimen's were divided into 6 groups.

Drugs	No of Samples
Cefazolin(group 1)	n=10
Cefuroxime (group 2)	n= 10
Cefoperazone (group 3)	n=10
Cefipime (group 4)	n=10
Sodium hypochlorite (+ve control)	n=10
Saline (-ve control)	n=10

CONTAMINATION WITH E.FAECALIS:

1) PREPARATION OF E.FAECALIS SUSPENSION:

A loop full of growth from overnight incubated E.faecalis (American type culture collection) on BHI agar was used to inoculate BHI broth. The inoculated broth was incubated overnight at 37˚C. The turbidity was matched to mac farland's 0.5 standard. This suspension was used for inoculation.

2) INOCULATION OF SPECIMEN VIALS:

The teeth samples of all groups following sterility checking, were subjected for contamination with E.faecalis. from each vial 2 ml sterile BHI broth was discarded and replaced with 2ml of E.faecalis suspension prepared as stated previously under sterile conditions. The inoculated vials were incubated at 37˚C for 14 days at aerobic environment to favour continuous growth of E.faecalis in inoculated vials, 1ml of the inoculated medium was removed and replaced with 1 ml of the sterile BHI broth, once in every 2 days under sterile conditions. Following the completion of incubation period the tooth samples were subjected for antibacterial assessment.

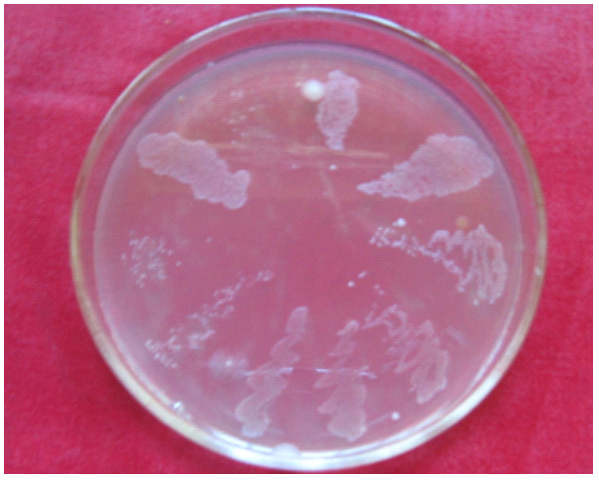
ANTIBACTERIALASSESSMENT:
Each of the tooth specimen was taken from their respective vial under sterile conditions and were rinsed with 5 ml of sterile saline solution. The teeth were air dried and its outer surface was covered with 2 layers of nail varnish in order to prevent contact with the medicament with the external surface.

IRRIGATION OF TEETH SAMPLES:
0.5% of the irrigating solution were instilled into the canal with sterile syringes using 27 gauge needle. The irrigant was instilled until the dentinal tubes were completely filled, the irrigant was allowed to remain for 10 minutes in the dentin tubes after which they were removed using sterile paper points.

From each group tooth samples were taken on day 1,3 and 5 for antibacterial assessment. The dentin samples were removed from the canals with k-file size 50 on 1,3and 5 days. The file removed 0.1mm of dentin approximately. The powdered dentin samples obtained with each file were immediately collected in separate sterile vials containing 3ml of sterile saline

EVALUATION OF E.FAECALIS LOAD IN DENTIN SAMPLE:
The dentinal chips were collected in a vial containing sterile saline. The content were vortexed to dislodge the bacteria from the specimen. The samples were diluted using sterile saline.100µl of sample from each each dilutions was spread on the surface of dry BHI agar plates. The plates were allowed to dry and incubated at 37˚c for an overnight period at aerobic condition. After the incubation period the colonies were counted and expressed as cfu/ml.

E.faecalis ATCC29212 used for inoculating tooth samples



RESULTS
In this present study the tooth samples were subjected to inoculation with e.faecalis, followed by irrigation with 4 different generations of cephalosporin based irrigants. The minimum inhibitory concentration by serial dilution method and the residual antibacterial activity was evaluated by the number of colony forming units.

The statistical analysis was done using one way ANOVA analysis and post multiple comparison was done with t-test, showed significant difference in mean values of groups 1,2,3 and 4 when compared. In this present study group 4 exihibited statistically significant values of high residual antibacterial activity when compared to group 1,2 and 3. The results of the present study showed group 2 (cefuroxime) had least antimicrobial activity against e.faecalis. group 3 (cefoperazone) has better antimicrobial activity against e.faecalis compared to group 2 (cefuroxime) but had less antibacterial property when compared to two other groups group 1 (cefazolin) and group 4 (cefipime) for 1,3 and 5 days. Group 4 (cefipime) has better antibacterial property when compared to other groups.

