

Original Article

Biofilm forming capacity of *Enterococcus faecalis* on Gutta-percha points treated with four disinfectants using confocal scanning laser microscope: An *in vitro* study

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ABSTRACT

Background: The aim of this study was to evaluate and compare the *in vitro* biofilm forming capacity of *Enterococcus faecalis* on Gutta-percha points disinfected with four disinfectants.

Materials and Methods: A total of 50 Gutta-percha points used in this study were divided into four test groups based on disinfectant (5.25% sodium hypochlorite, 2% chlorhexidine gluconate, 20% neem, 13% benzalkonium chloride [BAK]), and one control group. The Gutta-percha points were initially treated with corresponding disinfectants followed by anaerobic incubation in Brain Heart Infusion broth suspended with human serum and *E. faecalis* strain for 14 days. After incubation, these Gutta-percha points were stained with Acridine Orange (Sigma – Aldrich Co., St. Louis, MO, USA) and 0.5 mm thick cross section samples were prepared. The biofilm thickness of *E. faecalis* was analyzed quantitatively using a confocal scanning laser microscope. Results statistically analyzed using analysis of variance. $P < 0.05$ was considered to be significant.

Results: Confocal scanning laser microscope showed reduced amount of *E. faecalis* biofilm on Gutta-percha points treated with BAK and sodium hypochlorite. *Post-hoc* (least square differences) test revealed that there is no statistically significant difference between BAK and sodium hypochlorite groups ($P > 0.05$).

Conclusion: This study illustrates that the Gutta-percha points disinfected with sodium hypochlorite and BAK showed minimal biofilm growth on its surface.

Key Words: Benzalkonium chloride, biofilm, chlorhexidine gluconate, confocal scanning laser microscope, *Enterococcus faecalis*, sodium hypochlorite

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INTRODUCTION

Endodontic failures can be caused by secondary invasion of oral bacteria into the root canals during treatment, after the breakdown of temporary restorations between appointments, or after fracture of the permanent restoration.^[1] Newly invading microorganisms might come across a variety of

situations that may favor their establishment as biofilms or their inclusion into preexisting biofilms.^[2] It has been reported that bacteria might be able to survive inflammatory responses within periapical lesions and the concept of extra radicular infection and biomaterial-centered infection has received considerable attention as main etiological factor of refractory periapical periodontitis.^[3-5] Implanted biomaterials (i.e., Gutta-percha points) provide surface for bacterial adherence and formation of biofilm, eventually leading to biomaterial-centered infections. In previous studies, authors observed extruded root filling Gutta-percha points associated with refractory periapical periodontitis, using scanning electron microscopy.^[6]

Irrespective of thorough cleaning and shaping, complete elimination of microorganisms from

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the root canal system is not possible and these microorganisms may remain in dentinal tubules, apical ramifications and periapical areas.^[7,8] These microorganisms have the ability to form intra radicular, extra radicular, and foreign body associated biofilms leading to failure of the endodontic treatment.^[3] One of the possible modes of preventing these failures is by thorough disinfection of Gutta-percha points before obturation or by making Gutta-percha points resist the formation of biofilm on its surface.^[9] Biofilm formation is initiated by bacterial deposition on a surface and irreversible adhesion to the substratum. In the initial stages of biofilm formation, the adhesive property of bacterial cells play a major role for irreversible attachment to surfaces and is also influenced by the formation of a conditioning film on the surface as a result of interactions between the substratum and the surrounding environment.^[10,11]

Enterococcus faecalis is one of the most prominent bacterial species isolated from root canals of treatment failed teeth.^[12] Studies have showed *E. faecalis* in 30–89% of teeth with postendodontic treatment failures, mostly as monoculture.^[13,14] *E. faecalis* has the ability to survive harsh environmental conditions present in the root canals of endodontically treated teeth with Gutta-percha and sealer and it could survive endodontic irrigant by resisting high concentrations of intra-canal medicaments and wide variations in pH.^[15-17] The aim of the present study was to evaluate and compare the *in vitro* biofilm forming capacity of *E. faecalis* on disinfected Gutta-percha points after incubating them in Brain Heart Infusion (BHI) broth supplemented with human serum and *E. faecalis* suspension.

MATERIALS AND METHODS

A total number of 50 Gutta-percha points of size F2 (Dentsply Maillefer, Ballaigues, Switzerland) of the same batch sterilized with ethylene oxide were used in this study. These Gutta-percha points were treated with four disinfectants before incubation with *E. faecalis* and they were divided into four test groups ($n = 10$) based on disinfectant and one control group.

