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DEPTH OF PENETRATION AND BIOVOLUME OF ENTEROCOCCUS FAECALIS AND FUSOBACTERIUM NUCLEATUM BIOFILM INTO DENTINAL TUBULES USING CONFOCAL MICROSCOPE AND CO-AGGREGATION ASSAY

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ABSTRACT

Introduction: Formation of biofilms in dentinal tubules is the major cause for the bacterial survival and virulence. The purpose of the study was to evaluate the depth of penetration and a biovolume of a mono and dual species of gram positive facultative anaerobe *Enterococcus. faecalis* and gram negative obligate anaerobic *Fusobacterium. nucleatum* into the dentinal tubules under Confocal microscope and coaggregation of bacteria using visual coaggregation assay. **Methods:** Root segment with a length of about 7 to 8 mm were prepared from Forty five caries-free single rooted lower premolar. Root canal was enlarged to a size of a Gates Glidden bur #2 (70mm).Irrigation protocol followed. The teeth were washed and was split in to two half Segments. The specimens were sterilized by autoclave for 20 minutes at 121°C. The strains *F. nucleatum* American Type Culture Collection (ATCC) 25586 and *E.Faecalis* ATCC 29212 were anaerobically incubated to an optical density at 600 nm (OD_{600}) of 0.2 with 2×10^7 colony-forming units (CFU) per milliliter. Bacterial suspension was inoculated as mono and dual species into the root canal for 21 days with sterile thioglycolate medium changed every 48 hours in an anaerobic condition. Depth of penetration and biovolume of mono(control group A and group B –fusobacterium nucleatum) and dual species biofilm (group C) into dentinal tubules was evaluated. Visual Coaggregation assays were performed and a coaggregation score was given and confirmed with a bacterial smear. **Results**: The depth of penetration and biovolume of dual species biofilm into the dentinal tubules was statistically higher than monospecies biofilm((p <0.1). Coaggregation assay showed maximum coaggregation (+4) of *E. faecalis* and *F. nucleatum.* **Conclusion***:* Dual species biofilm penetration and biovolume is dentinal tubules can be one of the major factors in failure of root canal treatment.

KEYWORDS: *Enterococcus. Faecalis, Fusobacterium. Nucleatum.*

INTRODUCTION

Biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other and are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced. They exhibit an altered phenotype with respect to growth rate and gene transcription.[1] In most biofilms, the bacterial populations are less than 10% of the dry mass while the EPS matrix are over 90%.[2] Bacterial biofilm is responsible for primary and persistent infections by invading and penetrating into root canal dentinal tubules.[3,4] The survival and virulence factors capable of causing infection are collagen–binding protein, serine proteases and lytic enzymes. It also utilizes serum as nutritional source, binds to dentinal tubules, withstands nutritional deprivation and resists the activity of antimicrobial agents due to biofilm formation.[5,6] The penetration of bacteria protects it from endodontic medicaments.[7]

E. faecalis is a facultative anaerobe, gram-positive coccus,non-motile is isolated from teeth with 40% primary endodontic infections and frequently in persistent periradicular disease[8,9] Commonly 2 to 8 bacterial species are isolated from infected root canals

with *F. nucleatum*, *Prevotella intermedia* and Streptococci being dominant. A strong association between *F.nucleatum* with *Peptostreptococcus micros, Porphyromonas endodontalis* and *Camylobacter rectus* has been described.[10] Johnson *et al.* suggested that intergeneric coaggregation between *E. faecalis* and *F. nucleatum* plays a potential role in endodontic infections.[11]

In mixed infections, *F.nucleatum* forms a bridge between early and late colonizers in root canal biofilm.[12,13] Difficulties in cultivating these bacteria in strict anaerobic conditions leads to difficulty in detecting fastidious anaerobes in invaded coronal or radicular dentin.

Thus, the aim of our study was to evaluate the depth of penetration and biovolume of a mono and dual species of gram positive facultative anaerobe *E.faecalis* and gram negative anaerobic *F. nucleatum* into the dentinal tubules under Confocal microscope and coaggregation of bacteria using visual coaggregation assay.

MATERIALS AND METHODS

Specimen preparation

Forty-five caries-free single-rooted lower premolar human teeth were collected under a protocol approved by the ethics committee of Sri Ramachandra University (IEC-NI/12/MAR/27/13). Dentin root segments were sectioned at root tip and the crown at 2 to 3 mm below the cementoenamel junction to get a length of 7 to 8 mm. (9). Each canal was enlarged to a size of a Gates Glidden bur #2 (70mm).Irrigation was performed with 1 mL 3% NaOCl solution after each instrument change. The smear layer was removed using 17% EDTA. The teeth were washed with distilled water in ultrasonic bath for 10 minutes and stored in sterile water for 1 week to remove any residual chemical compound. Superficial longitudinal grooves were made in the buccal and lingual surface to facilitate the fracture of the specimens with chisel into two half segments and then flattened with silicon carbide grinding paper.Nail varnish was applied to avoid penetration of bacteria in to the dentinal tubules other than the root canal. Sterilization of the samples was done by autoclave for 15 minutes at 121°C.