MIC, MBC and zone of inhibition of antibiotic based irrigants

S.NO		MIC	MBC	ZONE OF INIHIBITION
1	CEFZOLINE	0.5µg/ml	2µg/ml	29mm
2	CEFUROXIM	1µg/ml	2µg/ml	24mm
3	CEFOPERAZONE	2µg/ml	2µg/ml	26mm
4	CEFIPIME	0.5µg/ml	2µg/ml	31mm

Mean and standard deviation of CFU/ml of E.faecalis following irrigation with antibiotic based irrigants and Naocl (day 1, 3 and 5)

Table 1

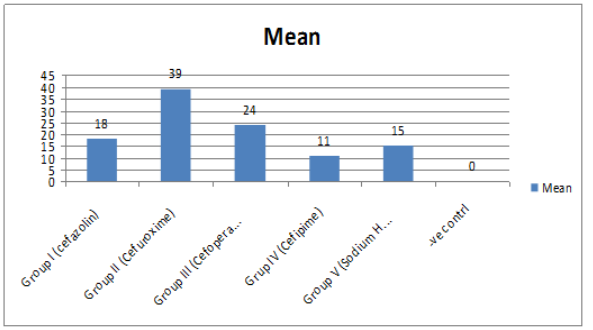
	Day 1	Day 3	Day 5
Group I (cefazolin)	185	145	97
Group II (Cefuroxime)	396	2811	189
Group III (Cefoperazone)	2413	189	124
Grup IV (Cefipime)	114	8.74	5.72
Group V (Sodium Hypochlrite)	158	10.27	7.44
-ve control	-	-	-

ANOVA of CFU/ml of E.faecalis following irrigation with antibiotic based irrigants, positive control and negative control at day 1

Table 2

Groups	N	Mean	SD	F - Value	P - Value
Group I (cefazolin)	10	18	5	7.59	0.001 (P<0.01)
Group II (Cefuroxime)	10	39	6		
Group III (Cefoperazone)	10	24	13		
Grup IV (Cefipime)	10	11	4		
Group V (Sodium Hypochlrite)	10	15	8		
-ve control	10	0			

Mean value of CFU/ml of E.faecalis following irrigation with antibiotic based irrigants, positive control and negative control at day 1

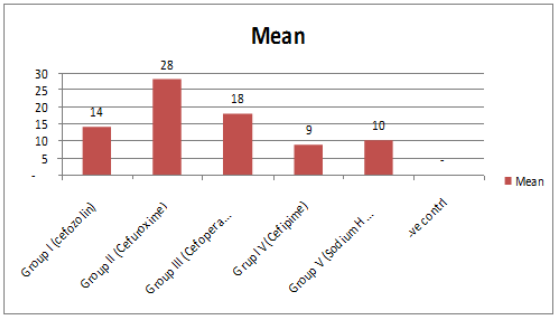


ANOVA of CFU/ml OF E.faecalis following irrigation with antibiotic based irrigants, positive control and negative control at day 3

Table 3

Groups	N	Mean	SD	F - Value	P - Value
Group I (cefozolin)	10	14	5	3.795	0.001 (P<0.01)
Group II (Cefuroxime)	10	28	11		
Group III (Cefoperazone)	10	18	9		
Grup IV (Cefipime)	10	9	4		
Group V (Sodium Hypochlrite)	10	10	7		
-ve control	10	-	-		

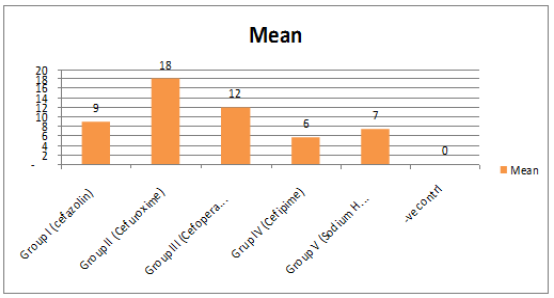
Mean of CFU/ml of E.faecalis following irrigation with antibiotic based irrigant, positive control and negative control at day 3.



