Comparative Evaluation and Efficacy of Linezolid, Vancomycin and Ciprofloxacin on Enterococcal induced biofilm using Scanning Electron Microscopy an in vitro study

Musab Hamed Saeed[1]*, Manu Zacharias[2], Krishna Prasad Shetty[1], Alexender M Luke[3], Simy Mathew[4]

1. Department of Restorative Dentistry, College of Dentistry, Ajman University, Ajman, UAE.
2. Consultant Endodontist, Government Corporate Hospital, Kaduthuruthy, Kerala, India.
3. Department of Surgical Sciences, College of Dentistry, Ajman University, Ajman, UAE.
4. Department of Growth and Science, College of Dentistry, Ajman University, Ajman, UAE.

Abstract

To evaluate and compare the effect and efficacy of linezolid, vancomycin and ciprofloxacin on enterococcal induced biofilm using scanning electron microscopy.

Fifty mandibular premolars were selected for use in this study. Access cavity preparation was done. Working length was determined using periapical radiographs. Coronal third of the canals were flared using Gates Glidden drills. The canals were prepared up to size # 25 K file, 1mm short of apical foramen. Step back preparation was completed up to size # 50 K file. During instrumentation of the canals, canals were irrigated using sodium hypochlorite 5.25% and 17% EDTA. The teeth were then air-dried and steam autoclaved at 121°C for 30 minutes. Two longitudinal grooves were made along the entire length on opposite sides of the outer surface of the teeth to act as a guide for the subsequent splitting of the tooth into two halves. All the samples were divided into 5 groups (n=10). GROUP 1: Negative control. GROUP 2:Positive control. GROUP 3: Linezolid. GROUP 4: Vancomycin. GROUP 5: Ciprofloxacin. Residual aliquots were removed and spread on brain heart infusion broth and send for SEM to determine the percentage of negative culture obtained.

Results were analysed using one-way ANOVA test and Duncan's test. A p-value less than 0.05was considered significant.

The antimicrobial efficacy of linezolid was found to be higher than that of ciprofloxacin and vancomycin.

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Introduction

The existence of microorganism are present in some of the critical areas of the tooth such as ramifications, isthmus, deltas, irregularities and dentinal tubules which may not be eliminated by mechanical means alone, hence different canal irrigants and antibacterial medication plays an important role in elimination and disinfection of this bacteria in the root canal system.[1] Biofilm consists of infectious bacteria usually attached to substratum or surface ,it often becomes difficult to treat if this layer of biofilm forms at later stage it can also results in pathogenesis of oral diseases.[2]

Disinfectants and antiseptics are the antimicrobial agents that are used to inanimate object or surface and antiseptics are used on living tissues in order to prevent any infection and kill bacterial growth or pathogenic microorganisms from potential tissue damage.[3]

E. faecalis has often been isolated from the root canal after endodontic treatment (post-treatment) in apical periodontitis and frequently in chronic periapical pathology due to the growth of single kind of organism which is free from other organism (pure culture).[4]

Linezolid (LZ) is the other agent used in this study it is an oxazolidine component acts by inhibiting the initiation of bacterial protein synthesis, its action are against gram positive organisms including vancomycin resistant E. faecalis.[5]

Corresponding author:
Musab Hamed Saeed,
Department of Restorative Dentistry,
College of Dentistry, Ajman University , Ajman, UAE.
E-mail: m.saeed@ajman.ac.ae

Vancomycin (VC) is necessarily used in this study as it does helps in eradication of biofilm, vancomycin found to be effective in retreatment conditions as study conducted by krishnaraj etal showed vancomycin irrigation with filing is more effective than just irrigation.[4]

On the other hand, Ciprofloxacin (CFN) obtained least effective against gram positive bacteria as well as Streptococcus pneumoniae, Staphylococcus aureus and Enterococcus faecalis, but it is a broad spectrum antibiotic, more sensitive to gram-negative bacteria and structurally related to nalidixic acid as it is a fluorinated quinolones.[6]

In the present study, we compare the effect of Linezolid, Vancomycin, Ciprofloxacin and concentrate mainly on the efficiency of three different antibacterial drugs on E.faecalis induced biofilm, as it is noted from various other studies that E.faecalis is resistant to certain drugs used commonly.

