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Short Communication

ENTEROCOCCUS FAECALIS CAUSE FOR PERSISTING INFECTION A CONFOCAL ANALYSIS

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Abstract :

Aim : to know ability of Enterococcus faecalis invasion into root dentin.

Methodology : Forty single rooted human intact teeth were selected, after access opening and canal debridement, all the samples were subjected for gamma sterilization to ensure complete absence of microorganisms, then exposed to Enterococcus faecalis broth, broth is placed with the help of micro pipette into root canal and also at the same time apical 1/3 of tooth were immersed into broth for 8 weeks, biomechanical preparation, obturation and coronal sealing done using GIC followed by examination under confocal laser scanning microscope after splitting the teeth samples into two halfs buccolingually.

Results : This study shows invasion of Enterococcus faecalis upto 160 µm deep in to root dentin.

Conclusion : penetration and survival of Enterococcus faecalis deep into dentin in extreme conditions may be the possible reason for persisting infection after root canal treatment.

Keywords: Enterococcus faecalis, persisting infection, root dentin, confocal laser scanning microscope.

Introduction:

Because apical periodontitis is usually caused by bacteria, a major objective of root canal treatment is to eliminate bacteria from infected root canals. Although bacterial infection can be substantially reduced by standard intracanal procedures[1], it is difficult to render the root canal free from bacteria. Bacteria are located in inaccessible areas such as complicated root canal anatomy and dentinal tubules, and it is difficult to deliver antibacterial agents to these locations[2]. Bacteria may survive and recolonize the root canal space whenever there is opportunity, and this may become a focal source for persistent infection. Bacteria are commonly found within dentinal tubules of clinically infected canals[3]. Amongst these bacteria, Enterococcus faecalis is of interest because it is the most frequently detected species in root filled teeth with persistent lesions[4]. Some possible factors facilitating its long-term survival in the root canal system are its ability to invade dentinal tubules[5], where it can survive for a prolonged period under adverse conditions such as starvation[6] and the high pH of calcium hydroxide medication[7]. Although the mechanism of bacterial invasion is not completely understood, bacterial adhesion to dentinal tubule walls (TWs) is a logical early step in the process. Collagen is widely considered to be the primary substrate for specific binding of E. faecalis to dentine, and the collagen binding protein of E. faecalis (Ace) and a serine protease (Spr) have been proposed to play significant roles in binding to the root canal wall[8]. Ace also promotes E. Faecalis binding to collagen type I [9,10] and in vitro ace gene expression at 37 _C was enhanced in the presence of collagen[11]. However, in my study the interaction of E. faecalis specifically with dentinal tubules has been investigated.

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Methodology:

The present in-vitro study was conducted in the Department of Conservative Dentistry and Endodontics, and central Research Laboratories. Teeth sterilization (gamma irradiation) done at Microtol, Bangalore. Data collection done using inverted confocal laser scanning microscope (ZEISS LSM 510 META. GmbH, Mannheim, Germany) at Indian Institute of Sciences (IISc) Bangalore.

Selection of Samples

Forty human single rooted teeth recently extracted for orthodontic reasons were collected for the study. After extraction the teeth were stored in chlorhexidine solution, until collection of all teeth.

Inclusion Criteria

Single rooted caries free teeth, examined under 20x magnification under a microscope to rule out any cracks, caries, fractures or craze lines and radiographed to determine the presence of a single canal were included for the present study.

Exclusion Criteria :

Teeth that had already undergone root canal treatment or teeth with more than one canal, immature root apices, teeth with root caries, restorations, fracture or craze lines, thin curved roots, calcified canals were excluded from the study.

Methodology:

The teeth were cleaned off soft tissue, calculus and stains with the help of sharp hand scalers and thoroughly washed under running tap water to remove any remaining tissue remnants sticking to the tooth surface and were stored in normal saline solution at room temperature until further use.

Procedure:

All the specimens were exposed to gamma irradiation (25 kGy)[12] after access opening and canal debridement followed by culturing with E.faecalis broth placed within root canal with the help of pippette and apical one third of teeth submerged in the cultured broth for 8 weeks.