ANOVA of CFU/ml of E.faecalis following irrigation with antibiotic based irrigants, positive control and negative control at day 5.
Table 4

Groups	N	Mean	SD	F - Value	P - Value
Group I (cefazolin)	10	9	7	7.234	0.001 (P<0.01)
Group II (Cefuroxime)	10	18	9		
Group III (Cefoperazone)	10	12	4		
Grup IV (Cefipime)	10	6	2		
Group V (Sodium Hypochlrite)	10	7	4		
-ve control	10	0	-		

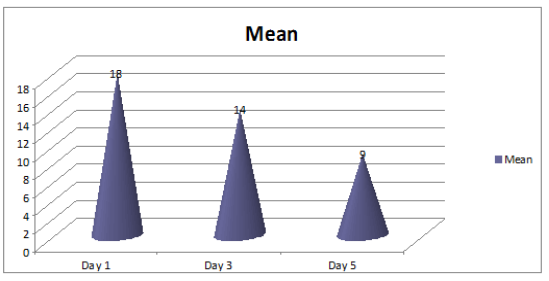
Mean of CFU/ml of E.faecalis following irrigation with antibiotic based irrigants, positive control and negative control at day 5.



ANOVA of CFU/ml of E.faecalis following irrigation with cefazolin at different days
Table 5
Group I cefazolin at different days

Days	N	Mean	SD	F - Value	P - Value
Day 1	10	18	5	3.654	0.039
Day 3	10	14	5		
Day 5	10	9	7		

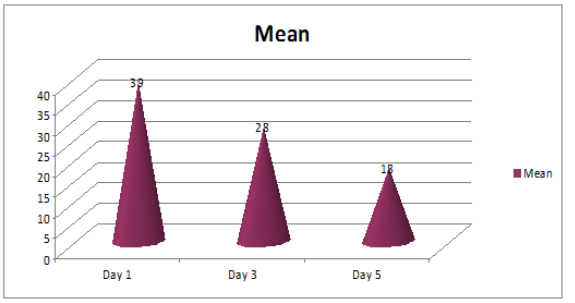
Mean of CFU/ml of E.faecalis following irrigation with cefazolin at different days.



ANOVA of CFU/ml OF E.faecalis following irrigation with cefuroxime at different days (day 1,3 and 5).
Table-6
Group II (Cefuroxime) at different days

Days	N	Mean	SD	F - Value	P - Value
Day 1	10	39	6	20.87	0.03
Day 3	10	28	11		
Day 5	10	18	9		

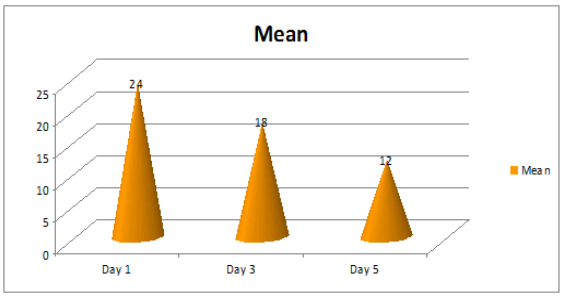
Mean of CFU/ml of E.faecalis following irrigation with cefuroxime at different days (day 1,3 and 5).



ANOVA of CFU/ml of E.faecalis following irrigation with cefoperazone at different days
Table 7
Group III (Cefoperazone) at different days

Days	N	Mean	SD	F - Value	P - Value
Day 1	10	24	13	5.78	0.001 (P<0.01)
Day 3	10	18	9		
Day 5	10	12	4		

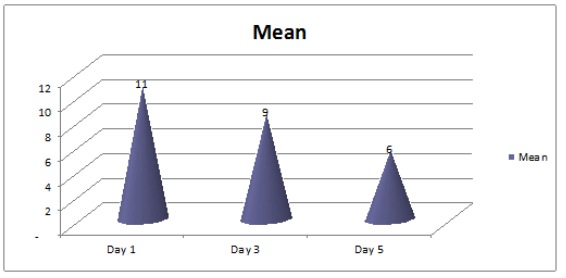
Mean of CFU/ml of E.faecalis following irrigation with cefoperazone at different days.



