EVALUATION OF ANTIMICROBIAL EFFECT OF OCTENIDINE, TRICLOSAN AND CHLORHEXIDINE AGAINST *E.FAECALIS* IN ROOT CANAL DISINFECTION - AN EXVIVO STUDY

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CERTIFICATE

This is to certify that this dissertation titled **"EVALUATION OF ANTIMICROBIAL EFFECT OF OCTENIDINE, TRICLOSAN AND CHLORHEXIDINE AGAINST *E.FAECALIS* IN ROOT CANAL DISINFECTION – AN EXVIVO STUDY"** is a bonafide record of work done by **B. ANURADHA** under our guidance during the study period between 2008-2011.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES/PHOTOGRAPHS

INTRODUCTION

Endodontics has advanced and developed since the 17th century and, research has proceeded continuously without pause.

Endodontic therapy is a sequence of treatment for the diseased pulp of a tooth with the goal to eliminate its infection and protect the decontaminated tooth from future microbial invasion. This goal may be accomplished using mechanical instrumentation and chemical irrigation, in conjunction with medication of the root canal between treatment sessions.

Complete debridement and disinfection of the pulpal space are considered to be essential for predictable long-term success in endodontic treatment. Residual pulpal tissue, bacteria, and dentine debris may persist in the irregularities of root canal systems, even after meticulous mechanical preparation (Abou-Rass).[38] Therefore, several irrigant solutions have been recommended for use in combination with canal preparation. However, the efficacy of these procedures also depends upon the vulnerability of the involved species.

The selection of effective microbial control of infected root canals requires detailed knowledge of the microorganisms responsible

for pulp and periapical pathology associated with knowledge of the mechanism of action of the antimicrobial solution.

Microorganisms most commonly infect the root canal system by ingress from the oral cavity through dental caries or defective restorations. The dentine-pulp complex of the tooth may react in a number of ways to the presence of microorganisms, but irreversible inflammatory changes may ultimately occur with the development of an inflammatory front in the periradicular tissues causing a chronic periradicular periodontitis.[20]

In the past few years, *Enterococcus faecalis* has been mentioned with increased frequency with regard to teeth with post-treatment disease (PTD), where it has also been detected as monocultures. The prominence of *E. faecalis* in root-filled teeth with apical periodontitis has made it a focus of attention as an etiological factor of PTD. It has been speculated that the presence of *E. faecalis* is encouraged by the conventional endodontic techniques.[34]

Throughout the history of endodontics, endeavors have continuously been made to develop more effective irrigant delivery system for root canal irrigation. So far, there is no one unique irrigant that can meet all the requirements, even with the use of methods such

as lowering the pH, increasing the temperature, as well as addition of surfactants to increase the wetting efficacy of the irrigant.[50]

Octenisept is an antiseptic for skin burns, wound disinfection and mouth rinses consisting of octenidine hydrochloride and phenoxyethanol. It demonstrates broad spectrum antimicrobial effects covering both Gram-positive and Gram-negative bacteria, fungi and several viral species.[47]

Identification of the root canal isolates from previous studies has traditionally been performed using standard microbiological and biochemical techniques. These methods have shown that the polymicrobial infections are mainly caused by obligate and facultative anaerobes. However, correlation of the microbiological findings from these studies is affected by certain limitations of the culture techniques, leading to the underestimation of bacterial diversity within the root canal system.[37]

The SYTO dyes can be used to stain RNA and DNA in both live and dead eukaryotic cells, as well as in Gram positive and Gram negative bacteria. The SYTO dyes are compatible with a variety of fluorescence-based instruments that use either laser excitation or a conventional broadband illumination source (e.g., mercury- and

xenon-arc lamps). SYTO 9, a nucleic acid stain have been used in diverse applications from staining DNA spotted on microarrays to staining live and fixed cells.[55]

The aim of this study was to evaluate and compare the antimicrobial effect of **Octenidine, Triclosan and Chlorhexidine** as a root canal irrigant in the elimination of E. *faecalis.*

The objective of this study was:

(1) To investigate the antimicrobial activity of the irrigants on dentine model

(2) To quantify the surviving bacteria by colony forming units

(3) To confirm the vitality status of bacteria by fluorescence labelling.

(4) To compare the efficacy of octenidine, Triclosan, chlorhexidine and sodium hypochlorite.

REVIEW OF LITERATURE

Sedlockl and Bailey **(1985)[40],** demonstrated that Octenidine hydrochloride (OCT), WIN 41464-2;[N,N'-(1,10 decanediyldi - 1[4H] -pyridinyl - 4 - ylidene) bis (1octanamine) dihydrochloride when used as a topical microbicide in concentrations of <1.5 ,LM (0.94,ug/ml) caused a greater than 99% reduction of each microbial population within 15 min and concluded from their study that OCT is a broad spectrum antibiotic agent which is an active biocide at low concentrations. The compound is bactericidal as well as candidicidal and is effective against resident skin microflora.

Siqueira et al (1997)[39] Evaluated the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal and concluded that NaOCl applied by the three methods tested, was significantly more effective than the saline solution (control group) in disinfecting the root canal.

Rass et al (1998)[38] investigated the microorganisms of strictly selected closed periapical lesions associated with both refractory endodontic therapy and pulpal calcification and concluded that closed periapical lesions associated with calcified teeth or those resistant to

root canal treatment harbour bacteria. The inability to eradicate all root canal microorganisms during root canal treatment, along with anatomical factors, may allow further bacterial colonization of the root apex and surrounding periapical tissues, and consequently prevent healing.

Heling et al (1998)[19] investigated sodium hypochlorite (with and without EDTA), chlorhexidine, and hydrogen peroxide in varying concentrations when used in sequence or in combination as endodontic irrigants. Sterile saline served as the control and concluded that All irrigant regimens were more effective than saline in killing bacteria. Chlorhexidine and sodium hypochlorite were similarly effective.

Molander et al (1998)[29] examined the microbiological status of 100 root-filled teeth with radiographically verified apical periodontitis concluded that the microflora of the obturated canal differs from that found normally in the untreated necrotic dental pulp, quantitatively as well as qualitatively.

Lenet et al (2000)[26] assessed the efficacy of two CHX delivery vehicles, a controlled-release device and a gel, to affect antimicrobial substantivity of bovine root dentin and suggests that suggest that bovine root canals medicated with 2% CHX gel for 7 days acquire antimicrobial properties for at least 21 days.

Peciuliene et al(2000)[35] studied the occurrence of Enterococcus faecalis in root canals of previously root filled teeth with apical periodontitis requiring retreatment and concluded that, rather than previous chemical treatment, it is the ecological conditions present in the incompletely filled root canal that are important for the presence of *E.faecalis* in these teeth.

***Tasman et al* (2000)**[48] evaluated the surface tension values of established and potential endodontic irrigants to which a surface active agent had not been added and concluded that Cetredixin displayed the lowest surface tension. A low surface tension agent should penetrate tubules better.

Komorowski et al (2000)[21] assessed the antimicrobial substantivity of chlorhexidine-treated bovine root dentin over a period of 21 days and concluded that Specimens treated with CHX for 7 days demonstrated significantly less dentin colonization by

E.faecalis than the other specimens. CHX has potential as an intracanal medicament, if it can be applied for a period of at least 7 days.

Gomes et al (2001)[15] studied, in vitro**,** the effectiveness of several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of chlorhexidine gluconate (gel and liquid) in three concentrations (0.2%, 1% and 2%) in the elimination of *E.faecalis* and concluded that even though all tested irrigants possessed antibacterial activity, the time required to eliminate *E.faecalis* depended on the concentration and type of irrigant used.

Heling et al (2001)[20] evaluated the antimicrobial and cytotoxic effects of sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) and concluded that both agents were very effective in killing bacteria, and their cytotoxicity to fibroblasts in tissue culture was similar.

Buck et al (2001)[36] compared three endodontic irrigants for efficiency in killing bacteria established within human dentinal tubules and concluded that sodium hypochlorite seemed to be superior. Tubulicid and Peridex were better than water. More bacteria remained viable at greater distances from the pulp.

Marley et al (2001)[30] assessed whether chlorhexidine gluconate (0.12%), used as an endodontic irrigating solution would affect the apical seal obtained when using three different root canal cements and concluded that there is no significant difference in seal related to the irrigant at both the 90 and 180 day observation periods.

Ferraz et al (2001)[14] assessed the chlorhexidine gluconate gel as an endodontic irrigant. The ability of chlorhexidine gel to disinfect root canals contaminated in vitro with *Enterococcus faecalis* was investigated and concluded that chlorhexidine gluconate in gel form has potential for use as an endodontic irrigant.

Rolph et al (2001)[37] stated that a relatively wide range of bacteria have been isolated from root canals using standard culture techniques. It is estimated that less than 20% of bacteria in the environment are cultivable, with that percentage increasing to 50% for clinical cultivation techniques for bacteria from the oral cavity, leading to the suggestion that a large number of bacteria are still uncultivable using conventional techniques

Lima et al (2001)[25] evaluated the effectiveness of alternative medications in eliminating *E.faecalis* biofilms and concluded that the association of clindamycin with metronidazole significantly reduced

the number of cells in 1day biofilms. However of all medications tested, only 2% chlorhexidine-containing medications were able to thoroughly eliminate most of both 1-day and 3 day *E.faecalis* biofilms.

Siqueira et al (2002)[45] evaluated the prevalence of *Actinomyces species, streptococci* and *Enterococcus faecalis* in primary root canal infections by using a molecular genetics method and concluded that the most prevalent species were members of the Streptococcus anginosus group. With regard to the asymptomatic lesions, the most prevalent species were *S.intermedius ,E.faecalis , and S.anginosus,S.constellatus* was the most prevalent species in pus samples.

Sunde et al (2002)[43] investigated the periapical microbiota of 36 teeth with refractory apical periodontitis and demonstrated that a wide variety of microorganisms, particularly Gram-positive ones, in the periapical lesions of teeth with refractory apical periodontitis

Estrela et al (2002)[10] stated that, the choice of an irrigating solution for use in infected root canals requires previous knowledge of the microorganisms responsible for the infectious process as well as the properties of different irrigating solutions. Irrigating solutions

must have expressive antimicrobial action and tissue dissolution capacity. Sodium hypochlorite is the most used irrigating solution in endodontics.

Estrela et al (2003)[12] analyzed the antimicrobial effect of 2% sodium hypochlorite (NaOCl) and 2% chlorhexidine (CHX) by agar diffusion test and by direct exposure test. Five microorganisms: *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans* and one mixture of these were used and concluded that the best performance of antimicrobial effectiveness of NaOCl was observed in the direct exposure test, and of CHX was observed in the agar diffusion test.

Ferguson et al (2003)[13] studied the Effect of Chlorhexidine Gluconate as an Endodontic Irrigant on the Apical Seal and concluded that chlorhexidine gluconate irrigant did not adversely affect the apical seal of three root canal cements at 270 and 360 days.

Sundqvist et al (2003)[44] stated that infection of the root canal is not a random event. The type and mix of the microbial flora develop in response to the surrounding environment. Factors that influence whether species die or survive are the particular ecological

niche, nutrition, anaerobiosis, pH and competition or cooperation with other microorganisms.

Portenier et al (2003)[34] stated that, *Enterococcus faecalis* is probably the species that can best adapt to and tolerate the ecologically demanding conditions in the filled root canal and survive harsh conditions. In endodontics, *E.faecalis* is rarely present in primary apical periodontitis, but it is the dominant microorganism in root-filled teeth presenting with post-treatment apical periodontitis. Eradication of *E.faecalis* from the root canal with chemomechanical preparation and using disinfecting irrigants and antibacterial dressings is difficult.

Lynne et al (2003)[24] evaluated the antimicrobial activity of several medication preparations in root canal dentin infected with *Enterococcus faecalis* and suggests that suggest that 10% $Ca(OH)_2$ may be more effective than Peridex or 10% $Ca(OH)_2$ in Peridex for the elimination of *E.faecalis* from dentin tubules

Ercan et al (2004)[11] studied Antibacterial Activity of 2% Chlorhexidine Gluconate and 5.25% Sodium Hypochlorite in Infected Root Canal and concluded that both chlorhexidine gluconate and sodium hypochlorite were significantly effective to reduce the

microorganisms in the teeth with necrotic pulp, periapical pathologies, or both, and could be used successfully as an irrigant solution.

Amorium et al (2004)[3] studied the minimum inhibitory concentrations of chlorhexidine digluconate and paramono-chlorophenol against microorganisms commonly found in endodontic infections. Both agents were tested by agar dilution tests. The MIC of chlorhexidine for *e. Faecalis* was 3.33µg/ml.

Orstavik et al (2004)[33] reviewed the virulence factors of *E. faecalis* that may be related to endodontic infection and the periradicular inflammatory response and stated that the most-cited virulence factors are aggregation substance, surface adhesins, sexpheromones, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin. Each of them may be associated with various stages of an endodontic infection as well as with periapical inflammation.