- Group 1: Control group (Gutta-percha points without disinfection).
- Group 2: Gutta-percha points treated with 5.25% sodium hypochlorite (NaOCl) (Asian Acrylates, Mumbai, India).

- Group 3: Gutta-percha points treated with 2% chlorhexidine gluconate (CHX) (V-Concept, Vishal Dentocare Pvt. Ltd., Ahmedabad, India).
- Group 4: Gutta-percha points treated with 20% neem (Baidyanath Ayurved Bhawan, Nagpur, India).
- Group 5: Gutta-percha points treated with 13% benzalkonium chloride (BAK) (SDFCL, SD Fine-Chem Limited, Mumbai, India).

Five groups were labeled properly on five sterile test tubes of 10 mL volume and each group consisted of 10 Gutta-percha points. All the Gutta-percha points of each experimental group were treated with respective disinfectants for 1 min except control group. After 1 min of disinfection, the solutions were removed from the test tubes with sterile plastic droppers (US Associates, Uttar Pradesh, India) and then Gutta-percha points were washed with distilled water and they were allowed to dry in their corresponding test tubes. In this study, BHI broth was used as a nutrient for culture of *E. faecalis*. The total experiment was divided into four steps.

Preparation of culture media

The *E. faecalis* bacterial strain ATCC 29212 was harvested during stationary phase and 100 μ L of each bacterial suspension was inoculated into the test tube containing 2 mL of BHI (HiMedia Laboratories Pvt. Ltd. Mumbai, India) broth supplemented with 50% (vol/vol) of human serum for each group.

Preparation of Gutta-percha point for biofilm formation

The prepared culture media was placed into five test tubes and all the Gutta-percha points were incubated for 14 days at 37°C in an anaerobic jar (Dynamicro GR, Thane, India). The medium was changed every 24 h and these Gutta-percha points were subsequently subjected to staining.

Staining of biofilm

The test tubes with *E. faecalis* contaminated Gutta-percha points were stained with 1 mL of 0.01% Acridine Orange (Sigma – Aldrich Co., St. Louis, MO, USA) in a dark environment for 30 min and finally rinsed with distilled water to remove excess dye from the Gutta-percha points and were allowed to dry. Acridine Orange was selected for this study because this dye has the ability to bind with bacterial nucleic acids emitting red fluorescence under excitation and emission wavelength of 460 nm and 650 nm, respectively. After the staining procedure, the

corresponding specimens were immediately subjected to confocal scanning laser microscope (CSLM) under $\times 40$ magnifications (Leica Microsystems GmbH, Mannheim, Germany).

Biofilm thickness measurements

Cross section of 0.5 mm thick sample from each Gutta-percha point at around 5 mm from the tip is prepared on glass slab with a custom made acrylic block containing two parallel razor blades (Gillette do Brasil & Cia, Riode Janeiro, Brazil) of 0.5 mm separation and hence that each group has 10 samples and all the samples were observed using an inverted Leica TCS-SPE Confocal Microscope (Leica Microsystems GmbH, Mannheim, Germany) under $\times 40$ magnifications in a format of 1024×1024 pixels.

The images were acquired and evaluated using the Leica Application Suite - Advanced Fluorescence software (Leica LF, Leica Mannheim, Germany) [Figure 1].

Statistical analysis was performed using SPSS software (version 19.0.1, SPSS Inc., Chicago, IL, USA) by applying mean values using analysis of variance with *post-hoc* least square differences (LSD) method. $P < 0.05$ was considered to be significant.

RESULTS

Confocal scanning laser microscope images confirmed *E. faecalis* biofilm formation in all groups. Biofilm thickness (μm) on the cross section samples were randomly measured at twelve reference points [Figure 2]. The mean thickness of biofilm on the

samples was calculated. Results were expressed as mean \pm standard deviation. CSLM showed reduced amount of *E. faecalis* biofilm on Gutta-percha points treated with BAK and NaOCl compared with other groups [Table 1]. *Post-hoc* (LSD) test revealed that there is no statistically significant difference between BAK (Group 5) and NaOCl group (Group 2) ($P > 0.05$).