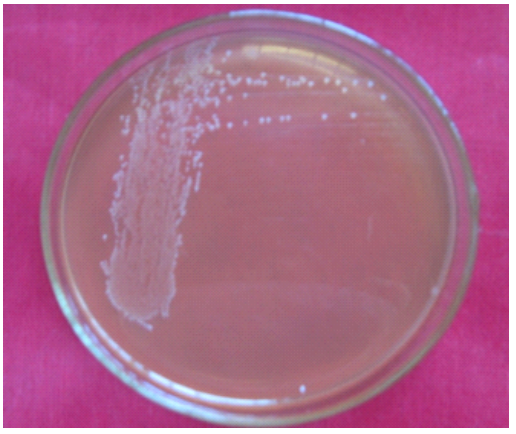
ANOVA of CFU/ml of E.faecalis following irrigation with cefipime at different days.
Table 8
Group IV (Cefipime) at different days

Days	N	Mean	SD	F - Value	P - Value
Day 1	10	11	4	8.523	0.001 (P<0.01)
Day 3	10	9	4		
Day 5	10	6	2		

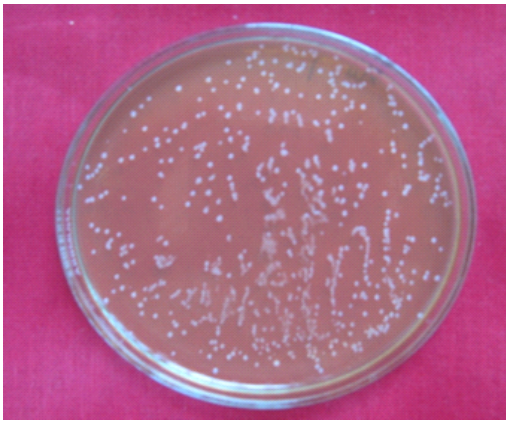
Mean of CFU/ml of E.faecalis following irrigation with cefipime at different days



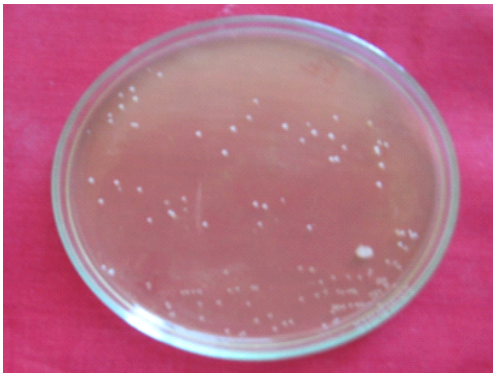
This photo shows the reduction in the colony counts of E.faecalis following irrigation with cefazolin.



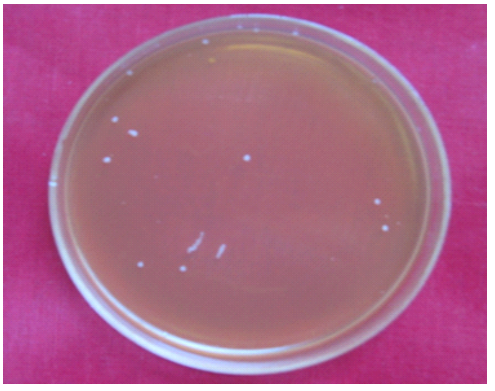
This photo shows the reduction in the colony counts of E.faecalis following irrigation with cefuroxime



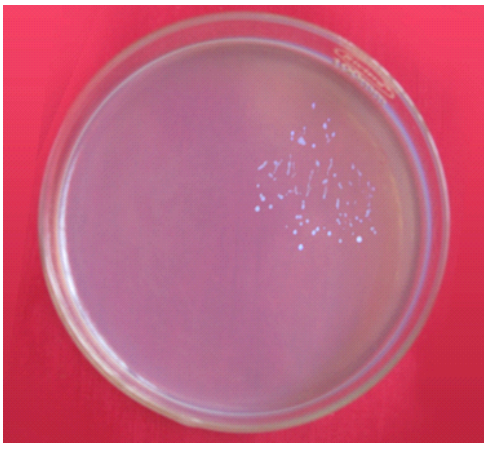
This photos shows the reduction in the colony counts of E.faecalis following irrigation with cefoperazone



This photo shows the reduction in colony counts of E.faecalis following irrigation with cefipime



This photo shows the reduction in the colony counts of E.faecalis following iriigation with Naocl



DISCUSSION

One of the important requirements of an ideal irrigant is its ability to eliminate microorganisms from the root canal system. This antimicrobial effect can be a direct chemical effect or indirectly by facilitating the mechanical disinfection through lubrication, tissue dissolving, and flushing of contaminated debris accumulated during root canal preparation. In addition, the root canal irrigants should be biocompatible with oral tissues (31).