The strains *F. nucleatum* American Type Culture Collection (ATCC) 25586 and *E.faecalis* (ATCC) 29212 (Himedia laboratories) were incubated at 37°C in 10 ml in a Thioglycolate medium (Himedia Labs, Mumbai) supplemented with 0.5 mg/l vitamin K and 5 mg/l hemin in a anaerobic condition. After overnight incubation the suspensions were diluted at 1:10 with fresh medium and then incubated up to an optical density at 600 nm (OD600) of 0.2 with 2×10^7 colony-forming units (CFU) per milliliter. The cell suspension was adjusted to match the turbidity equivalent to 0.5 McFarland standard.

For dentin infection, samples were transferred individually into 96 well plate. 200µl of *E.faecalis* suspension and *F.nucleatum* suspension was inoculated in 15 samples each (Control –Group A -15) (Group B-15) and 100µl of *E.faecalis* and 100µl of *F.nucleatum* were inoculated together in 15 samples (Group C -15) for 21 days in anaerobic condition. The anaerobic environment was produced by activating Gas Pak Anaerobe System with indicator (Becton-Dickinson) in anaerobic jars (Becton-Dickinson Microbiology Systems). Sterile Thioglycolate medium was continuously changed every 48 hours and the indicator was monitored. After the incubation period of 21 days, Phosphate Buffered Saline (PBS) was used to wash dentin segments to remove non-adherent bacteria Root samples was stained with 50µl fluorescein diacetate (FDA; Sigma) and 50µlpropidium iodide (PI; Sigma). The specimens were immediately analyzed by inverted Carl zeiss Confocal microscope(CLSM) using a 20x magnification oil lens. The 9Z stack images were obtained by using sections of 1µm step size, and the pictures was standardized to a format of 524×524 pixels. For an objective analysis, the CLSM images were exported to the Image J software in order to quantify the amounts total mean biovolume of bacteria. A 3D biofilm structure was reconstructed using the same software.

Visual Coaggregation assay

Coaggregation assays was performed as described by Cisar in coaggregation buffer (CAB; 150mM NaCl, 1mM Tris, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$• H2O [pH 7.5]). Cells were pelleted and re-suspended in CAB to $2x10^7$ cells. Coaggregation assay was done in a reaction tube by mixing 200µl of each strain to a total mix of 400µl. The reaction mixtures were mixed for 5 seconds when the second partner strain was added. 10 mins incubation was done atleast 10 min prior to evaluation. Visual scoring system by Cisar was done ranging from 0 to 4. A coaggregation score 0 - there is no change in turbidity and no visible coaggregates. Score +4-maximum coaggregation and large coaggregates settled immediately, leaving a water-clear supernatant. Score +3 - formation of large settling coaggregates but a slightly turbid supernatant. Score+2 - definite coaggregates were visible but did not settle immediately. Score +1 indicated finely dispersed coaggregates in a turbid background.

Bacterial coaggregation –smear

Overnight cultures of *E. faecalis* and f.nucleatum were used to prepare the smear. A loopful of culture was spread evenly on a clean glass slide and air-dried. The smear was heat fixed and slides were stained with Propidium Iodide(PI) and the slides were viewed under Confocal microscopy.

RESULTS

F. nucleatum and *E. faecalis* monocultures were inoculated in 96 well plates in dentin samples and their depth of invasion were quantified after 21 days of incubation. Representative confocal micrographs of *E. faecalis* (Fig.IA) and *F. nucleatum* (Fig. IB) individually. Fig I C shows the mutualistic growth of *F. nucleatum*

and *E. faecalis*.The mean depth of mono bacterial invasion in control Group-A (*E.faecalis*)was 283.44µm and Group-B (*F.nucleatum*)257.027µmand Group-C had greater depth of penetration of dual species bacteria at a mean value of 325µm (P<0.1) (Table. I). The mean biovolume of dual species bacteria of *E.faecalis* and *F. nucleatum* was 3781.33 in the dentinal tubules compared to mono species biofilm(P<0.1).(Table II). The least biovolume was in group B (555.13). 3D biofilm structure was reconstructed (Fig.II). The results of coaggregation assay of *E.faecalis* and *F.* nucleatum resulted in +4 score. This assay highlights the maximum coaggregation of *F. nucleatum* and *E.*faecalis which was confirmed with the bacterial smear(Fig III).