Goldberg et al (2004)[16] evaluated the effect on root dentin microhardness of 2.5% and 6% sodium hypochlorite solutions for various irrigation periods and concluded that a decrease in

microhardness was found at 500 µm between the control and samples irrigated with 6% NaOCl and 2.5% NaOCl at all irrigation periods.

Ari et al (2004)[4] evaluated the effect of 0.2% chlorhexidine gluconate on the microhardness and roughness of root canal dentin compared with widely used irrigation solutions and concluded that all the irrigation solutions except chlorhexidine significantly decreased microhardness of root canal dentin ($p < 0.05$); 3% H_2O_2 and 0.2% chlorhexidine gluconate had no effect on roughness of the root canal dentin.

Luis Cha´Vez De Paz et al (2004)[9] stated that recent years have seen a renewed interest in Gram-positive facultatives as these organisms are common in samples from root-filled teeth affected by apical periodontitis. Structural components of the robust bacterial cell wall of Gram positives protect them from noxious environmental factors. Additionally, the majority of these organisms express fast-adaptive properties when exposed to extreme conditions, thus making them potentially interesting as causal elements in post-treatment endodontic disease.

Love et al (2004)[23], concluded that bacterial invasion of coronal dentinal tubules occurs when the dentine is exposed to the

oral environment and of radicular dentinal tubules subsequent to infection of the root canal system or as a consequence of periodontal disease. The content and architecture of a dentinal tubule can influence bacterial invasion, with tubule patency being important.

Gulabivala et al (2005)[17] stated that the principal aim of root canal preparation is to obtain and maintain access to the apical anatomy, for the purpose of delivering antimicrobial agents to the infection in this site. A combination of NaOCl and EDTA remains the irrigant of choice for both smear layer removal and bacterial debridement; however, their effectiveness in the apical anatomy depends upon a careful regimen and adequate mechanical preparation.

Haapasalo et al (2005)[18] concluded that debridement of the root canal by instrumentation and irrigation is considered the most important single factor in the prevention and treatment of endodontic diseases and the effects of instrumentation and irrigation on intracanal infection have been a focus of increased activity in endodontic research. Although sterility of the root canal can occasionally be achieved by instrumentation and irrigation with antibacterial

solutions, the protocols used today cannot predictably provide sterile canals.

Tandjung et al (2007)[47] investigated the antimicrobial activity of octenidine on *Enterococcus faecalis* ATCC 29212. Octenidine-phenoxyethanol gel (1:1) was applied for different timing and concluded that Octenidine was effective against *E.faecalis* in dentin disinfection.

Estrela et al (2008)[12] evaluated the efficacy of the sodium hypochlorite (NaOCl) and chlorhexidine (CHX) on *Enterococcus faecalis* by systematic review and meta-analysis. They concluded that, NaOCl or CHX showed low ability to eliminate *E.faecalis* when evaluated either by PCR or culture techniques.

Dogan et al (2008)[8] evaluated the efficacy of common antiseptic mouth rinses and octenidine dihydrochloride (OCT) and concluded that OCT had a significantly greater inhibitory effect on the studied bacteria than 0.2% chlorhexidine gluconate (CHX) and 7.5% polyvinylpyrrolidone-iodine complex (PVP-I) from 15 min to 120 min following the application ($p < 0.01$).

Mohammadi (2008)[31] stated that Sodium hypochlorite, an excellent non-specific proteolytic and antimicrobial agent, is the most common irrigation solution used during root canal therapy. It has a wide range activity against both Gram positive and Gram negative bacteria. Sodium hypochlorite is the strongest antifungal agent among root canal irrigations and medications.

Tirali (2009)[46] compared the in vitro effectiveness of sodium hypochlorite (NaOCl), and octenidine hydrochloride (Octenisept) at different concentrations in the elimination of resistant microorganisms including *S. aureus, E. faecalis*, and *C. albicans* over a range of time intervals and concluded that The antimicrobial action is related to type and concentration of the irrigants as well as the microbial susceptibility.

MATERIALS AND METHODS

MATERIALS

1. 50 extracted intact human mandibular premolar
2. 0.1%Octenidine dihydrochloride
3. 2% Chlorhexidine
4. 99.0%pure Triclosan
5. 5% sodium Hypochlorite
6. Normal Saline(0.9%w/w)
7. 0.2% Sodium azide solution
8. 1% Sodium Hypochlorite
9. 10% citric acid
10. Syto -9 dye
11. Light cure composite
12. Nail varnish
13. Aerobic mixture
14. Mueller-Hinton Broth
15. Mueller-Hinton Agar
16. Sheep blood

ARMAMENTARIUM

1. Vortex mixer
2. Airoter
3. Contra-angled latch type hand piece
4. Straight hand piece
5. Micromotor
6. Abrasive trimmer
7. K-files(15-40)
8. Gates Gliden drill no. 2 and no.3
9. Autoclave
10. Incubator
11. Sonicator
12. Epifluorescence microscope
13. Laminar flow chamber
14. Eppendorf tubes

For microbiological testing

1. *Enterococcus faecalis* ATCC 29212
2. Micropipettes- 5µl-50µl and 100µl-1000µl
3. Disposable micropipette tips-50µl
4. Forceps

5. 5ml syringes and 27 gauge disposable needles
6. Glass beaker
7. Glass Test tubes
8. Cotton plugger
9. Tin foil
10. Gates Gliden drill no.3 and 4
11. Sterilized disposable Petridish
12. Bacteriological loop

Preparation of tooth specimens:

Fifty single-rooted human intact mandibular premolar teeth were collected. The teeth had no carious lesions and were previously not root filled. Digital images of the teeth were captured to eliminate teeth other than single root with single canal. Calculus and tissue remnants were removed using ultra sonic scalers. The teeth were incubated in 0.2% sodium azide solution for 14 days before their preparation. The teeth were de-coronated with a diamond disc under water cooling, and the teeth were shortened, resulting in 10 mm of remaining root length. The specimens were kept in tap water during all procedures to avoid dehydration. The root canals were enlarged to a size 40 with k files under constant irrigation with a total of 10 ml

NaOCl (1%).It was then rinsed with 10 ml citric acid (10%) for 5 min to remove smear layer and, finally with 20 ml of physiological saline. The root surface was coated with nail varnish, and apex was sealed using light cure composite resin.

Sterilization of tooth specimens:

The tooth specimens were sterilized in an autoclave at 121^0C for 15 min under 15lbs of pressure. The teeth were then transferred to individual sterile Eppendorf tubes and 1ml of Mueller Hinton broth was added to the tubes using a sterile pipette. The EP tubes were sealed with paraffin strips. The EP tubes containing the tooth samples and Mueller Hinton broth were re-sterilized in the autoclave.

Preparation of Media:

Mueller Hinton Broth:

Ingredients	Gms / litre
Beef, infusion form	300.00
Casein acid hydrolysate	17.50
Starch	1.50

pH (at 25^0C) 7.4±0.2

The Mueller Hinton powder was mixed with distilled water in a conical flask. The conical flask was plugged with cotton and

sterilized in an autoclave at 121^0C for 15 min under 15 lbs of pressure.

Mueller Hinton Agar:

Beef extract	2.0 gm
Acidicase Peptone	7.5 gm
Starch	1.5 gm
Agar	17.0 gm

Distilled water

The ingredients were dissolved in distilled water and mixed thoroughly. It was heated with frequent agitation and was boiled for one min. The pH was adjusted to 7.4±2. The agar was sterilized in an autoclave at 121^0C for 15 min under 15 lbs of pressure.

Preparation of Media plate:

The prepared sterilized Mueller Hinton agar was poured into sterilized petridish to a depth of 5mm, under laminar flow. For every 100ml of the medium, 5 plates were poured. The poured plates were allowed to solidify and refrigerated. For every batch of prepared plates, one plate was kept for sterility check for 24 hrs in the incubator at 37^0C.

Preparation of Broth Tubes:

The prepared sterilized Mueller-Hinton broth was dispersed in quantity of 20 ml in sterile screw capped test tubes. One tube was kept for sterility checking at 37 ˚C for 48hrs and one tube was used as control to check the performance to support the growth of *E.faecalis.*

Inoculation of the dentin blocks with *E.faecalis*

Isolated 48 hour colonies of *E.faecalis* (ATCC 29212) grown on sheep blood agar were suspended in 5ml of Mueller Hinton broth and was adjusted resulting in a total density of approximately 2.4 x 10^5cfu ml^{-1} (McFarland's Standard 0.5). 50µl of the inoculum was transferred to individual Eppendorf tubes containing 1 ml of Mueller Hinton broth and dentin block. The blocks were incubated at 37°C for 7 days and every second day the blocks were transferred to fresh tubes containing 1 ml of broth contaminated with 50µl of *E.faecalis*. Broth purity was checked by sub culturing 5µl on sheep blood agar.

ANTIBACTERIAL ASSESSMENT

DENTINE SAMPLING:

Dentin blocks were incubated at 37°C in Mueller- Hinton broth for 7 days. The blocks were divided into 5 groups containing 10 teeth each

Group I- Octinidine Dihydrochloride (0.1%)

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of 0.1% Octenidine dihydrochloride for 1 min. After irrigation, the dentin block was dried with sterile paper points. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 &No.3. The fine dentine chips obtained were collected in an Eppendorf tube containing 1 ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min.The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

Group II- Triclosan (99.0% pure)

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of 99.0%pure Triclosan for 1 min. After irrigation, the dentin block was dried with sterile paper points. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 &No.3. The fine dentine chips obtained were collected in an Eppendorf tube containing 1ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

Group III- Chlorhexidine (2%)

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of 2% Chlorhexidine gluconate for 1 min. After irrigation, the dentin block was dried with sterile paper points. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 &No.3. The fine dentine chips obtained were collected in an Eppendorf tube containing 1 ml of phosphate buffered saline and 3

small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

Group IV- Sodium hypochlorite(5%)

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of 5% Sodium hypochlorite for 1 min. After irrigation, the dentin block was dried with sterile paper points. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 &No.3.The fine dentine chips obtained were collected in an Eppendorf tube containing 1 ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

Group V- Control (The control was not subjected to any irrigation)

Each block was carefully removed from the broth and held with artery forceps. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 &No.3. The fine dentine chips obtained were collected in an Eppendorf tube

containing 1 ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

MICROBIOLOGICAL ANALYSIS:

Proportion of viable bacteria (PVB) using epifluorescence microscope:

0.5 ml of the undiluted sample from each specimen was vortexed for 5 min, washed once with sterile saline, harvested by centrifugation and resuspended with 100µl of staining solution (syto 9 and propidium iodide) using live/dead backlight bacterial viability kit. After incubation in a dark chamber for 15 min, the sample was centrifuged, and the supernatant was discarded. The pellet was resuspended in 10µl of distilled water. It was then immediately analysed under epifluorescence microscope which labels viable bacteria by green fluorescence (excitation FITC 450-490nm) and propidium iodide which marks dead bacteria by red fluorescence (excitation Rhodamine 540nm). Visual fields at a magnification of 1000X were recorded. PVB was calculated as the number of bacterial

cells associated with green fluorescence divided by the total number of bacteria emitting either green/ red fluorescence.

Colony count by CFU:

From 0.5 ml aliquot of each specimen, 10µl was diluted as $10^{-}1$, $10^{-}2$, $10^{-}3$, $10^{-}4$, $10^{-}5$ and $10^{-}6$ in saline. Subsequently from each tube 10µl of the diluted sample inoculated on to the Mueller-Hinton blood agar plate and inoculums was uniformly spread on the surface of the plate .The plates were incubated at 37˚C for 48 hrs. The no of colonies were counted and final CFU was expressed after taking into consideration the dilution factors and expressed as CFU/ml.

Minimum Inhibitory Concentration (MIC) & Mini mum Bactericidal Concentration (MBC)

The minimum concentration of an antimicrobial used is µg per ml.

Octenidine(0.1%)

Minimum inhibitory concentration of octenidine was determined by working out the initial concentration of 100 µg/ml. A further dilution of 1/10 was made using saline to arrive at a concentration of 10 µg/ml. A serial dilution was made by mixing 0.5ml of 10 µg/ml of octenidine with 0.5ml Mueller-Hinton broth. Further doubling dilution were made by transferring 0.5ml into

subsequent tubes. The range thus obtained was from 5µg/ml through 0.0015 µg/ml. control tube was prepared by adding 0.5ml of Mueller-Hinton broth. To all the tubes 0.05 ml of *E.faecalis* which was grown at 37˚Cfor 48 hrs and opacity adjusted to McFarland standard of 0.5 was added .The tubes were incubated at 37˚C for 18 hrs. Minimum inhibitory concentration was determined by as the lowest concentration of octenidine showing inhibition of growth by absence of turbidity.

After the MIC readings were recorded, 5µl of suspension was subcultured from all tubes including control on Mueller-Hinton blood agar plates and incubated at 37˚C for 48 hrs. MBC was determined as the lowest concentration of the antimicrobial agent in µg/ml showing no growth.