Materials and methods

Selection and Preparation of the Samples

Fifty mandibular premolars with mature apices and single root canals were selected (fig 1) for use in this study. All teeth were cleaned using 2.5% sodium hypochlorite followed by ultrasonic scaling. Access cavity preparation was done using BR 45 round bur and Endo Z bur (Dentsply USA). working length was determined, followed by instrumentation with step back preparation canals were irrigated with sodium hypochlorite 5.25% and 17% EDTA All the samples were then decoronated at cemento enamel junction using high speed diamond disc (fig 2) All the samples were dried and autoclaved at 121°C for 30 minutes.

Figure 1. Freshly extracted 50 single rooted premolars.

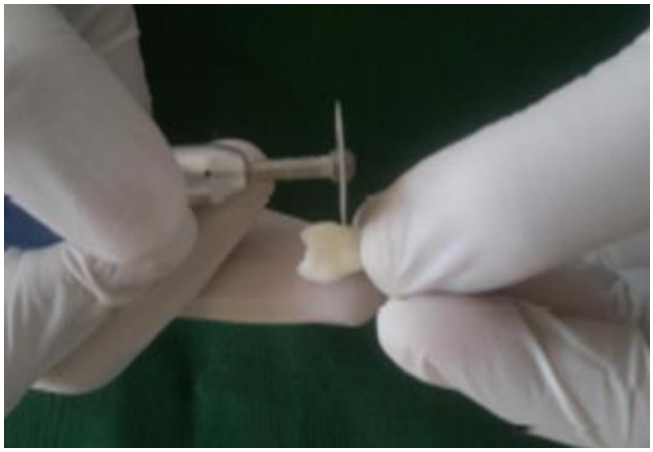
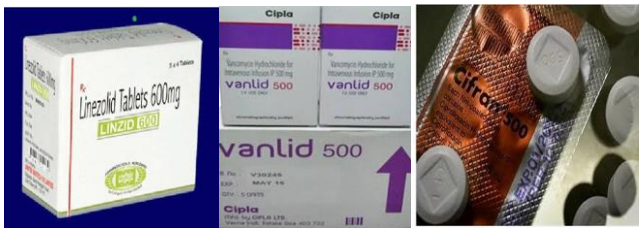


Figure 2. Decoronation of premolars.

Classification of the Samples

Fifty samples were included in the study and were divided into five groups (n=10).
Group 1: Positive control group (n=10).
Group 2: Negative control group (n=10).
Group 3: Linezolid group (fig 3a) (LINZID 600mg®Unitec Biotech India).
Group 4: Vancomycin group (fig 3b) (VANLIDinj 500mg® Cipla LTD India).
Group 5: Ciprofloxacin group (fig 3c) (CIFRAN 500mg® Ranbaxy LTD India)

The positive control group was used to check for bacterial viability throughout the experiment, while the negative control group was used to check for sterility of the procedures.



(a) **(b)** **(c)**

Figure 3a. Linezolid group (LINZID 600mg® Unitec Biotech India).
Figure 3b. Vancomycin group (VANLID inj 500mg® Cipla LTD India).
Figure 3c. Ciprofloxacin group (CIFRAN 500mg® Ranbaxy LTD Ind).

Biofilm Development

A clinical isolate of *E. faecalis* (fig 4)was used for biofilm formation Samples from the experimental groups and the positive control group were immersed in a 24-hour pure culture

suspension of *E. faecalis* grown in Brain Heart Infusion broth (fig 5) and adjusted to No. 1MacFarland turbidity standard; all teeth were incubated at 37ºC in sealed vials(fig 6) .This procedure was repeated every 72 hours using a 24-hourpure culture(fig 7) prepared and adjusted to the No. 1MacFarland turbidity standard to obtain a fresh and pure biofilm of E. *faecalis* inside the root canal The negative control samples were immersed in sterile BHI broth replenished with sterile saline every 72 hours to test for sterility of the procedures. The teeth were maintained in a humid environment at 37°C for 30days.