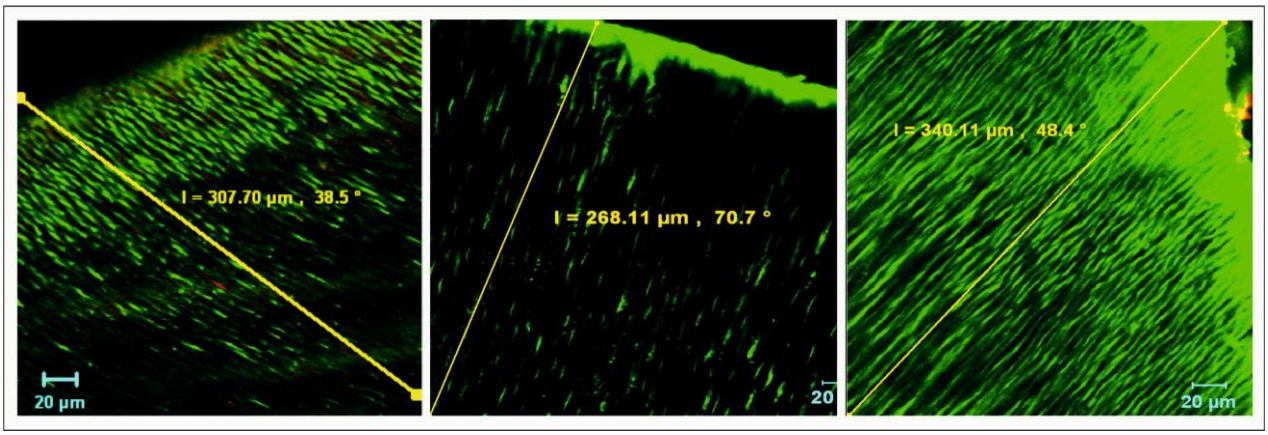

Fig I: Representative confocal scanning images of depth of penetration of bacteria into dentinal tubules (A) *E.faecalis* (B) *F.nucleatum* (C) *E.faecalis&F.nucleatum* biofilm.

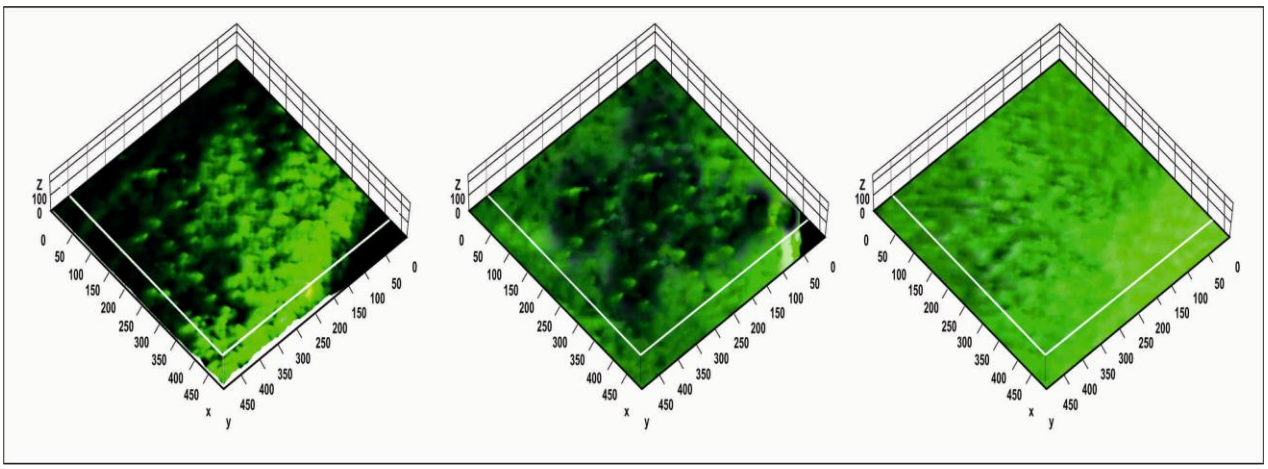

Fig II: 3D Biofilm reconstruction of A.*E.faecalis*, B.*F.nucleatum* and C.dual species biofilm of E.faecalis and F.nucleatum.

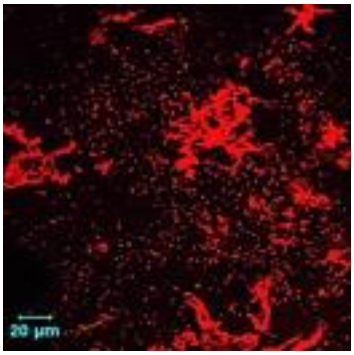

Fig III: Bacterial smear of coaggregation between *E.faecalis* and *F.nucleatum*.