Triclosan (99.0% pure)

Minimum inhibitory concentration of triclosan was determined by working out the initial concentration of 400 µg/ml from the pure substance, along with equal volume of absolute alcohol and distilled water. A further dilution was made using saline to arrive at a concentration of 200µg/ml. A serial dilution was made by mixing 0.5ml of µg/ml of triclosan with 1 ml Mueller-Hinton broth .Further

doubling dilution were made by transferring 0.5 ml into subsequent tubes. The range thus obtained was from 200µg/ml through 6.25µg/ml. Control tube was prepared by adding 0.5 ml of Mueller-Hinton broth. To all the tubes 0.05ml of *E.faecalis* which was grown at 37˚C for 48 hrs and opacity adjusted to McFarland standard of 0.5 was added .The tubes were incubated at 37˚C for 18 hrs . Minimum inhibitory concentration was determined by the lowest concentration of triclosan showing inhibition of growth by absence of turbidity.

After the MIC readings were recorded, 5µl of suspension was subcultured from all tubes including control on Mueller-Hinton blood agar plates and incubated at 37˚C for 48 hrs. MBC was determined as the lowest concentration of the antimicrobial agent in µg/ml showing no growth.

Chlorhexidine (2%)

Minimum inhibitory concentration of chlorhexidine was determined by working out the initial concentration of 200 µg/ml. A further dilution of 1/75 was made using saline to arrive at a concentration of 26.64 µg/ml. A serial dilution was made by mixing 0.5ml of 26.64 µg/ml of chlorhexidine with 0.5 ml Mueller-Hinton broth .Further doubling dilution were made by transferring 0.5ml into

subsequent tubes. The range thus obtained were from 13.32µg/ml through 0.83µg/ml. control tube was prepared by adding 0.5ml of Mueller-Hinton broth. To all the tubes 0.05ml of *E.faecalis* which was grown at 37˚C for 48 hrs and opacity adjusted to McFarland standard of 0.5 was added .The tubes were incubated at 37˚C for 18 hrs . Minimum inhibitory concentration was determined by the lowest concentration of chlorhexidine showing inhibition of growth by absence of turbidity.

After the MIC readings were recorded, 5µl of suspension was subcultured from all tubes including control on Mueller-Hinton blood agar plates and incubated at 37˚C for 48 hrs. MBC was determined as the lowest concentration of the antimicrobial agent in µg/ml showing no growth.

Sodium hypochlorite (5%)

Minimum inhibitory concentration of Sodium Hypochlorite was determined by working out the initial concentration of 5000 µg/ml. A further dilution of 1/ 2.5ml was made using saline to arrive at a concentration of 2000µg/ml. A serial dilution was made by mixing 0.5ml of 2000µg/ml of NaOCl with 0.5ml Mueller-Hinton broth. Further doubling dilutions were made by transferring 0.5ml

into subsequent tubes. The range thus obtained was from 2000µg/ml through 0.49µg/ml. Control tube was prepared by adding 0.5ml of Mueller-Hinton broth. To all the tubes 0.5ml of *E.faecalis* which was grown at 37˚C for 48 hrs and opacity adjusted to McFarland standard of 0.5 was added .The tubes were incubated at 37˚C for 18 hrs. Minimum inhibitory concentration was determined by the lowest concentration of NaOCl showing inhibition of growth by absence of turbidity.

After the MIC readings were recorded, 5µl of suspension was subcultured from all tubes including control on Mueller-Hinton blood agar plates and incubated at 37˚C for 48 hrs. MBC was determined as the lowest concentration of the antimicrobial agent in µg/ml showing no growth.

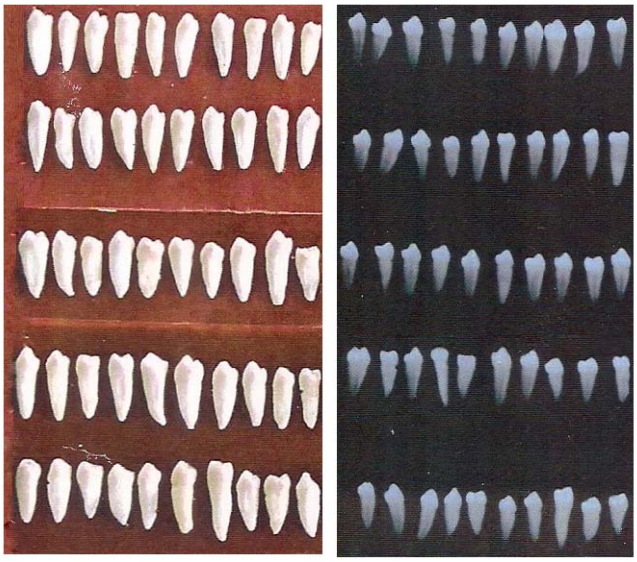


Fig.1: EXTRACTED HUMAN MANDIBULAR PREMOLAR

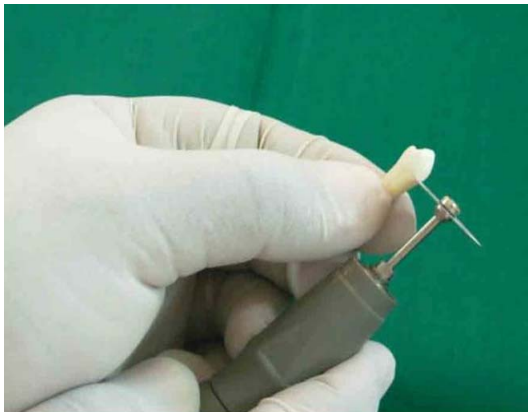


Fig.2 : DECORONATION

Fig.3: ARMAMENTARIUM USED FOR MICROBIOLOGICAL ANALYSIS

Fig. 4 : AUTOCLAVE

Fig. 5: ELECTRONIC BALANCE

Fig.6: CO_2 INCUBATOR

Fig. 7: VORTEX MIXER

Fig.8: CENTRIFUGE

Fig.9:BIO SAFETY INOCULATION HOOD(LAMINAR AIR FLOW CHAMBER)