Culturing Procedure:

Enterococcus feacalis streptomycin resistant strains were cultured in Trypton Soyabean Agar broth. Broth is prepared by mixing the 1.8 grams powder in 60ml of distilled water. The prepared broth is sterilized in Autoclave, after that the strains are inoculated in the broth and placed in an Incubator for the bacteria to grow at 37ºC for 24-48hrs. For confirmation of the bacteria Gram staining was done. Then the cultured broth was inoculated within root canal of the teeth samples with a micropipette and also apical one third submerged in the broth. The whole process is refreshed every alternative two days for a period of 8weeks.

Biomechanical Preparation & Obturation

After 8 weeks of culturing all the specimens were subjected to biomechanical preparation followed by obturation up to the working length. Working length was determined by using Root ZX II (J.Morita, Japan.). The root canal instrumentation was done using Protaper Ni-Ti rotary instrument system in a contra angle gear reduction hand piece (X-Smart Dentsply), finally obturated with gutta preach single cone using AH plus as sealer.

Culture and Observation:

After biomechanical preparation & obturation all the teeth specimens were again immersed into Enterococcus Faecalis broth for 8 weeks [12] after incubation period the entire tooth washed using 1 ml phosphate buffered saline (PBS) to remove nonadherent bacteria. A vertical groove is made on bucco lingual surface starting from occlusal surface to apical tip using tapered fissure diamond point, than with the help of chisel tooth is splitted in two half's segments (fig 1). splitted segments were observed under confocal laser scanning microscope(ZEISS LSM 510 META GmbH, Mannheim, Germany). After coding the samples, teeth were stained with 50 µL fluorescein diacetate (FDA sigma, st Louis, MO) and 50 µL of propidium iodide[12] (PI, Sigma). FDA is nonfluorescent cell permeable dye that is converted to fluorescein (green) by intracellular esterases produced by metabolically active microorganism[13]. PI is a fluorescent molecule impermeable to the cellular membrane and generally excluded from viable cell thus live

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bacterial cells are fluorescent green, whereas dead bacteria with damaged membranes are fluorescent red [14].The samples were examined on inverted confocal laser scanning microscope for the presence of E. Faecalis in root dentin. Data was subjected to appropriate statistical analysis. We have used SPSS software for statistical analysis.

Table 1: Data table. Score for ADHESION is 1, for NO ADHESION is 0 and for PENETRATION of E Faecalis in dentin measured in µm.

Samples / Groups	Group I	Group II
Sample 1	0	145
Sample 2	0	132
Sample 3	0	146
Sample 4	0	157
Sample 5	0	144
Sample 6	0	160
Sample 7	0	148
Sample 8	0	124
Sample 9	0	148
Sample10	0	134
Sample 11	0	153
Sample 12	0	122
Sample 13	0	144
Sample 14	0	160
Sample 15	0	156
Sample 16	0	142
Sample 17	0	125
Sample 18	0	160
Sample 19	0	142
Sample 20	0	158

TABLE 2 : MEAN, SD AND STUDENT'S T –VALUE FOR PENETRATION INTO DENTIN

GROUP	I	II
MEAN	0	145
S.D	0	11.9833
T-VALUE	52.74	

Conclusion: T - Value Shows Sginificant Difference For P>0.05

Results :

FDA/PI staining technique showed absence of any dead or alive E Faecalis in group I samples(fig 2).On other hand group II samples showed green and red colour coccoidal structure observed under confocal laser scanning microscope (fig 3,4). samples showed presence of Enterococcus faecalis up to 160 µm deep in root dentin (fig 5) in all the samples of group II (table 1). Results were subjected to statistical analysis using T test. T value (table 2) is 52.74. To conclude, T value is 52.74 (T-value shows significant difference for p>0.05) this shows significant presence of Enterococcus Faecalis in root dentin (Graph 1).

Discussion :

In this study E Faecalis was chosen as the test organism because Enterococcus faecalis found to penetrate deep into dentinal tubules in vitro[2], and enterococcus faecalis is one among the micro organisms found in reinfection[4] and also Enterococcus faecalis produces protein during stress like Ace and serine protease (spr) protein[15] which in turn help enterococcus faecalis adhere to type I & IV collagen present in root dentin [8,9].

The adhesion of E Faecalis to type I and IV collagen is the basis of my study because, root dentin consist of mainly type I and type IV collagen. Other factors facilitating Enterococcus faecalis presence in obturated root canals with persistent lesions include, it can survive in high pH[7] and has long starvation period and in presence of serum ability to recover[15]. Enterococcus faecalis has ability to survive long term in root canal without nutrients[16].