DISCUSSION

Successful endodontic therapy relies upon thorough cleaning and shaping, disinfection and three dimensional obturation of the root canal system.^[18] Irrespective of thorough cleaning and shaping, the ability of bacteria to form biofilms in harsh environments pose a challenge to the outcome of endodontic treatment and failures associated with biomaterial-centered infections, which are a common entity in endodontic therapy.^[7,8]

Biofilms are defined as polysaccharide matrix enclosed bacterial population's adherent to each other and/or to surfaces or interfaces.^[19] Formation of a

Table 1: Biofilm thickness (μm) on the Gutta-percha points

Groups	Mean \pm SD
Control	12.1960 \pm 0.68874
Sodium hypochlorite	3.7670 \pm 0.66361*
Chlorhexidine	5.8880 \pm 0.35568
Neem extract	8.6040 \pm 0.49259
BAK	3.6640 \pm 0.45783*

Results are expressed as mean \pm SD (μm). *Symbol are statistically not significant ($P > 0.05$). SD: Standard deviation, BAK: Benzalkonium chloride

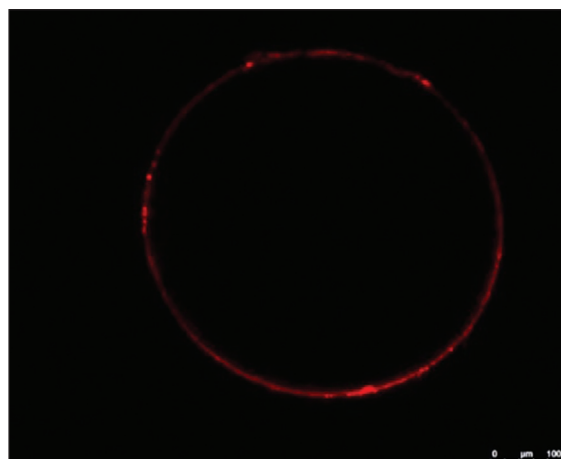


Figure 1: Sample under inverted Leica TCS-SPE Confocal Microscope.

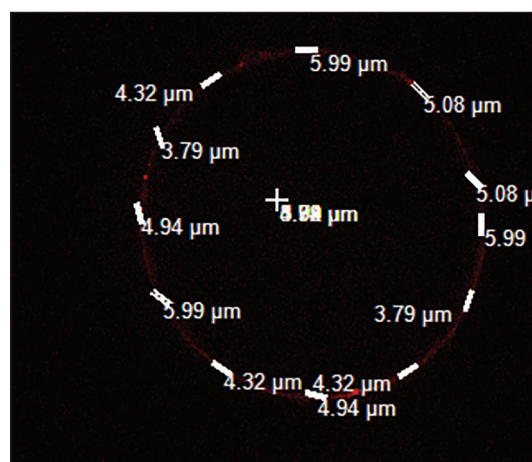


Figure 2: Biofilm thickness calculated at 12 points on a sample using the Leica Application Suite - advanced Fluorescence software.

biofilm is a step-wise procedure, which goes through following phases:

1. Deposition of conditioning film.
2. Adhesion and colonization of planktonic microorganisms
3. Bacterial growth and biofilm expansion
4. Detachment of biofilm microorganisms into their surroundings.^[20]

Endodontic biofilm can be classified as:

- (1) Intra radicular biofilm.
- (2) Extra radicular biofilm.
- (3) Periapical biofilm and
- (4) Foreign body centered biofilm.

Foreign body – centered biofilm is seen when bacteria adheres to an artificial biomaterial surface and forms biofilm structures, also known as biomaterial-centered infection.^[21] Bacterial adherence to a biomaterial can be described in three phases – transport of bacteria to biomaterial surface; initial, nonspecific adhesion phase; specific adhesion phase.^[20]

In endodontics, biomaterial-centered biofilm can be intraradicular or extraradicular depending upon the position of obturating material. Takemura *et al.*^[22] suggested Gram-positive facultative anaerobes have the ability to colonize and form extracellular polymeric matrix surrounding Gutta-percha in the presence of serum. Serum provides a variety of proteins and glycoproteins.^[23,24] When exposed to high concentrations of serum, Gutta-percha point surfaces are thought to become coated with serum pellicle and it is possible that proteins and glycoproteins in the serum pellicle serve as receptors that are recognized by specific bacterial species, that increases the surface hydrophobicity of planktonic bacteria, and that this elevated hydrophobicity promotes bacterial adherence.^[22]

Enterococcus faecalis is a Gram-positive cocci, facultative anaerobe. It is associated with infections in root canal and also they are seen in cases with chronic periapical pathology and failed root canal cases.^[12,15,25] *E. faecalis* has many survival and virulence factors capable of causing mono-infection, utilize serum as a nutritional source, bind to dentinal tubules, produces collagen – binding protein and serine protease that alter host responses, and suppresses the action of lymphocytes.^[13,14,17,26] Among all the survival and virulence factors, *E. faecalis* has the unique property of biofilm formation and the physiochemical properties of these organisms help them to modify according to the prevailing environmental and nutrient

conditions.^[27] Irrespective of type and technique of sealer application, *E. faecalis* can form biofilm on the Gutta-percha points in the root canal system.^[28]