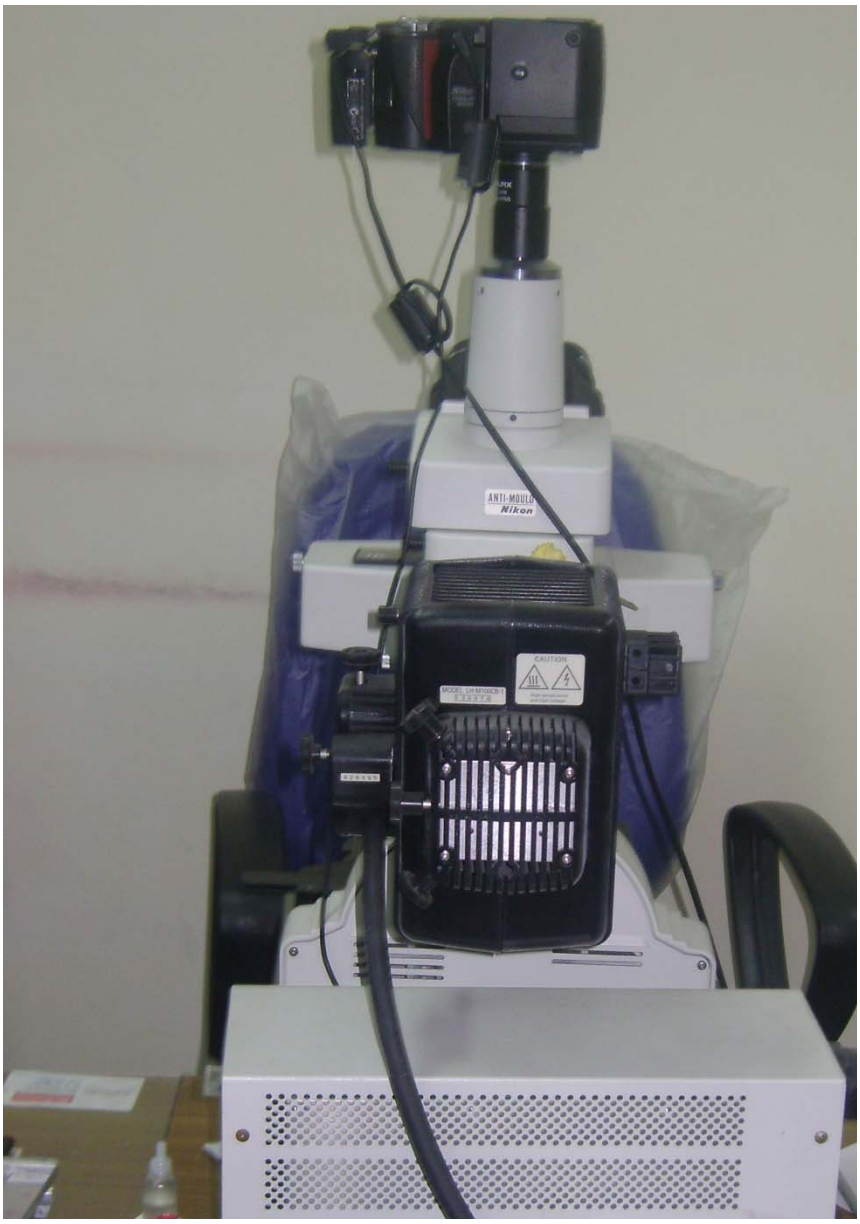


Fig. 10: EPIFLUORESCENCE MICROSCOPE

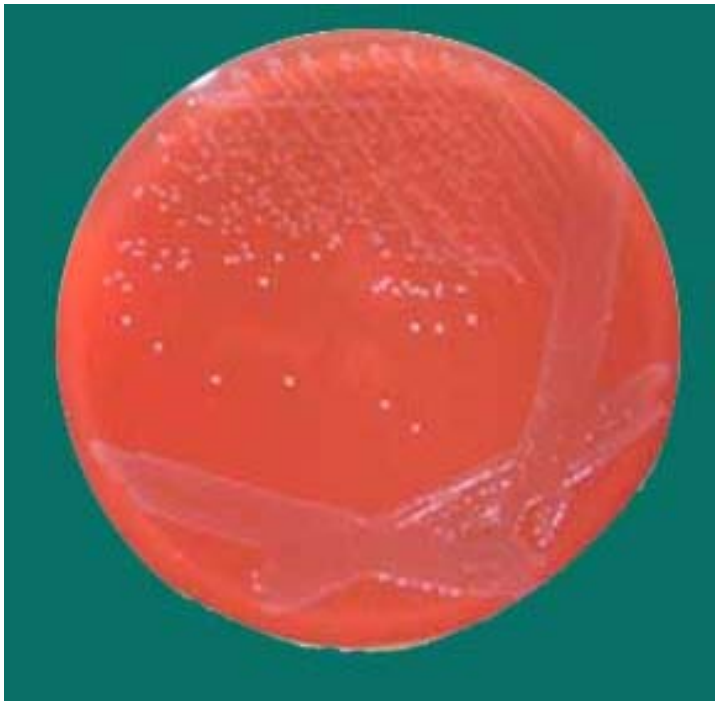


Fig.11: ENTEROCOCCUS FAECALIS ATCC 29212

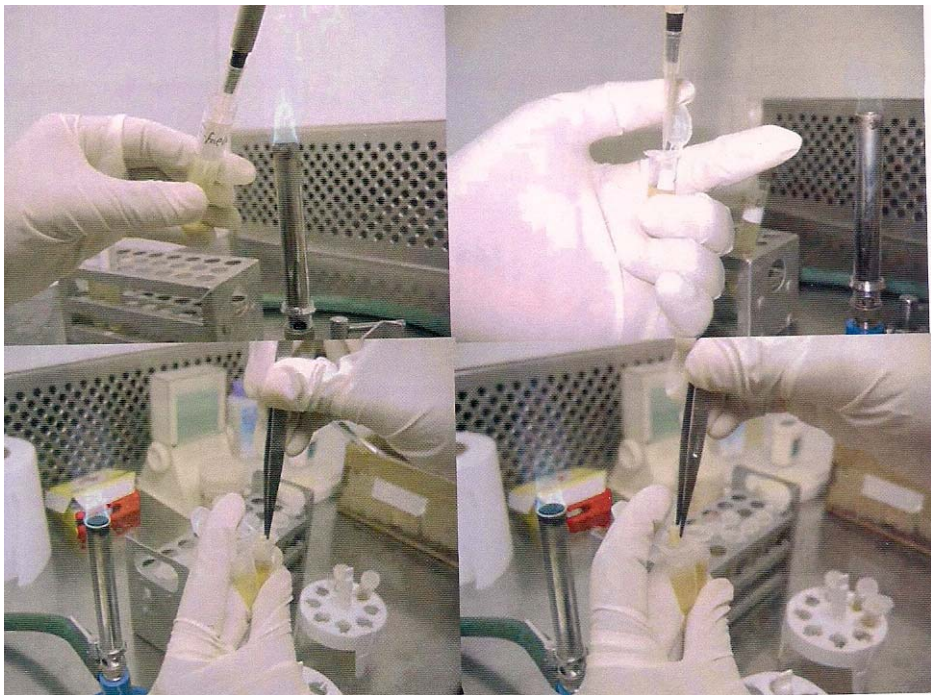


Fig.12: INOCULATION OF THE TEETH WITH E.FAECALIS

Fig.13: DENTIN BLOCKS IN MUELLER HINTON BROTH

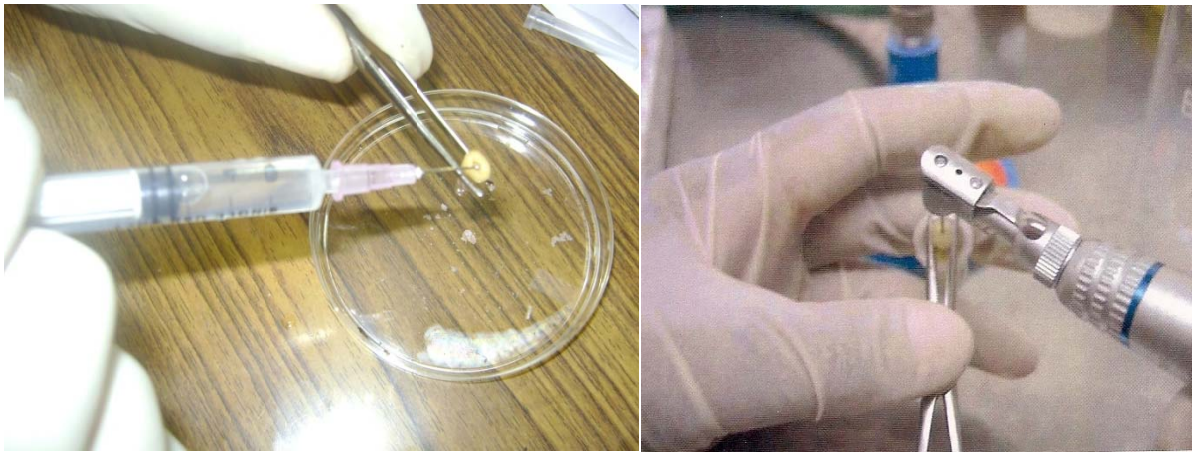


Fig.14: IRRIGATION AND COLLECTING OF DENTINE DEBRIS

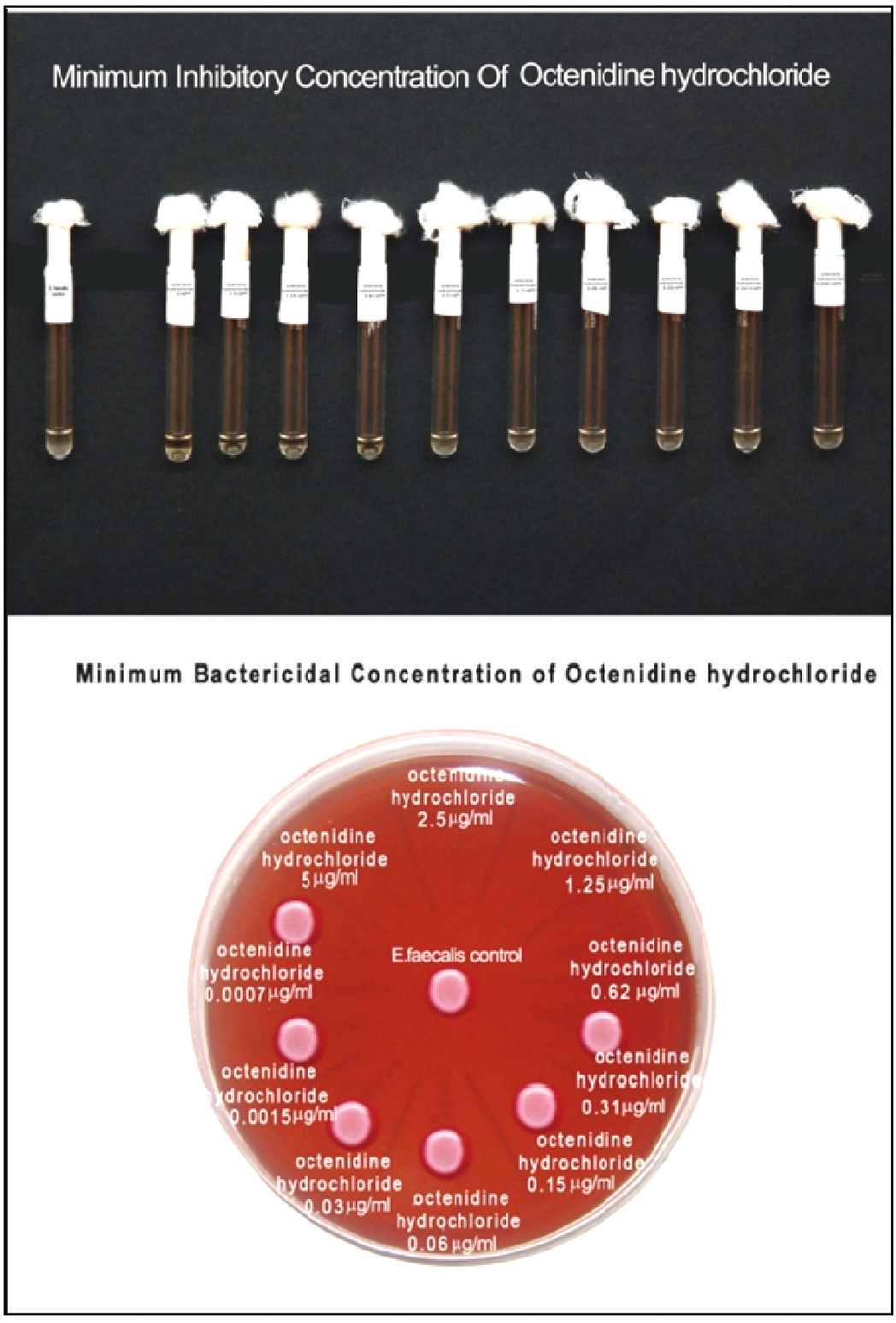


Fig.15: MIC AND MBC OF OCTENIDINE

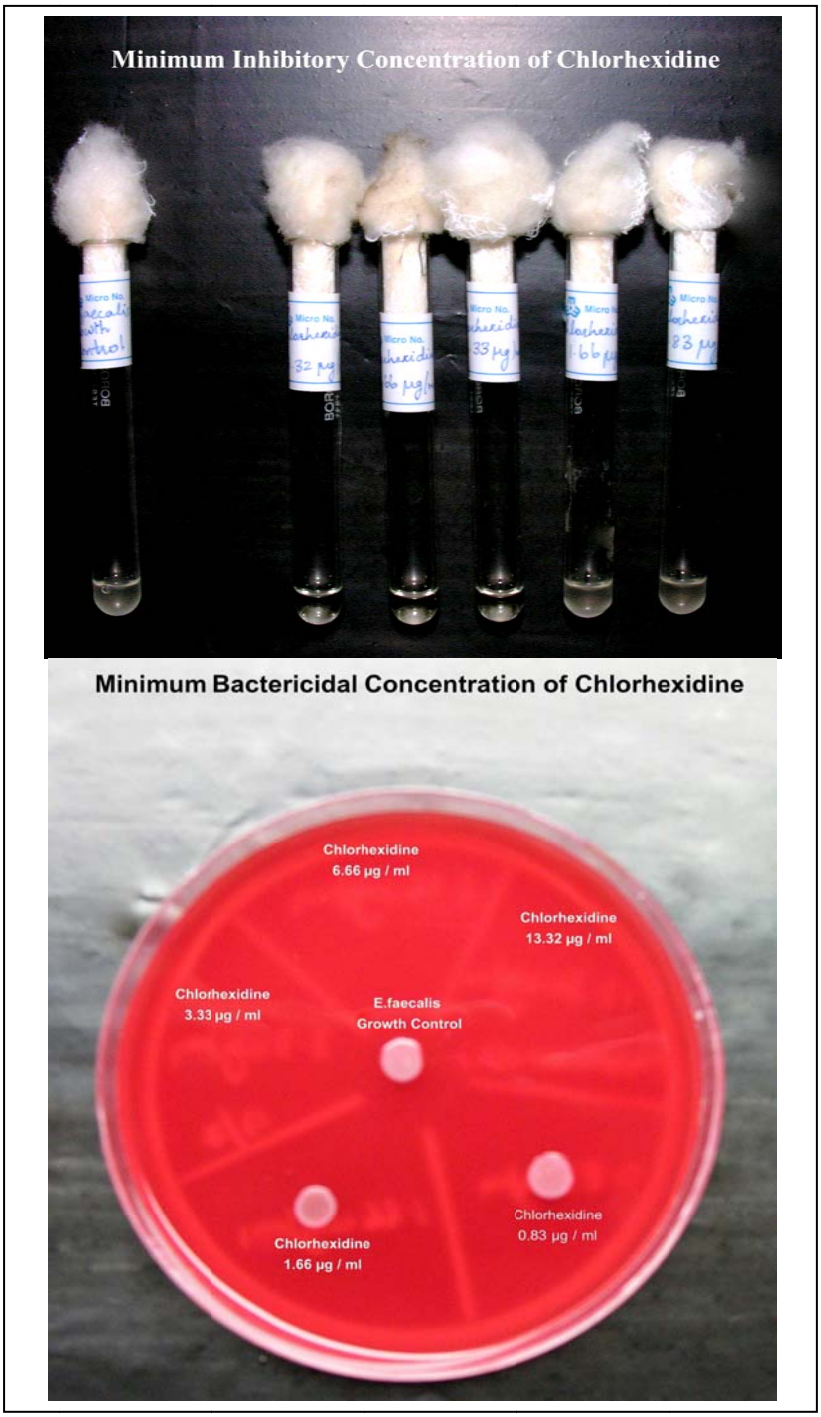


Fig.16: MIC AND MBC OF CHLORHEXIDINE

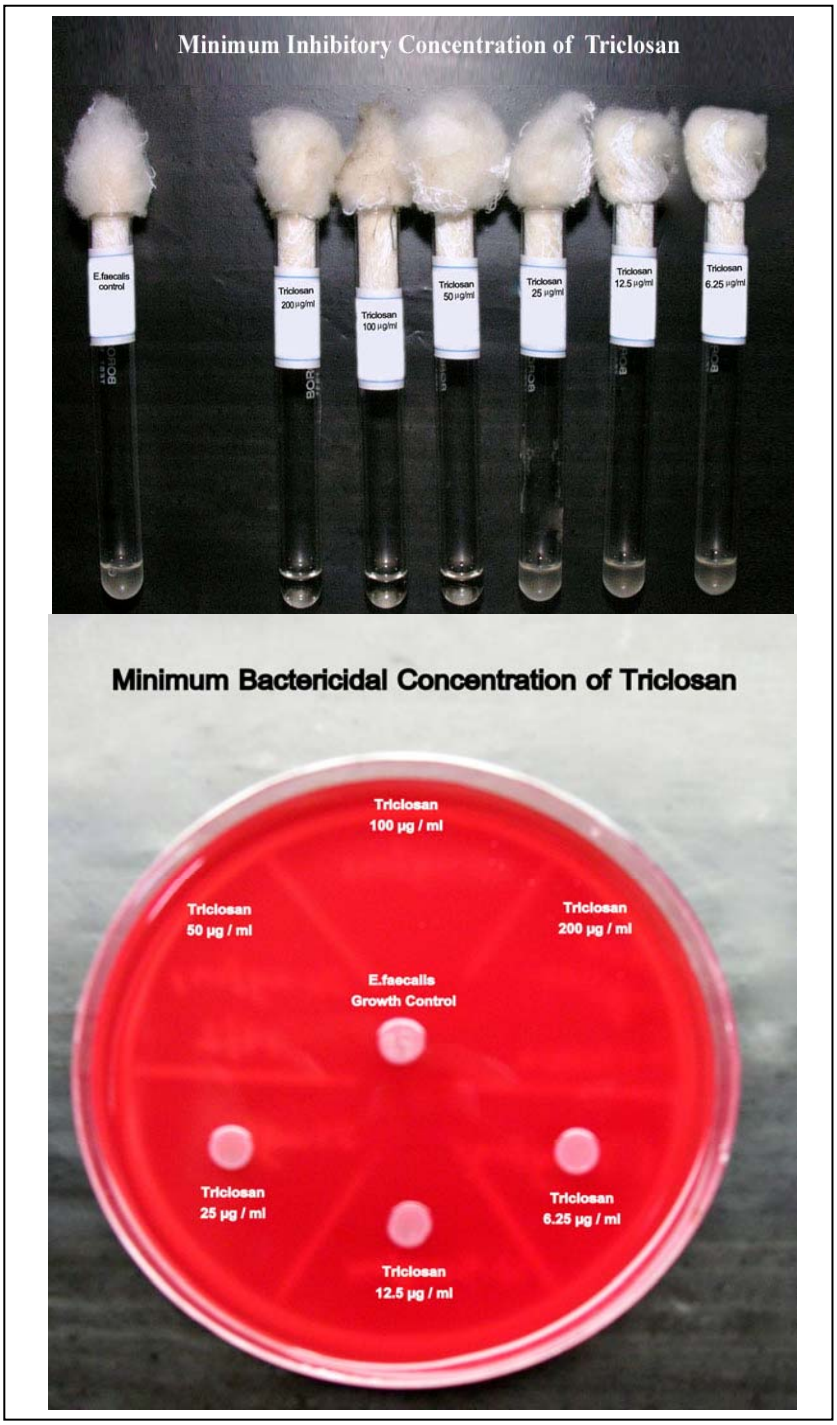