Table I: Shows the mean depth of bacterial invasion in Group-A (*E.faecalis*), Group-B (*F. nucleatum*) and Group-C (*E.faecalis* and *F. nucleatum*).

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	15	275.7093	31.76093	8.20064	258.1207	293.2979	208.16	307.36
Group B	15	248.7447	10.87772	2.80861	242.7208	254.7685	233.10	268.18
Group C	15	335.4877	7.54461	1.94801	331.3096	339.6657	321.77	351.13
Total	45	286.6472	41.47850	6.18325	274.1857	299.1087	208.16	351.13

Table II: Biovolume of bacterial invasion in Group-A (*E.faecalis*), Group-B (*F. nucleatum*) and Group-C (*E.faecalis* and *F. nucleatum*).

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	15	1972.0000	607.03048	156.73460	1635.8377	2308.1623	1075.00	2846.00
Group B	15	555.1333	235.22174	60.73399	424.8719	685.3948	240.00	849.00
Group C	15	3781.3333	1140.69357	294.52581	3149.6383	4413.0284	2094.00	6023.00
Total	45	2102.8222	1527.01270	227.63361	1644.0568	2561.5876	240.00	6023.00

DISCUSSION

Bacteria entry in to the root canal system is responsible for persistent root canal infection.[14] Dentinal tubules work as a great reservoir of bacteria completely outside immunological control. Biofilm formation in the dentinal tubules includes adherence of the organisms, formation of microcolonies and co-adhesion and coaggregation.[15] As bacteria approaches the root canal surface, both attractive and repulsive forces help in adhesion. When the bacteria is away at 10–20 nm distance from the root surface, negative charges on the bacterial surface and root surfaces are repelled. This is followed by mechanical attachment due to attractive van der waals forces, fimbriae and flagella.[16] Proteinaceous adhesions of one species recognizes and adheres to polysaccharide receptor on the cell surface of other species. The coadhesion and coaggregation can be unimodal and multimodal.[17] Coaggregation of facultative anaerobe to obligate anaerobe results in consumption of oxygen, which results in survival of strict oral anaerobes in an aerobic environment.[18]

Our results showed greater depth of penetration and biovolume of dual species biofilm in the dentinal tubules. The depth of penetration of the organisms in to the dentinal tubules may be due to the adherence to collagen and intertubular cell growth. Average oral streptococcal cell and related microorganisms (0.5-0.7 mm) is smaller than a dentinal tubule and can penetrate dentinal tubules by changing its appearance based their size and dentinal tubules status.[19] Adherence to type I collagen in dentinal tubules invasion is mediated by adhesins like collagen binding protein of *E.*faecalis (Ace) and a serine protease (spr). They also adhere to the mineral part probably through Lipoteichoic acid.[20,21] Adhesion of *F.* nucleatum to other bacteria and to tooth surface may be due to fimbrial adhesions and non fimbrial adhesions. Fimbrial adhesions like FadA is important for attachment and invasion.[22] Deeper penetration in to the dentinal tubules may be due to intertubular cell growth and does not require specific binding.[19] *F. nucleatum* activates multiple cell signalling systems that lead to stimulation of collagenase MMP 3 which can also help in bacterial invasion.[23]

The increased penetration and biovolume of coaggregated bacteria of gram positive and gram negative biofilm into the dentinal tubules than single species biofilm may be also due to *E.* faecalis with the help of with cytolysin, AS (aggregation substance) and bacteriocins prevents the other gram positive bacterial growth[21] but it does not inhibit gram-negative organisms. *F. nucleatum*, an obligate anaerobe adapts in higher oxygen content in aerobic environment by increasing in number, length and generates an oxidoreduction potential low enough for the survival of the strict anaerobes.[24] The presence of *F. nucleatum*, critical in physical interactions between aerobic and anaerobic bacteria during biofilm formation increases 4-fold when total bacteria increase was about 10%. [25] Five adhesins are involved in biofilm formation by *F.nucleatum* have been described. FomA, 300-350 kDa Galactose-binding Adhesin, N-acetylneuraminic Acid Specific *F. nucleatum* Adhesin, RadD.[26]

The ability of *E. faecalis* and *F. nucleatum* to penetrate deeply and form increased biovolume in the dentinal tubules was demonstrated in this confocal study.Variations observed in depth of penetration and biovolume of bacteria in dual species biofilm might be a considering factor in failure of root canal treatment.

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