Enterococci are facultative anaerobes which account for 12% of infections. It is found in 4-40% of primary endodontic infections. However, its frequency in persistence periradicular lesions has been shown to be nine times higher. It relies more upon its ability to survive and persist in the root canal of teeth. (1)

In this study, E.faecalis was chosen as a test organism because it is a facultative organism that is non-fastidious, easy to grow, and efficiently and rapidly colonizes tubules (8). It has been used extensively in endodontic research because it has been found to be present in 63% of teeth with post treatment disease. (4)

Enterococci particularly E.faecalis, have been frequently found in obturated root canals exhibiting signs of chronic apical periodontitis, isolated in 23-70% of the positive cultures and often occur in monoculture(11). Most strains are non hemolytic. It is a non-motile microorganism and facultatively anaerobic, it ferments glucose without gas production, and does not produce a catalase reaction with hydrogen peroxide.

Enterococcal surface protein is a high molecular weight surface protein. The contribution of the surface protein is to colonization and persistence of e.faecalis in tooth, heart and urinary tract infection. Enterococcal surface protein is also associated with promotion of primary attachment and biofilm formation of E.faecalis on abiotic surfaces (11)

Adherence to a solid substrate enables microbes to evade the normal flushing actions of tissue fluids and allows them to survive difficult environmental conditions (11). Microbial adherence to a substrate has been suggested to occur in 2 distinct phases (3). In phase 1, the interaction between microbial cell and substrate is mediated by the attractive force and/or repulsive force as the function of separating distance between the surfaces involved. Hydrophobic interactions between microbial cell and substrate are thought to contribute largely to attractive forces, whereas repulsive forces are due to the net negative charges (zeta potential) on the surfaces of both microbial cell and substrate. The phase one interaction is the initial interaction(in seconds to minutes) that occurs as the cell approaches the substrate surface, whereas phase 2 interaction (in hours to days)occurs between the polymeric microbial cell surface structures on which adhesion molecules are expressed (eg, fimbriae and pili), and the substrate. The phase 2 interaction makes adherence between bacteria and the substrate firmer.(3)

Adherence of bacteria to hard tissues or artificial biomaterial s is the first step toward biofilm-mediated infections. E.faecalis possess

different virulence factors that enable them to adhere to dentin and invade dentinal tubules (15). Biofilm-mediated infections are not resolved by the host immune system, whereas the bacteria residing in a biofilm are difficult target to treat with local or systemic antimicrobials. Thus mechanical removal of biofilm is indicated to be the ideal. The ability to form distinct biofilm under difficult growth conditions is considered as the major factor attributing to their survival in postendodontic environment approach to treat biofilm-mediated infections (15).

Sodium hypochlorite is at present the most popular irrigant. It is a broad spectrum antimicrobial agent that has proven to be effective against bacteria (9). The antimicrobial efficacy of Naocl is due to its ability to oxidize and hydrolyse cell proteins and to some extent, osmotically draw fluids out of cells due to its hypertonicity. Sodium hypochlorite has a pH of approximately 11 to 12 and when hypochlorite contacts tissue proteins, nitrogen, formaldehyde, and acetaldehyde are formed within a short time and peptide links are broken resulting in dissolution of proteins. During the process, hydrogen in the amino groups is replaced by chlorine, thereby forming chloramines, which plays an important role in antimicrobial effectiveness. (2)

Antibiotics are valuable adjunct to the armamentarium available to the dentist for management of bacterial infections. During endodontic treatment it can be used as an intracanal medicament or as an irrigant (27).

Antibiotics are classified mainly into 2 categories –bactericidal, those kill bacteria rapidly and bacteriostatic, that prevent multiplication of bacteria by retarding protein synthesis. Faster killing antibiotics are more desirable. Bactericidal drugs are more desirable and effective for local application as an irrigant (27).

Enterococci, particularly stains of E.faecalis, expressing β-lactamase enzyme and having high resistance to penicillin and amphicillin have been reported from various locations (72). Cephalosporins when compared with penicillins are less susceptible to inactivation by β-lactamase (72).

Cephalosporins are beta-lactam compounds in which the beta lactam ring is fused to a 6- membered dihydrothiazine ring, thus forming the cephem nucleus (67). Side chain modification to the cephem nucleus confers-an improved spectrum of antibacterial activity, pharmacokinetic advantages and rapid action (67). They prevent cell wall synthesis by binding to enzymes called penicillin binding proteins (67). These enzymes are essential for the synthesis of the bacterial cell wall. They have concentration –independent bactericidal activity, with maximal killing 4-5 times the MIC of the organism (67).