FIG.17: MIC AND MBC OF TRICLOSAN

Fig.18: MIC AND MBC OF SODIUM HYPOCHLORITE

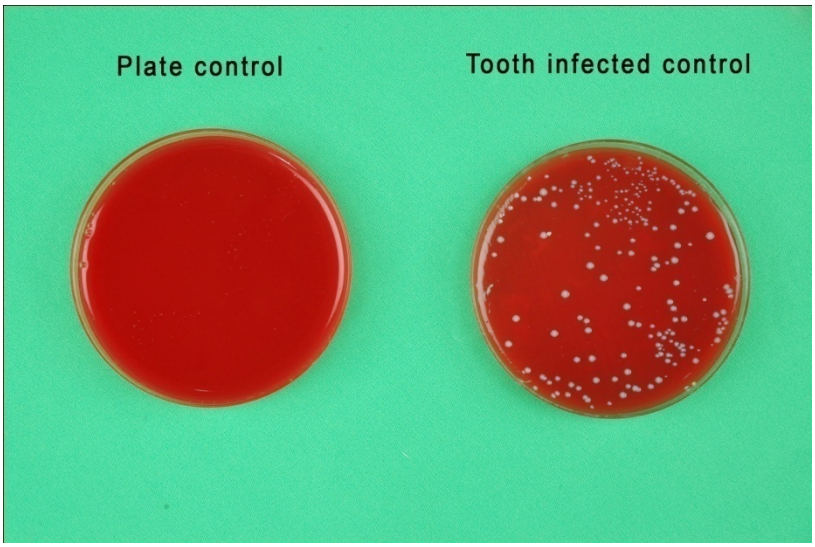


Fig. 19 : CULTURE PLATES OF CONTROL GROUP BEFORE AND AFTER INOCULATION

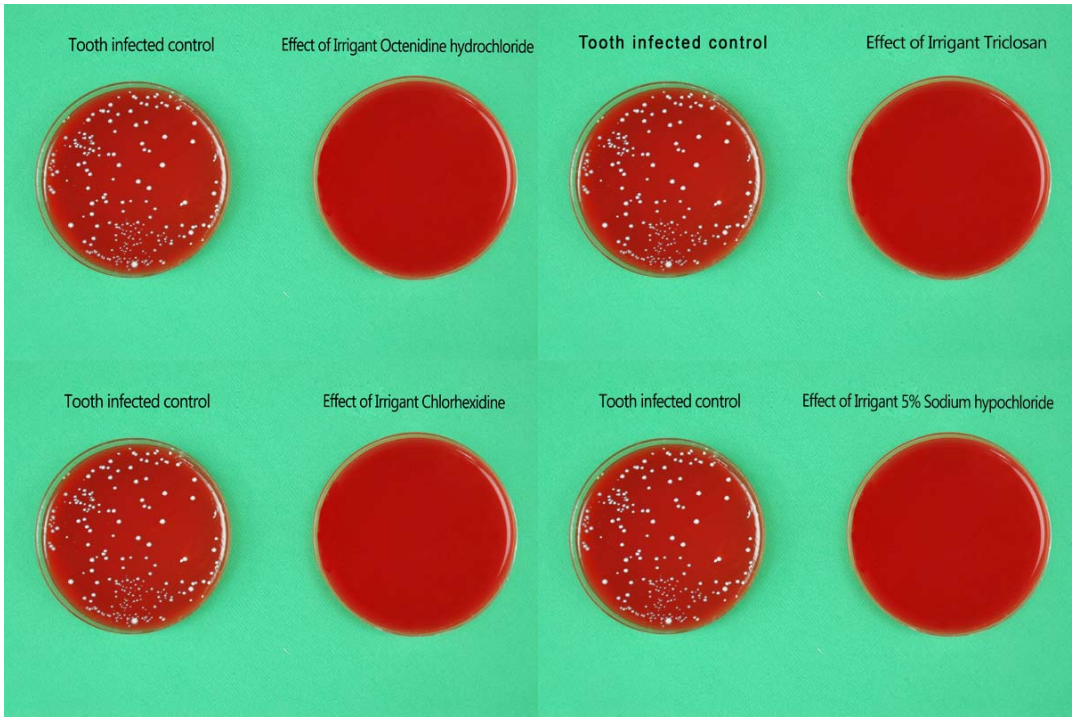


Fig.20: RESULTS OF COLONY COUNT BEFORE AND AFTER IRRIGATION OF GROUP I, II, III & IV

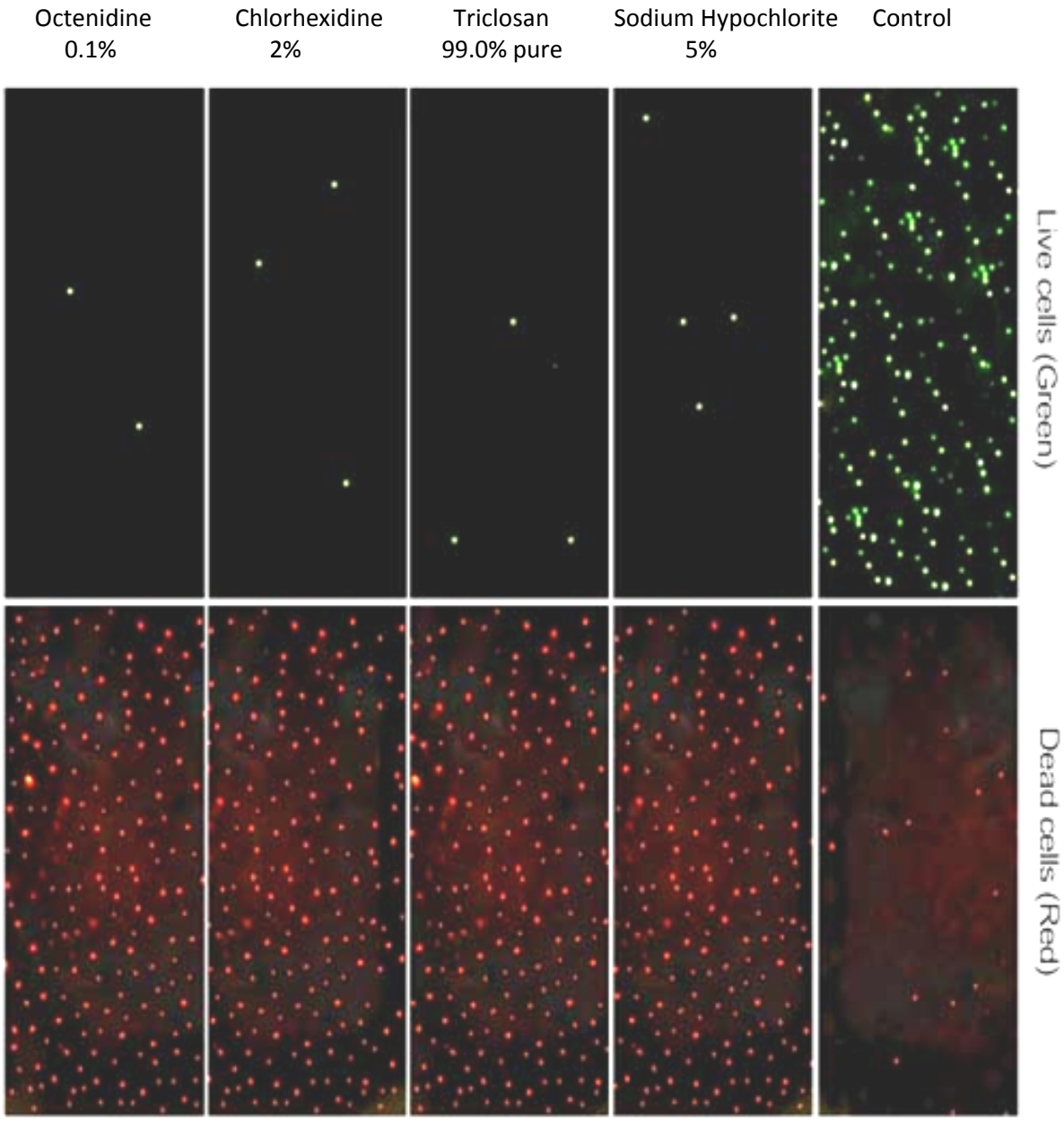
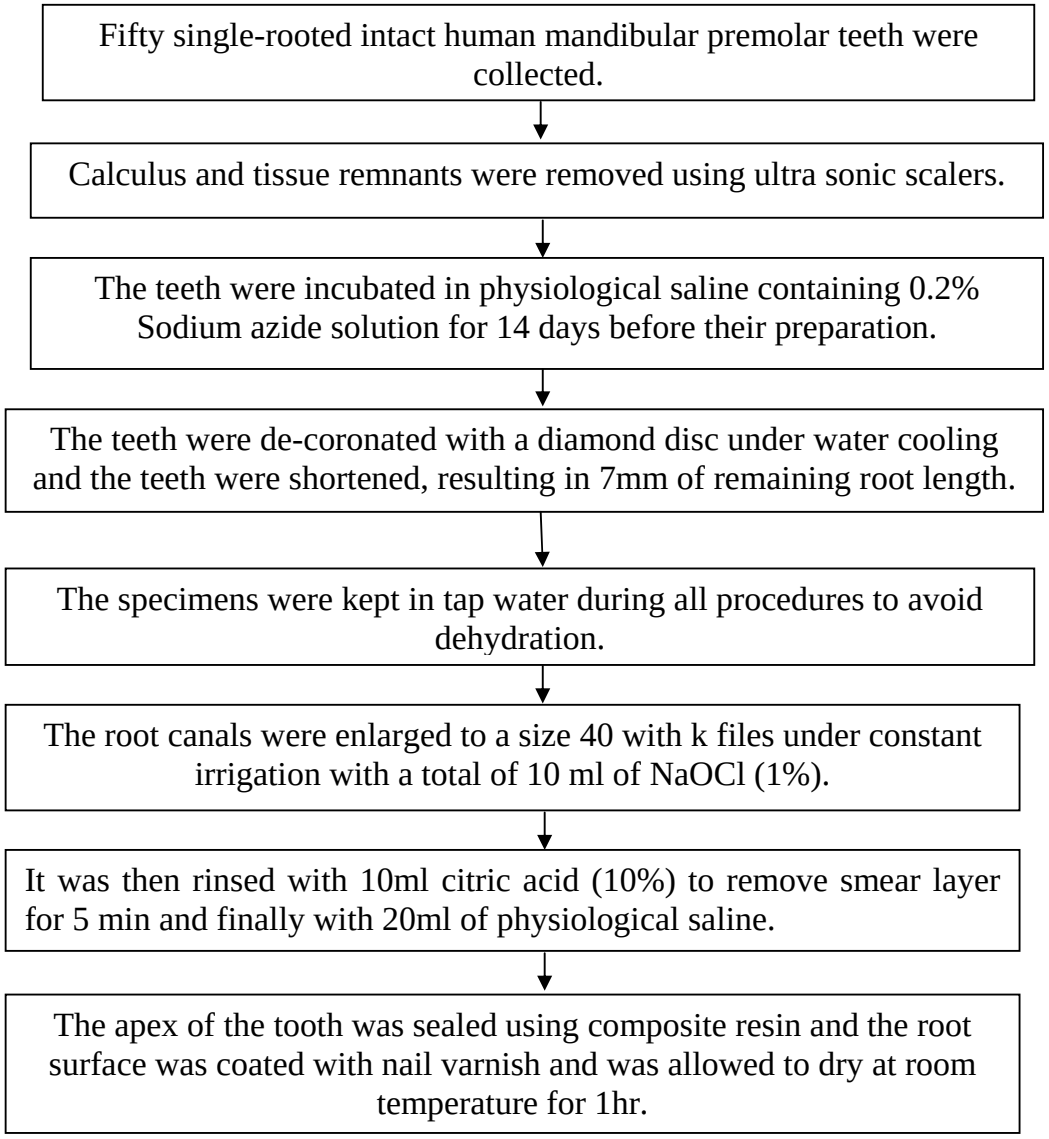


Fig. 21 : RESULTS OF SYTO 9 STAINING FOR PVB AFTER IRRIGATION

METHODOLOGY



The specimens were autoclaved at 121°C for 15 min. For sterility check, the tubes were incubated at 37°C for 48hrs.