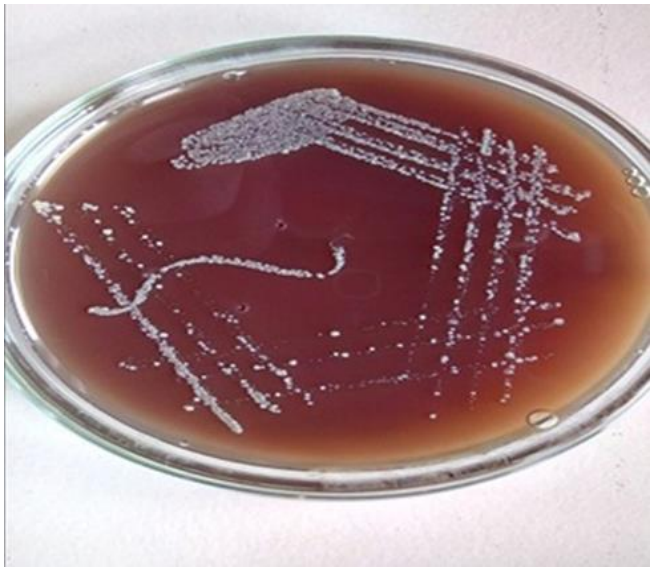


Figure 4. E faecalis isolates.

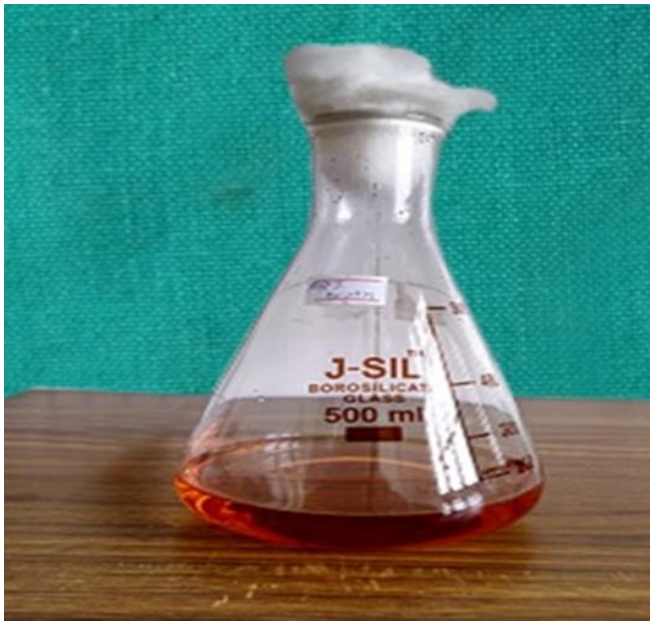


Figure 5. Brain Heart Infusiun Broth.

Figure 6. Incubator.

Figure 7. samples immersed in culture Suspension, suspension replenished every 24 hour.

Verification of Biofilm Development

Bacterial biofilm development onto root canal dentin was assessed by SEM examination (fig 8a-8d)at four time intervals (3, 10, 20 and 30days). Longitudinal Grooves(longitudinal) were cut along the length of selected samples before root canal preparation. Then, after immersion in the bacterial suspension, samples were split with a hammer and chisel into two halves. Each half was immersed in 2.5% gluteraldehyde for 24 hours at 4°C for fixation, washed with phosphate buffer saline for 15 min. Then, the samples were flushed with PBS and then dehydrated in an ascending acetone series (30%, 60% and 100%) for 10 minutes each. Finally, the samples were dried and observation of the whole canal was

performed by using a scanning electron microscope at 30 kV.