The antimicrobial susceptibility of each drug against E.faecalis was assessed by Kirby-bauer disk diffusion method. Minimum inhibitory concentration and minimum bactericidal concentration of each drug is calculated by serial dilution method. In order to simulate the in vivo conditions as a root canal irrigant colony forming units were calculated.

When compared with positive control group (sodium hypochlorite), group 4 (cefipime) shows better antimicrobial property. Other groups (1,2 and 3) had antimicrobial property lesser than that of positive control.

Group 2 (cefuroxime) had the least antimicrobial activity. The reason for this may be attributed to either reduced affinity of existing penicillin binding protein components or the acquisition of a supplementary beta lactam insensitive penicillin binding protein. The MIC and MBC of group 2 are better than that of group 3 (cefperazone) and less when compared with group 1(cefzolin) and group 4 (cefipime). The zone of inhibition is less when compared to group1 and group 4. When compared with group 3 its more or less equal. Action of this generation on gram positive bacteria is the same or a little bit less than that of the first generation.

Group 3 (cefoperazone) has better antimicrobial activity than group 2 but lesser antimicrobial activity than group 1 and group 4. Cefoperazone is an extended spectrum cephalosporin. As a piperazinyl derivative, it has structural characteristics similar to those aminopenicillin and is more active against enterococci. However, cefoperazone is less consistently active against certain strains of enterococci. This might be due to decreased antibiotic permeability through cell wall, antibiotic inactivation, alteration of target binding site and active antibiotic effiux. In general cefoperazone has increased antimicrobial activity against gram negative micro-organisms than gram positive microorganism due to extended gram negative spectrum. The MIC and MBC of group 3 (cefoperazone) inferior when compared to group 1,2 and 4. The zone of inhibition is more or less equal to group 2. When compared with group 1 and 2 zone of inhibition is less.

Group 1 (cefazolin) have a stronger antimicrobial action on gram positive bacteria than that of the other generations, but their action on gram positive bacteria is relatively less when compared to group 4 (cefipime). Comparatively, they are stable for beta-lactamase (penicillinase).group 1 (cefazolin) has MIC better than that of group2 (cefuroxime) and group3 (cefoperazone) but equal when compared with group4 (cefipime). MBC is equal when compared with other groups. The zone of inhibition of group 1 (cefazolin) is better than that of group 2 and group 3 but its more or less equal to that of group 4 (cefipime).

Group 4 (cefipime) are extended spectrum agents with activity greater than that of group 1. They also have a greater resistance to beta-lactamases than the third generation cephalosporins. Group 4 (cefipime) had the best antimicrobial action when compared with other groups. Group 4 (cefipime) has MIC better than that of group 2 and group 3 but equal when compared to group 1. The MBC of group 4 (cefipime) is the same when compared with the other groups. The zone of inhibition of group 4 (cefipime) is better than that of group 2 and group 3 but its more or less equal when compared with group 1 (cefazolin).

The fourth-generation cephalosporins are structurally related to the third-generation cephalosporins but, in addition, they possess a quaternary ammonium group at the C-3' position. They are zwitterionic compounds, which facilitates rapid penetration through the outer membrane of Gram-negative bacteria. This, together with their low affinity for clinically important P-lactamases, results in potent activity against many Gram-negative pathogens, including strains producing derepressed class I (AmpC) P-lactamase, resistant to most third generation cephalosporins. In addition, some fourth generation cephalosporins exhibit excellent activity in vitro against gram positive bacteria (73).

CONCLUSION

The results of the present study revealed:

1) Group 1 showed better antimicrobial activity the an group 2 and group 3. When compared with group 4 it showed lesser antimicrobial activity.
2) Group 2 showed least antimicrobial activity when compared with group 1, 3 and 5.
3) Group 3 showed better antimicrobial activity than group 2 and lesser antimicrobial activity than group 1 and 4.
4) Group 4 was the most effective irrigant in the study showing least number of colonies formed.

The use of cephalosporin based irrigant in endodontic treatment might be advantageous. preclinical and clinical trials are needed to evaluate biocompatibility and safety before cephalosporins can be exclusively recommended as intracanal irrigants.

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