↓

After sterilization the specimens were handled aseptically and all subsequent procedures took place in a laminar air flow chamber to avoid contamination.

↓

Inoculation of the dentine blocks with E.faecalis

Enterococcus faecalis was grown in Mueller-Hinton agar at 37°C for 48hr and was suspended in Mueller-Hinton broth, corresponding to an optical density of 0.5 on the McFarland scale.

↓

50 µl of the inoculum was transferred to individual Eppendorf tubes containing 1 ml of Mueller Hinton broth and dentin block.

↓

The blocks were incubated at 37°C for 7 days and every second day the blocks were transferred to fresh tubes containing 1 ml of broth contaminated with 50µl of *E.faecalis*.

↓

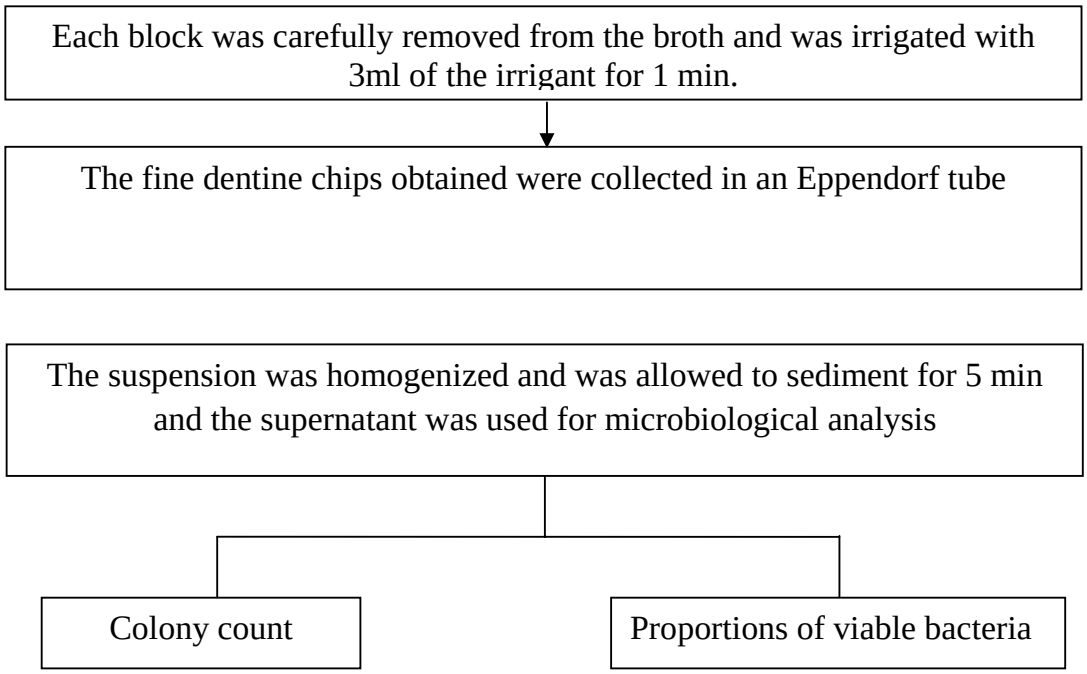
Broth purity was checked by sub culturing 5µl on sheep blood agar.

↓

Antibacterial assessment

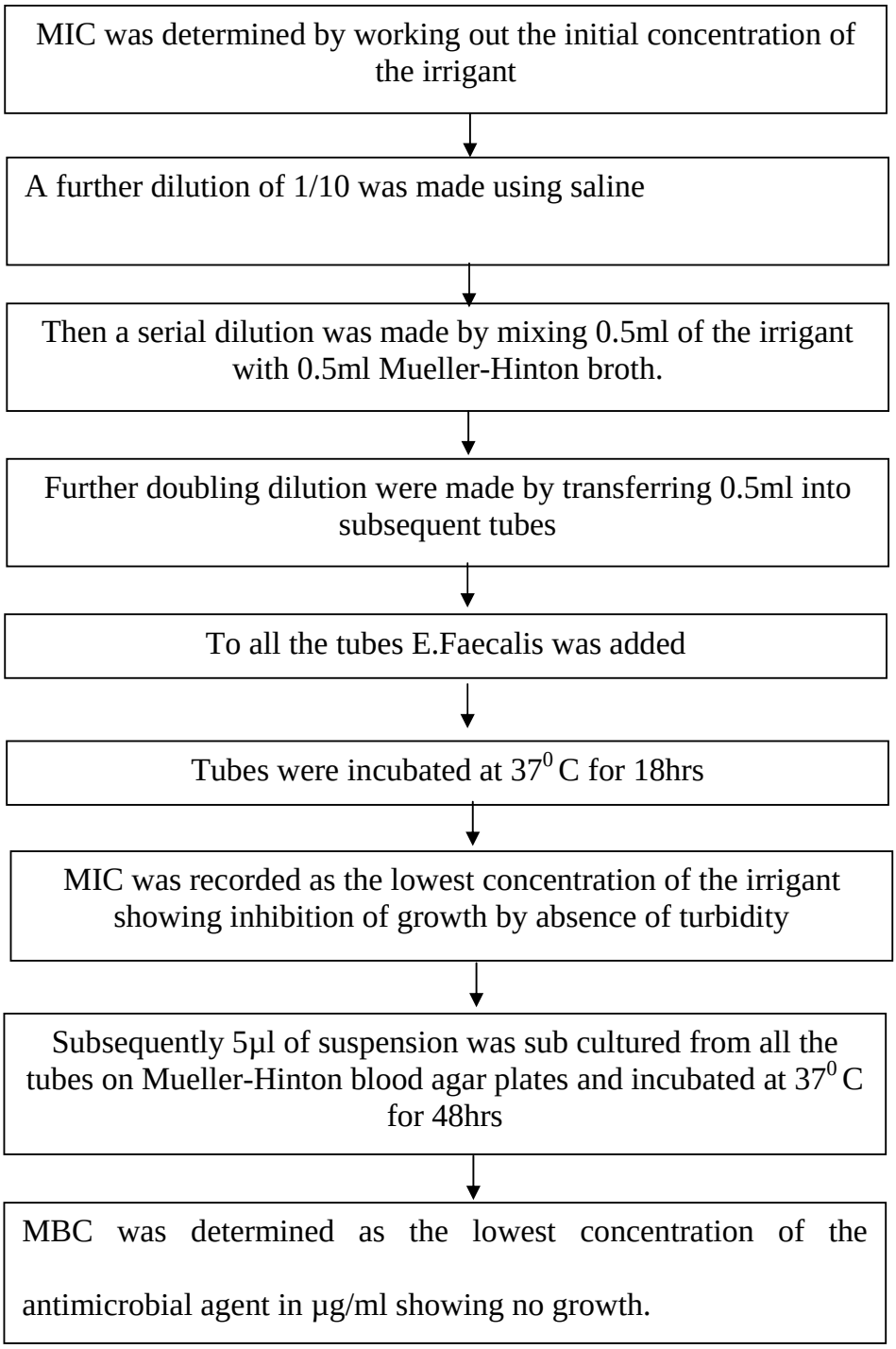
Dentin blocks were incubated at 37^0 C in Mueller- Hinton broth for 7 days. The blocks were divided into 5 groups containing 10 teeth each

Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Octenidine (0.1%)	Chlorhexidine (2%)	Triclosan (99.0%)	NaOCl(5%) (Control)	Negative Control (no irrigation)


Each block was carefully removed from the broth and was irrigated with 3ml of the irrigant for 1 min.
The fine dentine chips obtained were collected in an Eppendorf tube
The suspension was homogenized and was allowed to sediment for 5 min and the supernatant was used for microbiological analysis
Colony count
Proportions of viable bacteria

Minimum Inhibitory Concentration (MIC)&Minimum

Bactericidal Concentration (MBC)



RESULTS

The results of the present study were subjected to statistical analysis to interpret the significant differences among the intracanal irrigants.

The Mann-Whitney U test is a non parametric equivalent of the unpaired t-test and can be used when the sample size and group are balanced, that is the sample sizes in all the groups is the same or similar. The test ranks the values within each group and determines the median. Then by a process of ranking the medians, and measuring the distance between the median in relation to the range, it determines the significance of the difference between the medians. Each group is compared with every other group. This test was used in the present study since each group consisted of similar sample size.

Kruskal-Wallis test was used in this study to compare among the irrigants used. This is a non-parametric test. The results suggested that there was statistically significant difference between the control and the irrigants but there was no statistical difference between the irrigants used.

The chi-square test is a homogeneity test. This test is used to check whether the irrigants are similar, or equal or homogenous in some characteristics. The test was used in this study to check if the results were homogenous before and after irrigation ie Alive or Dead micro-organisms.

To summarize the results:

1. The inhibition of growth in all the groups was statistically significant in comparison to control group(no irrigation)
2. Group 1(octenidine) was the most effective among the irrigants against E. faecalis.
3. The inter-group comparison showed no statistical difference between the groups in inhibition.

Table 1: MIC values of Octenidine hydrochloride

Tube	1	2	3	4	5
Potency (μg/ml)	2.5	1.25	0.62	0.31	0.15
	-	-	-	+	+

Table 2: MIC values of Triclosan

Tube	1	2	3	4	5
Potency (μg/ml)	200	100	50	25	12.5
	-	-	-	+	+

Table 3: MIC values of Chlorhexidine

Tube	1	2	3	4	5
Potency (μg/ml)	13.32	6.66	3.33	1.66	0.83
	-	-	-	+	+

Table 4: MIC values of Sodium hypochlorite

Tube	1	2	3	4	5
Potency (μg/ml)	1000	500	250	125	62.5
	-	-	-	+	+

Table 5: comparison of the MIC values of the irrigants

Variables μg/ml	Octenidine (0.1%)	Triclosan (99.99%)	Chlorhexidine (2%)	Na hypochlorite (5%)
MIC	0.62	50	3.33	250

Table 6: comparison of the MBC values of the irrigants

Variables μg/ml	Octenidine (0.1%)	Triclosan (99.99%)	Chlorhexidine (2%)	Na hypochlorite (5%)
MBC	0.62	50	3.33	250

Table 7: Colony count for groups of 10 teeth before and after irrigation

Teeth Sample	Control (cfu/ml)	Octenidine (cfu/ml)	Chlorhexidine (cfu/ml)	Triclosan (cfu/ml)	NaOCl (cfu/ml)
Teeth 1	25000	0	0	0	0
Teeth 2	24500	0	0	0	0
Teeth 3	2500	0	0	0	0
Teeth 4	26000	0	0	0	0
Teeth 5	27000	0	0	0	0
Teeth 6	26500	0	0	0	0
Teeth 7	25500	0	0	0	0
Teeth 8	24000	0	0	0	0
Teeth 9	25000	0	0	0	0
Teeth 10	26000	0	0	0	0
Mean	**25450**	**0**	**0**	**0**	**0**

Table 8: Syto 9 values for teeth samples before and after irrigation [Proportion of viable (green) and dead (red) Enterococcus faecalis]

Teeth Sample	Control		Octenidine		Chlorhexidine		Triclosan		NaOCl	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**
Teeth 1	990	10	11	1250	14	1050	15	1400	16	1150
Teeth 2	1010	2	10	1100	12	1100	14	1450	14	1200
Teeth 3	1020	5	10	1200	11	1040	13	1350	13	1250
Teeth 4	1010	10	11	1250	10	1010	14	1400	14	1350
Teeth 5	1005	8	10	1150	11	1020	12	1250	15	1300
Teeth 6	990	10	10	1200	10	1030	13	1300	14	1200
Teeth 7	1010	5	11	1150	11	1030	14	1450	14	1250
Teeth 8	1005	6	10	1100	12	1020	13	1350	13	1300
Teeth 9	1010	10	11	1200	11	1040	12	1300	14	1250
Teeth 10	1010	5	10	1150	10	1010	13	1350	14	1200
Mean	**1006**	**7.10**	**10.40**	**1175**	**11.20**	**1135**	**13.30**	**1260**	**14.10**	**1245**

STATISTICAL ANALYSIS

COLONY COUNT

INTER-GROUP COMPARISON

Kruskal-Wallis Test

Kruskal Wallis test which is a non-parametric test is used to compare among the agents.