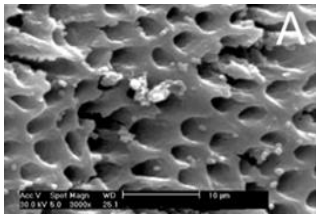


Figure 8 a

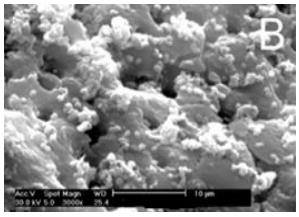


Figure 8 b

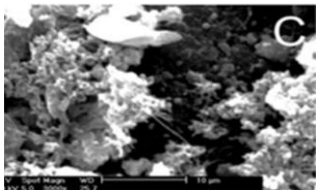


Figure 8 c

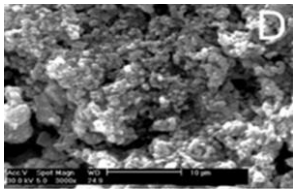


Figure 8 d

Figure 8 A-D. SEM images showing the stages of E.*faecalis* biofilm formation and maturation onto root canal dentin.

Preparation and Application of the Antimicrobial Agents

The drug concentrations were adjusted based on published data for minimum inhibitory concentrations for *E. faecalis.* Sterile saline solution was added in a drop-wise manner to the antibiotic powder and mixed to obtain a thick paste. A 2ml syringe was used to introduce the paste inside the infected canals. In group 4, vancomycin inj 500 mg solution was diluted using normal saline in accordance to maintain proper concentration of drug. To allow the antibacterial properties of the intracanal medicaments to be expressed under clinical conditions, wax was used to seal the apex as well as the coronal access cavity; then aluminium foil was used to envelop the samples. All samples were incubated for a week at 37°C under humid conditions.

Bacterial Sampling

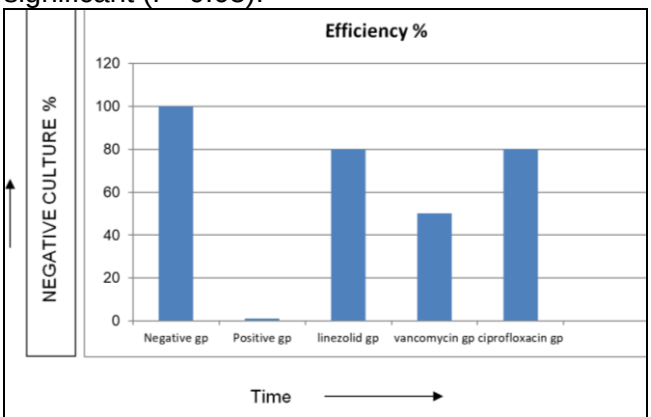
After one week, all of the samples were irrigated with 20 ml sterile saline solution to remove the root canal contents. Bacterial samples were taken using a standard method of collection. The root canals were filled with sterile saline as a transport fluid, then #15 K-file was placed into the canal to within1 mm of working length and circumferentially filed for 10 seconds before sterile absorbent paper points adsorbed the transport fluid and transferred it to a test tube containing 1.0 ml of saline. Aliquots of 0.1 ml were spread plated onto BHI agar plates, incubated at 37°C for 48 hours, and colony-forming units(CFU) per 1 mL were enumerated.

Statistical Analysis

One Way Analysis of Variance followed by Duncan's Multiple Range Test was used to test the effect of treatments on colony forming units.

Results

The antimicrobial effect of Linezolid (80% negative cultures), Ciprofloxacin (80% negative cultures) was significantly better than for Vancomycin (50% negative cultures) at P=0.05. However, the difference in the antimicrobial effectiveness among them was statistically non-significant (P=0.05).



Treatment	Mean	S.D	Min	Max	Negative culture	Dt
Negative Control Group	0	0	0	0	100%	A
Positive control group	$7.55*10^2$	$2.19*10^2$	$4.00*10^2$	$11.09*10^2$	0%	D
Linezolid	3.16*10	8.10*10	0	$3.00*10^2$	80%	B
Vancomycin	4.60*10	6.60*10	0	$3.60*10^2$	50%	C
Ciprofloxacin	4.10*10	8.80*10	0	$3.00*10^2$	80%	B

Table 1. Mean colony forming units and the percentage of negative cultures after application of the antimicrobial agent.