Earlier studies evaluated the effectiveness of various disinfectants like glutaraldehyde, povidone iodine, NaOCl and per acetic acid for disinfection of Gutta-percha points, with few noted disadvantages. Glutaraldehyde releases toxic vapors which can cause eye, nose, and throat irritation, allergy, contact dermatitis, asthma, and rhinitis.^[29] Povidone iodine tends to dry Gutta-percha points.^[30] Valois *et al.*^[31] observed aggressive deteriorative effects on Gutta-percha cone elasticity for 5.25% NaOCl at 1 min. Bounoure *et al.*^[32] reported that repeated exposure to per acetic acid is toxic. CHX, NaOCl, neem and BAK have been cited in literature as disinfecting irrigants for root canals.^[33,34] CHX is a salt of chlorhexidine and gluconic acid. It is used for better healing and regeneration of the oral tissues in conditions such as gingivitis, periodontitis, and as root canal disinfectant. It is a cationic bisguanide that acts by adsorbing onto the microorganism cell wall and causing intra cellular component leakage and is suggested as an effective irrigant and an intra-canal medicament because of its ability to disinfect dentinal tubules against *E. faecalis*.^[35-37] Neem is of particular interest to the field of dentistry for it has a long history of treating teeth and gingival problems. Nimbidin and nimbolide, which are constituents of neem cause lysis of bacterial cell walls.^[38] Neem is highly effective in the treatment of oral and periodontal disease because of good antibacterial, antifungal, antiviral, antioxidant, antiinflammatory, antipyretic, analgesic, and immune-stimulant activity. Furthermore, it also has an antiadherence activity by altering bacterial adhesion and colonization.^[39,40] NaOCl solution has been used for >70 years because of its well-known antimicrobial action and its ability to dissolve tissue. It is also an effective antimicrobial agent against *E. faecalis*. NaOCl is by far the most commonly used irrigant in endodontic therapy.^[41] It provides gross debridement, lubrication, destruction of microbes, and dissolution of tissues.^[41]

Benzalkonium chloride is a nitrogenous cationic surface-acting agent belonging to the quaternary ammonium group. It has been considered as one of the safest synthetic biocides known, and has a long history of efficacious use in eyewashes, hand, and face washes, mouthwashes, spermicidal creams, and in various other cleaners, sanitizers, and disinfectants. BAK has

positively charged molecules that bind strongly to the cell walls and membranes of bacteria because of their opposite, negative charge. The mode of action of BAK against bacterial cells is thought to involve a general perturbation of lipid bilayer membranes as found to constitute the bacterial cytoplasmic membrane and the outer-membrane of bacteria leading to a generalized and progressive leakage of cytoplasmic materials to the environment and/or that the repelling effect of surfactant coatings is focused in physicochemical and/or steric changes.^[42]

In previous studies, authors evaluated the efficacy of various disinfectants on Gutta-percha points. These studies involved initial contamination of Gutta-percha points with various microbial cultures and testing the efficacy of disinfectant.^[29,43,44] The unique feature of this study is that the Gutta-percha points were initially treated with corresponding disinfectants followed by contaminating with *E. faecalis* strain intentionally and evaluating resistance of Gutta-percha points against biofilm formation. The clinical relevance of this study is that in endodontically treated teeth, Gutta-percha points may get exposed to microbial species from various sources of root canal (dentinal tubules, apical ramifications, periapical lesions, and due to breakdown of short and long term restorations) irrespective of thorough treatment protocols.

Time for disinfection of Gutta-percha points was standardized for 1 min.^[32,43] Previous studies done on physical properties of Gutta-percha treated with different disinfectants concluded that disinfection for 1 min might not alter physical and chemical properties of Gutta-percha points.^[45,46]

CONCLUSION

The results of this study suggest that Gutta-percha points treated with BAK and NaOCl showed less amount of biofilm growth on their surfaces followed by, CHX and neem in comparison to control group. Based on the results of this study, there is no significant difference between BAK and NaOCl groups. The difference in biofilm formation on differently treated Gutta-percha points might be attributed to antimicrobial properties of the disinfectants and/or change in adhesive properties of treated Gutta-percha points against biofilm.

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