Table 9: inter-group comparison

Group		N	Mean Rank
Values	Control	10	45.50
	Octenidine	10	20.50
	Chlorhexidine	10	20.50
	Triclosan	10	20.50
	NaOCl	10	20.50
	Total	50	

Result:

The results indicate that there is a statistically significant difference among the agents (Chi- Square with 4 degrees of freedom = 48.214, P= 0.000)

Table 10: Over all Table for pair wise comparison:

No	Comparisons	Z - value	P value	Significance
1	Control Vs Octenidine	-4.042	0.000**	Yes
2	Control Vs Chlorhexidine	-4.042	0.000**	Yes
3	Control Vs Triclosan	-4.042	0.000**	Yes
4	Control Vs NaOCl	-4.042	0.000**	Yes
5	Octenidine Vs Chlorhexidine	0.000	1.000	No
6	Octenidine Vs Triclosan	0.000	1.000	No
7	Octenidine Vs NaOCl	0.000	1.000	No
8	Chlorhexidine Vs Triclosan	0.000	1.000	No
9	Chlorhexidine Vs NaOCl	0.000	1.000	No
10	Triclosan Vs NaOCl	0.000	1.000	No

**** - denotes significance at 1% level**

SYTO 9 VALUES:

1. Comparison among the Agents: (Chi Square Test)

Over all Table for pair wise comparison:

Pair wise comparison was done by using proportion test, and the significance level was compared with 0.005 (alpha= (0.05/10) = 0.005) instead of 0.05, because of 10 pair wise comparisons.

Table 11: Over all table for pair wise comparison

No	Comparisons	Z - value	P value	Significance
1	Control Vs Octenidine	-145.90	0.0000**	Yes
2	Control Vs Triclosan	-151.78	0.0000**	Yes
3	Control Vs Chlorhexidine	-140.95	0.0000**	Yes
4	Control Vs NaOCl	-147.90	0.0000**	Yes
5	Octenidine Vs Triclosan	0.75	0.4541	No
6	Octenidine Vs Chlorhexidine	1.45	0.1482	No
7	Octenidine Vs NaOCl	1.88	0.0599	No
8	Chlorhexidine Vs NaOCl	0.36	0.7167	No
9	Chlorhexidine Vs Triclosan	-0.77	0.4416	No
10	Triclosan Vs NaOCl	1.20	0.2312	No

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	-	-	-	+	+

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Tube	1	2	3	4	5
Potency (µg/ml)	1000	500	250	125	62.5
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Variables µg/ml	Octenidine (0.1%)	Triclosan (99.99%)	Chlorhexidine (2%)	Na hypochlorite (5%)
MIC	0.62	50	3.33	250

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	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**
Teeth 1	990	10	11	1250	14	1050	15	1400	16	1150
Teeth 2	1010	2	10	1100	12	1100	14	1450	14	1200
Teeth 3	1020	5	10	1200	11	1040	13	1350	13	1250
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Teeth 10	1010	5	10	1150	10	1010	13	1350	14	1200
Mean	**1006**	**7.10**	**10.40**	**1175**	**11.20**	**1135**	**13.30**	**1260**	**14.10**	**1245**

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6	Octenidine Vs Triclosan	0.000	1.000	No
7	Octenidine Vs NaOCl	0.000	1.000	No
8	Chlorhexidine Vs Triclosan	0.000	1.000	No
9	Chlorhexidine Vs NaOCl	0.000	1.000	No
10	Triclosan Vs NaOCl	0.000	1.000	No

**** - denotes significance at 1% level**

SYTO 9 VALUES:

1. Comparison among the Agents: (Chi Square Test)

Over all Table for pair wise comparison:

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4	Control Vs NaOCl	-147.90	0.0000**	Yes
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7	Octenidine Vs NaOCl	1.88	0.0599	No
8	Chlorhexidine Vs NaOCl	0.36	0.7167	No
9	Chlorhexidine Vs Triclosan	-0.77	0.4416	No
10	Triclosan Vs NaOCl	1.20	0.2312	No

**** - denotes significance at 1% level**

DISCUSSION

The pathogenicity of endodontic microorganisms responsible for stimulating apical periodontitis creates the need for finding effective antimicrobial medicaments.[12]

The complex anatomy of teeth and root canals creates an environment that is a challenge to instrument and clean. In addition, the complex chemical environment of the root canal prevents antimicrobial irrigating solutions and medicaments from exerting their full potential against all microorganisms found in endodontic infections. While the knowledge of persistent bacteria, disinfecting agents, and the chemical milieu of the necrotic root canal has greatly increased, there is no doubt that more innovative basic and clinical research is needed to optimize the use of existing methods and materials, and to find new techniques and materials, or combination of materials, to achieve the goal of predictable, complete disinfection of the root canal system in apical periodontitis.[18]

Invasion of radicular dentinal tubules by root canal bacteria is a multi-factorial event that a limited number of oral bacterial species have the necessary properties to participate in. Although the clinical

endodontic techniques of chemomechanical root canal preparation and inter-appointment root canal medication can manage infected radicular dentine, the complexity of the microbial interactions and bacterial resistance to treatment suggests that continued research into the pathologic events of invasion and development of the intra-tubular flora is warranted.[23]

Infection of the root canal is not a random event. The type and mix of the microbial flora develop in response to the surrounding environment. Factors that influence whether species die or survive are the particular ecological niche, nutrition, anaerobiosis, pH and competition or cooperation with other microorganisms. Species that establish a persistent root canal infection are selected by the phenotypic traits that they share in common and that are suited to the modified environment. Some of these shared characteristics include the capacity to penetrate and invade dentine, a growth pattern of chains or cohesive filaments, resistance to antimicrobials used in endodontic treatment, as well as an ability to grow in monoinfections, to survive periods of starvation and to evade the host response. Microorganisms that establish in the untreated root canal would

experience an environment of nutritional diversity that changes with time. In contrast, the well-filled root canal offers the microbial flora little more than shelter from the host and microbial competitors, but in a small, dry, nutritionally limited space. In all cases, it is the environment that selects for microorganisms that possess traits suited to establishing and sustaining the disease process.[44]

Numerous measures have been described to reduce the numbers of root canal microorganisms, including the use of various instrumentation techniques, irrigation regimens and intra-canal medicaments. There is no evidence in the literature to show that mechanical instrumentation alone results in a bacteria-free root canal system. Considering the complex anatomy of the root canal pulp space, this is not surprising. Furthermore, tissue remnants also impede the antimicrobial effects of root canal irrigants and medicaments. Therefore some sort of irrigation / disinfection is necessary to remove tissue from the root canals and to kill microorganisms.

Antibacterial irrigating solutions may reach canal ramifications and inaccessible areas and permeate completely through the dentinal tubules. Therefore, several irrigation solutions in different concentrations with antibacterial activity have been recommended for

use to irrigate and disinfect root canals in combination with root canal preparation.

An endodontic irrigant should ideally exhibit powerful antimicrobial activity, dissolve organic tissue remnants, disinfect the root canal space, flush out debris from the instrumented root canals, provide lubrication, and have no cytotoxic effects on the periradicular tissues. However, the efficacy of these procedures also depends upon the vulnerability of the involved species, as cited by Gomes et al.[15]

Enterococcus faecalis

For a bacterium to be pathogenic, it must essentially be able to adhere to, grow on, and invade the host. It must then survive host defense mechanisms, compete with other bacteria, and produce pathological changes. With the virulence factors described above, *E. faecalis* appears to possess the requisites to establish an endodontic infection and maintain an inflammatory response potentially detrimental to the host.[33]

Studies that have recovered microbes from filled root canals with persistent periapical disease have shown a high proportion of enterococci, ranging from29% to 77%. This contrasts with a rather low proportion of enterococci, around 5% or less, recovered from

untreated infected root canals and raises the question of how and when enterococci establish in the root canal.

Species belonging to the genus *Enterococcus* may be found in diverse environments, such as in the gastrointestinal tract of humans and other mammals and in birds, reptiles, insects, plants, water, and soil. They are also able to colonize the genitourinary tract and the oral cavity. Of the *Enterococcus* species, *Enterococcus faecalis* is the most commonly isolated or detected species from oral infections, including marginal periodontitis, infected root canals, and periradicular abscesses.[45]

Enterococcus faecalis is a nonspore-forming, fermentative, facultatively anaerobic, Gram-positive coccus. *E.faecalis* cells are ovoid and 0.5 to 1¯m in diameter. They occur singly, in pairs, or in short chains, and are frequently elongated in the direction of the chain. Most strains are nonhemolytic and nonmotile. Surface colonies on blood agar are circular, smooth, and entire.[45]

E.faecalis has some definite and candidate virulence factors, which may be involved in disease causation. They include cytolysin (lytic toward selected mammalian cells), lytic enzymes such as gelatinase and hyaluronidase (which may be involved in tissue

damage), aggregation substance (involved in binding to leukocytes and connective extracellular matrix), pheromones (small linear peptides involved in conjugative transfer of plasmids and chemoattractant for neutrophils), and lipoteichoic acid (involved in adhesion to host surfaces; stimulates cytokine production by monocytes). Data from culture studies have revealed that *Enterococcus faecalis* is occasionally isolated from primary endodontic infections but frequently recovered from treatment failures.[34]

Root canal irrigants

Historically, countless compounds in aqueous solution have been suggested as root canal irrigants, including inert substance such as sodium chloride (saline) or highly toxic and allergenic biocides such as formaldehyde.[50]

The primary function of an endodontic irrigant is to remove any debris loosened, but not removed, by instrumentation. Therefore, the irrigating solution must be brought into contact with the dentin wall and the debris. The intimacy of this contact depends on the wettability of the solutions on solid dentin.[48]

The simple act of irrigation flushes away loose, necrotic, contaminated materials before they are inadvertently pushed deeper into the canal and apical tissues. Irrigation solutions also provide gross debridement, lubrication, and destruction of microbes and dissolution of tissues.

Numerous studies have demonstrated that many of the commonly used irrigating solutions are ineffective in completely removing hard and soft tissue debris. The inadequacy of irrigating solutions has often been attributed to either the lack of mechanical flushing (i.e. flushing volume of irrigant) or their inability to remove organic and inorganic material simultaneously.[48]

An ideal root canal irrigant should:[50]

- Have a broad antimicrobial spectrum and high efficacy against anaerobic and facultative microorganism
- Dissolve necrotic pulp tissue remnants
- Inactivate endotoxin
- Prevent the formation of smear layer during instrumentation

Sodium Hypochlorite (NaOCl)

Hypochlorite solutions were first used as bleaching agents. Subsequently, sodium hypochlorite was recommended by Labarraque

to prevent childbed fever and other infectious diseases. Based on the controlled laboratory studies by Koch and Pasteur, hypochlorite then gained wide acceptance as a disinfectant by the end of the 19th century. Sodium hypochlorite has a wide range activity against both Gram positive and Gram negative bacteria

Sodium hypochlorite is the strongest antifungal agent among root canal irrigations and medications. It is a non-specific proteolytic agent with an excellent tissue dissolving ability. When heated, sodium hypochlorite tends to increase its antimicrobial and tissue dissolving abilities.[31]

Carlos Estrela et al[10] put forward the mechanism of action of Sodium hypochlorite. The actions are

- Saponification reaction

Sodium hypochlorite acts as an organic and fat solvent degrading fatty acids, transforming them into fatty acid salts (soap) and glycerol (alcohol), that reduces the surface tension of the remaining solution.

- Amino acid neutralization reaction

Sodium hypochlorite neutralizes amino acids forming water and salt

- Chloramination reaction.

With the exit of hydroxyl ions, there is a reduction of pH. Hypochlorous acid, a substance present in sodium hypochlorite solution, when in contact with organic tissue acts as solvent, releases chlorine that, combined with the protein amino group, forms chloramines. Hypochlorousacid (HOCl-) and hypochloriteions (OCl-) lead to amino acid degradation and hydrolysis.

Sodium hypochlorite exerts deteriorative effects on mechanical properties and chemical composition of dentine[16].Cytotoxicity and tissue toxicity of sodium hypochlorite is well documented. Most complications of the use of sodium hypochlorite appear to be the result of its inadvertent injection beyond the root apex which can cause violent tissue reactions (hypochlorite accident). In rare cases sodium hypochlorite may cause allergic reactions.[20]

Chlorhexidine (CHX)

Chlorhexidine in the chemical form is a cationic *bis*-biguanide that is usually marketed as a gluconate salt. A commercially available oral rinse contains 0.12% chlorhexidine gluconate in a base containing water, 11.6% alcohol, glycerine, flavoring agents, and saccharin. Approximately 30% of the active ingredient is retained in

the oral cavity after rinsing and is slowly released into the oral fluids.[30]

This substantive antimicrobial activity has been identified as a potentially protective element in the canal tissues for many hours after instrumentation. The potential for chlorhexidine gluconate use in endodontics has been clearly demonstrated by numerous investigators.[22,26,27]

At low chlorhexidine concentrations, small molecular weight substances will leak out, especifically potassium and phosphorous, resulting in a bacteriostatic effect. At higher concentrations, chlorhexidine has a bactericidal effect due to precipitation and/or coagulation of the cytoplasm, probably caused by protein cross-linking.