Discussion

The experimental model used in this study is concerned to mimic some clinical cases in which the clinicians have already completed the mechanical preparation, used all types of available irrigant combinations and the patients took more than one class of systemic antibiotics, but still there are some persistent clinical signs and symptoms in the form of slight pain, tenderness to percussion or mild exudate.[7]

The presence of *E.faecalis* in root canals can be detected either by culture or by molecular techniques. Cogulo et al confirmed that both culture and PCR methods were sensitive to detect E. *faecalis* in both deciduous and permanent teeth.[8]

Esterala et al suggested that three aspects must be considered for validation of biofilm models: the bacterial colonization structure, the biological indicator, and the time necessary for biofilm formation. All these aspects were achieved in this study.[9]

Dewa Ayu N.P.A etal pointed out that the average amount of E.faecalis and C.albicans is almost twice the amount seen in endodontic retreatment cases, possibility may be because of poor initial endodontic treatment and resistant to disinfection materials, study results indicate that E.faecalis and C.albicans are capable of invasion into dentinal tubule and are resistant to unfavourable conditions,which results in forming biofilms.[10]

Evans et al have reported that *E.faecalis* is resistant to killing by calcium hydroxide at or below a pH value of 11.1, so the pH was selected on the basis of this study.[11] Even after which it had no effect on the bacterial biofilms as evident by the high count obtained from the positive control group which was statistically significant in comparison with the other groups'.[12]

Sandoe et al stated that combination of gentamicin to linezolid or vancomycin produced a significant reduction in maximum bacterial concentration (MBC) and maximum bacterial inhibitory concentration (MBIC) in at least a third of the isolates tested.[13]. similar kind of study reported using Odontopaste, in reference to their study the concentration of clindamycin hydrochloride was 50,000 micrograms per ml showed significant effective against Enterococcus faecalis.[14]

However, all results obtained from in-vitro tests should be interpreted with caution, as they might not demonstrate the full clinical potential of the agents being tested. According to this study all of the chemotherapeutic agents used like amoxicillin+ clavulanic acid were significantly better than vancomycin in the elimination of biofilm bacteria. Our results are in agreement with previous studies. Study shown using calcium hydroxide by Taneja et al who reported less bactericidal activity of calcium hydroxide when used alone against *E. faecalis.*[15]

Stuart CH et al concluded that use of good aseptic technique, increased apical preparation sizes, and inclusion of 2% chlorhexidine in combination with linezolid is currently the most effective method to combat E. *faecalis* within the root canal systems of teeth.[16],however 2%chlorhexidine is proved to be effective against Entercoccus faecalis but found to be more toxic to certain cells, study conducted by Nilakesuma etal stated that use of 5% Ethanolic Extract of Propolis (EEP) which are nontoxic, natural resinous substance which are effective and an alternative irrigants in eliminating E.faecalis.[17]

Sharma D et al stated that antibiotic agents when added into endodontic sealers like Kerr sealer, Endomethasone, AH26, AH Plus, Roekoseal showed significant increase in their antibacterial properties in anaerobic & aerobic conditions. All the sealer-ciprofloxacin combination showed the maximum zone of inhibition where as all the sealer-metronidazole combination showed minimum zone of inhibition, which was statistically significant.[18] Rusdiana etal analysed the antibacterial effect of Bioceramic and MTA sealers against Enterococcus faecalis and accessed for 1 day (initial setting) and after 7 days of mixing both the sealers showed antibacterial effect against E.faecalis, MTA sealers had its antibacterial effect even up to 7 days.[19]

On the other hand, in this study none of them was able to achieve complete sterilization of the infected rootcanal system. The standard deviation was relatively high in some groups due to the presence of completely negative cultures in these groups. The higher percentage of negative culture obtained with Linezolid (80%)ciprofloxacin (80%) and vancomycin(50%) maybe because they are bactericidal agents.

Hence, additional research may be required in order to obtain the maximum clinical benefit of the anti-bacterial agents used and to investigate the best drug delivery form, substantivity and the feasibility of using drug combinations.

Conclusions

This method was carried out to determine the use of various antibacterial antibiotics against commonly occurring bacterial biofilm in root canal system and to determine its potential effects. In clinical application of this antibacterial antibiotic

does helps in restraining the periapical periodontitis hence, placement of this agents done after through microbial culture and antibiotic sensitivity test against the existing microbial flora.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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