Bacteria encounters with variety of stressful conditions in the root canal, such as nutrient deficiency, other bacterial toxins and intra canal medicaments, these conditions may regulate bacterial adhesin expression. Addition to it, when ever serum leakage into the root canal, inducing the expression of Aggregation substance (AS) and other carbohydrate moieties, thereby helping bacteria to adhere, even alkaline pH obtained by calcium hydroxide at the dentinal zone is ineffective due to deeper penetration of Enterococcus faecalis [17]. Other mechanisms of survival may be through Lipoteichoic acids (LTA), which resist the bacterium against many lethal conditions[18].

E. faecalis prevents the other bacteria growth with cytolysin, AS-48 (Aggregation substance), and bacteriocins, erythrocytes are the target cells of cytolysin [19] also PMNs and macrophages [20] and a broad range of Gram-positive but not Gram-negative organisms [21]. The bacteriocin effect of cytolysin of E. faecalis helps colonization of the Gram-negatives, there could be a shift of bacterial flora related

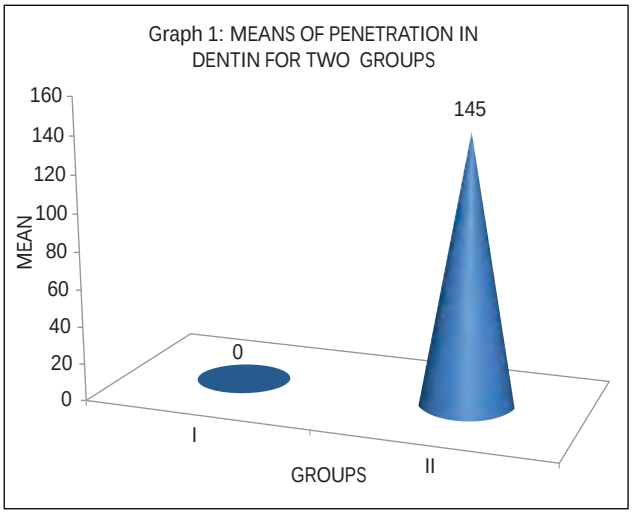


Fig 1: Indentation is made on bucco lingual surface occlusal apically using tapered fissure diamond point, spitted in two half's using chisel.

Fig 2: Group I samples showed no traces of Enterococcus faecalis presence in root dentin under confocal laser scanning microscope.

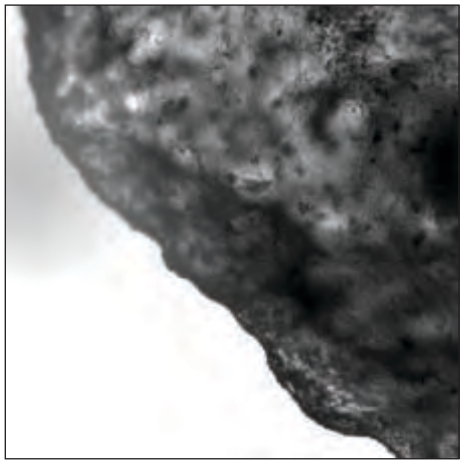


Fig 3: Group II samples showing presence of alive and dead Enterococcus faecalis into root dentin.

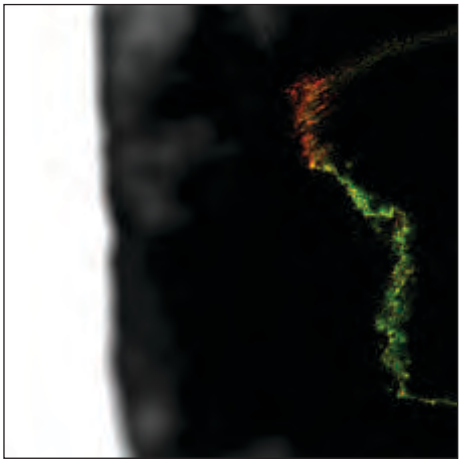


Fig 4: Group II samples showing presence of separate alive (green) and dead (red) Enterococcus faecalis into root dentin.