Octenidine hydrochloride (OCT)

Octenidine hydrochloride [N,N¢-(1,10 decanediyldi-1[4H]-pyridinyl-4-ylidene)bis(1-octanamine)dihydrochloride] belongs to the bipyridines carrying two cationic active centres per molecule and demonstrates broad spectrum antimicrobial effects covering both Gram-positive and Gram-negative bacteria, fungi and several viral species [47]

The mode of action is bactericidal/fungicidal by interfering with cell walls and membranes. Phenoxyethanol, an ethanol derivate, serves as a preservative component in Octenisept which is also supposed to improve the antibacterial activity of octenidine synergistically. Previous studies showed the efficacy of octenidine against dental plaque-associated bacteria, such as *Streptococcus mutans and Actinomyces viscosus* comparable to chlorhexidine digluconate. So far no carcinogenic or mutagenic effects have been registered.[47]

Triclosan:

Triclosan, a synthetic chemical, was first used in 1972 as a component of surgical scrub solutions. During the last decade, there has been a rapid increase in the use of triclosan-containing products in a wide variety of products including antibacterial soaps, deodorants, toothpastes, cosmetics, fabrics, plastics, and other products.

Zambon JJ et al[51], evaluated triclosan as a broad spectrum antimicrobial agent, active against gram-positive and gram-negative bacteria as well as some fungi and viruses. The antiplaque and antigingivitis efficacy of triclosan-containing dentifrices is well-established. Although triclosan is most often used for antisepsis to kill bacteria on the skin and other surfaces, it has also been incorporated into medical devices and used to protect products against microbial deterioration.

Triclosan inhibits bacterial fatty acid synthesis at the enoyl-acyl carrier protein reductase (EACPR) in the fatty acid biosynthetic (Fab) step. The Fab pathway is an excellent target for antimicrobial

agents. Because humans do not have the EACPR enzyme, triclosan is nontoxic to human cells. Triclosan is a very potent inhibitor, and only a small amount is needed for powerful antibiotic action.[32]

So far only one study has been cited in the literature for the use of Triclosan as an intracanal irrigant[32]. Hence in the present study, the efficacy of Triclosan against *E.faecalis* was explored.

The aim of this present study was to evaluate and compare the antimicrobial efficacy of **octenidine, triclosan** and **chlorhexidine** against *E.faecalis* in root canal disinfection.

The recommendations for dentin tubule disinfection model proposed by Haapasalo and Orstavik was modified in this study, the direct exposure method for testing antimicrobial agent was applied. The direct exposure method in comparison with dilution and agar diffusion methods correlated to the substance effectiveness and its direct contact with organisms. This method is independent of other variables and is feasible in the laboratory (Estrela et al).[12]

Intact human mandibilar premolars with complete root formation, extracted for orthodontic or periodontal reasons were selected for this study rather than bovine teeth. Basrani et al [5] pointed out that canal lumen of the standardized bovine blocks were three

times larger than that of standardized human blocks. This would result in a 10 fold increase in the canal volume than that of human root blocks of the same length. Substantive antimicrobial activity of certain medicaments would directly depend on the number of free molecule available to interact with dentin. Hence, studies using human dentin blocks would be more suitable to simulate the clinical scenario.

Radiovisiography was used to confirm single canal anatomy to facilitate standardization of specimens. This procedure was done to minimize the radiation exposure.

The dentine blocks were standardized to a measure of 7mm. This was in accordance with the study performed by L. Tandjung et al. [47]Standardization of the root canal diameter of the dentin blocks were achieved using k-files to a size 40. Subsequently the smear layer was removed using 10ml of 10% citric acid. The smear layer following instrumentation is 1 to 2µm thick on the dentin surface. The presence of smear layer acts as a diffusion barrier that can reduce the rate of diffusion by 25-30%. It was then rinsed with 20ml of

physiological saline. The root surface was coated with nail varnish, which was allowed to dry at room temperature for 1 hour.

Five test specimens were randomly assigned to one tube containing 20ml of Mueller-Hinton broth. In order to improve broth penetration into dentinal tubules, it was treated ultrasonically, for 30sec. Mueller-Hinton broth was used since it showed better infection of the dentinal tubules when compared to Brain heart infusion broth.[51]

The dentin blocks were sterilized in an autoclave at 121°C for 15 min to remove presence of microorganisms within the canal system. For sterility check, which was uniformly negative, the tubes were incubated at 37° C for 72 hrs.

Enterococcus *faecalis* (ATCC 29212) was used as the test organism as this Gram positive facultative anaerobic bacterium is the most common isolate found in failed cases. Almyroudi et al [1] found it easy to maintain and culture *E.faecalis* under laboratory conditions. Although this organism makes up a small percentage of the root canal flora, it may be favored by ecological challenges and establish infections difficult to treat and demand for retreatment.

Haapasalo and Orstavik [18] suggested that a shorter duration of infection can be used instead of the routine 3 weeks period of

incubation. Hence a 7 day infection period was used in this study. The broth was changed on alternate days to prevent saturation of broth with *E.faecalis* and to replenish the nutrient source for the organism. Following infection of the dentin blocks antibacterial testing was performed.

The various irrigants used in this study were, 0.1% octenidine, 2% chlorhexidine, triclosan (Pure Substance), and 5% Sodium Hypochlorite as positive control.

3ml of each irrigant was taken based on the studies by Gomes et al [13]and was irrigated for 1 min following the previous study done by Radcliff et al.

The positive control teeth were those which were irrigated with 5% sodium hypochlorite. The negative control was teeth which were exposed to *E.faecalis* but were not irrigated using any irrigants. This allowed inter-group comparison of the antibacterial activity of each group.

Dentin debris was harvested first with Gates Glidden drill No.3 followed by No.4, this allowed sampling to a depth of 400µm from the canal lumen[5]. Similar sampling was done by Krithika Datta et al[22]. The debris was collected in Eppendorf tubes containing 1ml of

phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

The results obtained from the present study revealed significant information on the newer intracanal irrigant used, against *E.faecalis*. 0.1% of octenidine was most effective against *E.faecalis*, followed by 2%chlorhexidine and Triclosan(99.0% pure). 5% Sodium hypochlorite was found to be the least effective against *E.faecalis* among all the groups.

0.1% octenidine was the most effective irrigant against *E.faecalis* in the present study, this is in accord with the findings of Resmiye Erbu Tirali et al [46] and L. Tandjung et al[47].

The probable reason for the enhanced antimicrobial effect of Octenidine can be attributed to its cation-active structure that tends to bind readily to the negatively charged bacterial cell envelope, automatically disrupting the vital functions of the cell membrane and killing the cell. Preliminary results point to a strong adherence particularly to lipid components (e.g. cardiolipin) prominent in

bacterial cell membranes explaining the high antimicrobial efficacy without adversely affecting human epithelia or wound tissue.[54]

The antibacterial potential of octenidine has been well documented and compared to some other disinfectants used in endodontics. The susceptibility of S.mutans and A.viscosus to octenidine has been reported to be comparable to chlorhexidine digluconate.[47]

Furthermore, it has been shown that octenidine resists an organic challenges, i.e. maintains its antimicrobial efficacy in the presence of organic material comparably to chlorhexidine and iodine. This is of interest, as, in a root canal system both organic and inorganic inhibitory factors are present that may weaken the antimicrobial efficacy (Haapasalo et al).[18]

In a study by Tirali et al Octenidine was found to be more effective than 5.25% NaOCl solution against *E.faecalis* as antimicrobial endodontic irrigants. There was a significant difference between the solutions in terms of producing negative cultures for the tested microorganisms.[46]

The efficacy observed in the present study indicates the performance of octenidine was sufficient in this biologically complex

environment. This indicates the justification for further comparative studies, including common antimicrobial agents used in endodontics.

Among the irrigants used in this study,the next effective irrigant was 2% Chlorhexidine. This result was similar to the studies done by Ferraz et al[14] and Ercan et al[11], who showed that 2% chlorhexidine was more effective in reducing the number of positive cultures and CFU than 5.25% sodium hypochlorite.

A possible clinical advantage of chlorhexidine gluconate over sodium hypochlorite is that, even though both are effective as antimicrobial agents, chlorhexidine gluconate is relatively nontoxic, is a broad-spectrum antimicrobial agent, and has residual action with less potential for adverse effects.

Chlorhexidine seem to act by adsorbing onto the cell wall of the microorganism and causing leakage of intracellular components. At low concentrations of chlorhexidine, small molecular weight substances will leak out, resulting in a bacteriostatic effect. At higher concentrations chlorhexidine has a bactericidal effect due to precipitation and/or coagulation of the cytoplasm, probably caused by protein cross-linking.[15]

However other investigations have shown that NaOCl presents better antimicrobial activity than CHX. Ayhan H et al [2], stated that the antimicrobial efficacy of NaOCl was greater when compared to CHX and other irrigants used in their study.

Triclosan was the next effective irrigant used in this study. Triclosan is a broad spectrum antimicrobial agent with less previously reported use in endodontics. Triclosan was dissolved in ethanol, which was done according to the study by Nudera et al [32]. He demonstrated that ethanol did not contribute to the growth inhibitory effect in the study. This conclusion is based on the fact that the MIC of ethanol for all test bacteria was at 1.44% or greater.

Among the irrigants used in this study the least effective was 5% Sodium hypochlorite. Though Sodium hypochlorite has excellent tissue dissolution properties[41], the antimicrobial efficacy seemed to be less when in comparison with newer irrigants. Jeansonne and White compared 2% CHX and 5.25% NaOCl as antimicrobial endodontic irrigants in freshly extracted human teeth with pulp pathosis. The results showed that the number of post-irrigant positive cultures and the number of colony-forming units in positive cultures obtained from CHX-treated teeth were lower than the numbers obtained from

NaOCl-treated teeth against *E.faecalis*[14]. Tirali et al studied the difference between NaOCl and Octenidine and concluded that octenidine showed better results when compared to NaOCl[46]. Thus proving that, the newer irrigants seem to have better antibacterial efficacy against *E.faecalis* than the gold standard sodium hypochlorite.

From the results of this study, it can be concluded that all the experimental irrigants used had good antibacterial efficacy in eliminating *E.faecalis*. Octenidine had the lowest MIC and MBC value suggesting it to be a potent irrigant. Sodium hypochlorite showed the highest MIC and MBC value, thus showing it to be the least effective irrigant used in this study. However antimicrobial effect is not the only requirement of an endodontic irrigant. Many studies have proved the tissue dissolving property of NaOCl, which is a significant attribute that the other irrigants do not posses. Further studies are required to find out the tissue dissolving properties of Octenidine.

SUMMARY

The present study evaluated the antimicrobial efficacy of octenidine, triclosan and chlorhexidine against Enterococcus faecalis in root canal disinfection. 50 single rooted pre-molar teeth with no caries and root fillings were collected. The dentine blocks were standardized by decoronating the teeth to measure 7mm long segments. The root canals were enlarged to size 40 using K files. The root surface were coated with nail varnish and was allowed to dry at the room temperature for 1hr and subsequently the root apex was sealed using light cure composite resin. Following sterilization of the blocks, they were infected with pure cultures of *E.faecalis*, which was grown in Muller-Hinton agar for a period of 7 days. The broth was changed every alternate day to maintain viable cultures. The specimens were divided into 5 distinctive groups containing 10 teeth each:

Group I with Octenidine, (0.1%)

Group II with Chlorhexidine (2%),

Group III with Triclosan,(99.0%)

Group IV with NaOCL (5%) (control)

Group IV with no irrigation (negative control)

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of the irrigant for 1 min. After irrigation, the dentine debris were harvested from the dentine blocks using Gates Glidden drills No.2 and No.3. The antibacterial assessment was carried out by finding out the MIC and MBC values by serial dilution method, the colony forming unit on Muller-Hinton agar plate and the proportions of viable bacteria under epifluorescence microscope using Syto9 dye. Statistical analysis comparing the inter-group was done using MANN-WHITNEY U test and KRUSKAL-WALLIS test. From the results it was observed that the antimicrobial efficacy of 0.1% Octenidine was the most, followed by 2% Chlorhexidine, 99.0% Triclosan and 5% Sodium hypochlorite against *E.faecalis*. Thus the irrigants do play a significant role in creating a “bacteria-free” root canal system.

CONCLUSION

Within the parametric limitations of this in vitro study, it can be inferred that:

1) The MIC values of Octenidine hydrochloride, Chlorhexidine digluconate and triclosan, were identical as compared to that of MBC, thereby revealing a good bactericidal property. Octenidine which showed good antibacterial property, in terms of very minimal concentration being required.
2) Results of the in-vitro evaluation by determination of Minimum Inhibitory concentration (MIC) and Minimal Bactericidal concentration (MBC) values correlated very well as compared with the mono infection using *Enterococcus faecalis* in an ex-vivo human dentine block model.
3) The mono infection using *Enterococcus faecalis* in the dentine block model worked very well with a short incubation period of 7 days, and showed efficacy in view of a statistically significant reduction in the colony count after irrigation for 3 mins.

4) The special staining dye Syto9 showed significant reduction in the proportion of viable bacteria thereby proving the efficacy of the 3 dental irrigants. This method however, is technically very sensitive and not within reach of all laboratories.
5) Octenidine which is not yet evaluated widely as an irrigant showed the maximum efficacy, against *E.faecalis* thus proving to be a potent irrigant.

Further in vivo studies are required to evaluate the tissue dissolving properties and also their safety in dental practice.

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