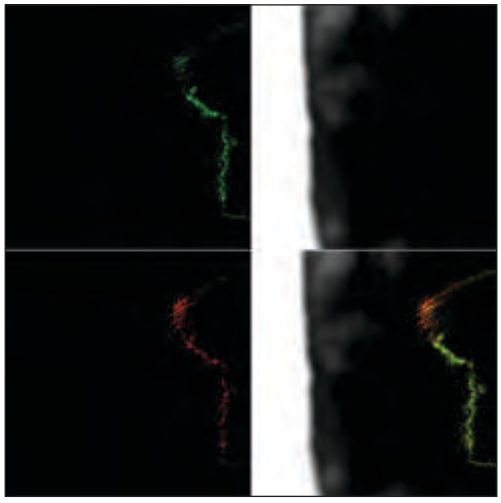
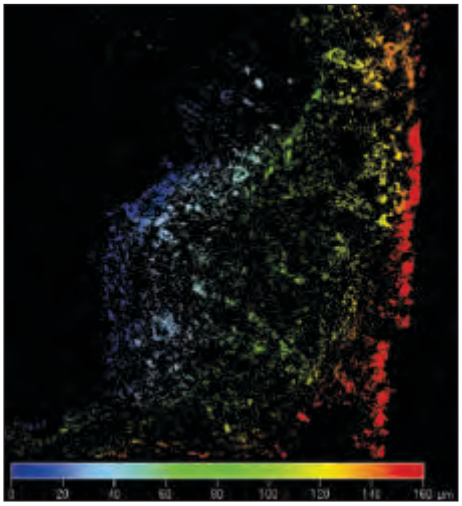


Fig 5: Group II samples showing shows invasion of Enterococcus faecalis upto 160 µm deep in to root dentin.



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with periodontal disease [22], the later factors is non pathogenic in humans. Along with cytolysin, they facilitate the dominance of E. faecalis in a mixed infection and serve as means to obtain ecological advantages which can result in disease in man. The root canal hardly contains any nutrient-rich medium, but when required E. faecalis may derive the energy it needs from the hyaluronan present in the dentin by breaking down hyaluronidase or E. faecalis may also feed on fluid present in the dentinal tubules. So it seems that even well obturated fluid tight seal does effect the survival of Enterococcus faecalis because it can generate energy to survive. The foci of infection is E Faecalis present deep inside the dentin or cementum that cannot be reached by host defence cells such as PNM, lymphocytes and macrophages etc. E faecalis elicits permanent provocative effect on the host defence cell mechanism which in turn damages periradicular region.

We used gamma irradiation method to sterilize the teeth because it does not alter collagen characteristics which is very important for E Faecalis to adhere to collagen, other methods of sterilization of teeth samples are by autoclave, hot air oven etc. Disadvantages of autoclave is it collapses the collagen strands and use of hot air oven makes teeth dehydrated and more brittle.

Previously most of the methods like fluorescent probes used failed to detect viability of bacterial and also spatial distribution[13,23] bacterial viability could be checked by using conventional fluorescence microscopy but the disadvantage is decalcification of the teeth samples, creating artificial condition, high background haze makes difficult to distinguish between individual cells.

In this context, better methodologies for the identification of bacterial viability in dentin are needed. The CLSM (ZEISS LSM 510 META GmbH, Mannheim, Germany) analysis[24] has advantage over the conventional fluorescence microscopy to visualize bacteria. In fact our research confirms ability of E Faecalis to infect root dentin and also shows the vitality of the bacteria in root dentin.

The visualisation of live and dead bacteria in root dentin using confocal microscopy, this method gives information about the root dentin infection and vitality of bacteria in the dentin determined in effective way at the cellular level.

Conclusion :

The mechanism of bacterial invasion is not completely understood, bacterial adhesion to dentinal tubule walls (TWs) is a logical early step in the process. Collagen is widely considered to be the primary substrate for specific binding of E. faecalis to dentin and the collagen binding protein of E. faecalis (Ace) and a serine protease (Spr) have been proposed to play significant roles in binding to the root canal wall[8]. Ace also promotes E. faecalis binding to collagen type I and IV[9,10] and in vitro ace gene expression at 37^0 c was enhanced in the presence of collagen[11]. There is 40% available collagen present in root dentin, collagen present in root dentin might help enterococcus faecalis to inhabit in dentin, and might be possible reason for treatment failure or in ability to control infection

Even after applying or using latest methods like rotary endodontics crown down technique and use of latest root sealers like, still we have failure or re infection cases. by our study we are able to examine viable and dead Enterococcus faecalis even after proper root canal treatment. To conclude we need to rethink in terms of sterilization of root canal, may be the use of any other methods of root canal disinfection may help in eradicating micro organisms from root canal to certain extent for predictable success in root canal treatment

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