ISSN- O: 2458 - 868X, ISSN–P: 2458 – 8687
Index Copernicus Value: 49. 23
PubMed - National Library of Medicine - ID: 101731606

International Journal of Medical Science and Innovative Research (IJMSIR)
IJMSIR : A Medical Publication Hub
Available Online at: www.ijmsir.com
Volume – 3, Issue – 5, October - 2018, Page No. : 170 - 176

Enterococcus faecalis biofilm formation on gutta-percha obturating material coated with or without sealer and conditioned using saliva or serum - an in vitro study.

[1]Charu Kapur, [2] Mantri Shivkumar P, [3] Bonny Paul, [4] Kavita Dube, [5] Mani Vishwakarma,

[1]Senior Lecturer, [2] Guide, Professor & Head, [3] Professor, [4] Professor

Department of Conservative Dentistry and Endodontics, Hitkarini Dental College and Hospital, Jabalpur

[5]Microbiologist, Narmada Gelatines Limited, Jabalpur

Corresponding Author: Dr Mantri Shivkumar P. Professor & Head, Dept.of Conservative dentistry & Endodontics, Hitkarini Dental College, Dumna Airport Road, Jabalpur, M.P. India.

Type of Publication: Original Research Paper

Conflicts of Interest: Nil

Abstract

Introduction: Presence of biomaterials in close proximity to the host immune system increases the susceptibility to biofilm formation. It could provide surface for bacterial adherence and formation of biofilm - leading to biomaterial-centered infections. Gutta percha is used as a root canal obturating biomaterial. The aim of this study is to assess and compare an Enterococcus faecalis (*E* Faecalis) biofilm formation on saliva/serum conditioned specimens of Gutta percha (GP) points coated with calcium hydroxide or zinc oxide eugenol sealer.

Methodology: Gutta percha point samples were divided into six groups based on the combinations of root canal sealers and conditioning liquids. Polyethylene tubes were used to prepare samples coated with sealer. Specimens, conditioned in saliva/serum for fifteen and thirty days, were incubated with Enterococcus faecalis for a period of 14 days in nutrient-rich media. Viable cell assay & scanning electron microscope examination was used to assess biofilm formation. Data obtained was statistically analyzed using ANOVA and Duncan's multiple range test. p-value <0.05 was considered as statistically significant.

Results: Statistically significant difference between the groups was observed (p=0.002). Saliva conditioned specimens of Gutta Percha points without sealer showed maximum mean Colony Forming Unit (CFU) count whereas Sealapex coated Gutta Percha points conditioned using serum for 15 days showed least biofilm formation.

Conclusion: All GP specimens exhibited biofilm formation. Conditioning medium, duration, and sealer affect biofilm formation.

Keywords: Enterococcus faecalis, Biofilm, Gutta Percha points, Saliva, Serum.

Introduction

Bacterial biofilms are cited as the primary cause of chronic and recurrent root canal infections. Endodontic biofilm can be categorized as- intracanal, extra-radicular, periapical and biomaterial centered bio films.[1,2] Presence of biomaterials in close proximity to the host immune system increases the susceptibility to biofilm formation. Implanted biomaterials could provide surface for bacterial adherence and formation of biofilm - leading to biomaterial-centered infections.[1]

E faecalis has been associated with a wide variety of human infections such as those of the urinary tract, the bloodstream, the abdomen, the endocardium and in situ foreign devices.[3] It is frequently found in obturated root canals, which exhibit signs of chronic apical periodontitis. 70% of positive cultures are noted and often occur in monoculture.[4,5] It can adhere to several surfaces with different kinetics in a distinct manner depending on the clinical isolate type. The adherence mechanism and subsequent biofilm formation have an important role for bacterial colonization and survival in the host and are implicated in chronic infections.[6] Some studies have held that if biofilm is removed completely with the help of chelating agents and proper cleaning of canals, the extent of obturation does not play a role. Achieving such an extent of biofilm conditioning or removal cannot be guaranteed in clinical practice.[7,8]

Orthograde or retrograde fluid leakage may occur in the root canal over a period of time. Endodontic treatment may fail due to coronal leakage. Obturated root canals may be contaminated from the oral cavity due to exposure of the root canal filling material because of breakdown or loss of the filling, fracture of the tooth or restoration, recurrent decay, microleakage through the material or delay in the placement of permanent restorations. In such situations, microorganisms may invade and recolonize the root canal system. If microbial cells and their products reach the periradicular tissues, they can induce and/or perpetuate periradicular disease.[9]

It is claimed that the percolation of periapical exudate into an incompletely filled root canal accounts for approximately 60% of endodontic failures. An investigation concluded that, there is a possibility of apical retrograde fluid movement as a result of chewing forces, which can provide an ideal environment for any surviving bacterial species to grow and cause persistent endodontic infections.[10]

Despite extensive research on the *E* faecalis biofilm, scanty literature is available on the role of saliva or serum conditioning on mono-species biofilm formation on gutta percha obturating material with or without sealer. Hence the aim of the present study was to compare E faecalis biofilm formation on saliva or serum conditioned gutta percha points without or with sealer.

Materials and Methods

One hundred and ninety-two gutta percha points, size 40 and taper 0.40 (Dentsply Maillefer, Ballaigues, Switzerland) were selected. The points were divided into six groups, each containing thirty-two GP (n=32), to prepare specimens as follows – GP (conditioned by saliva), GP (serum), GP with Tubliseal sealer (saliva), GP with Tubliseal sealer (serum), GP with Sealapex sealer (saliva) and GP with Sealapex sealer (serum).

Sample Preparation

GP points were disinfected by placing them in 3% sodium hypochlorite for 1 min, followed by rinsing in sterile distilled water and drying in a biohazard cabinet. Polyethylene tubes of 30 mm length and 3.5 mm diameter were selected to simulate root canal lumen. The tubes were disinfected with 90% isopropyl alcohol for 5 minutes and dried in a biohazard cabinet. Tubliseal (Kerr Dental Corporation, USA) was mixed as per the manufacturer's instructions. This was filled in the tube and a GP point was inserted in the filled tube to form a sample. The tubes with the gutta percha points coated with sealer were left undisturbed for eight days at 37^0C. Sixty-four samples were prepared using Tubliseal. Similar procedure was carried out for Sealapex sealer (Sybron Kerr, Orange,CA). After 8 days, the sealer- coated gutta percha points were removed gently from the tubes.

Conditioning of samples

Whole saliva collected from subject was filter- sterilized and was used as a conditioning fluid to simulate orthograde fluid leakage. Fetal bovine serum (HIMEDIA;

Ref- RM9955) was used as a conditioning liquid to simulate retrograde fluid leakage. The samples were subjected to conditioning with saliva or serum in a sterile glass container at 37^0C. The conditioning of the samples (n=32 per group) in respective liquids was carried out for 15 and 30 days. After a specific-time interval (i.e.12th day and 24th day) one sample was removed from each group to check the possibilities of contamination (i.e. two samples from each group).

Incubation with Enterococcus faecalis

Fourteen samples from each group, after 15 days and 30 days of conditioning, were incubated with bacteria (ATCC 29212) in separate sterile glass flasks under nutrient–rich medium at 37^0C for a period of 14 days. To remove the dead cells, and to replenish medium, 50% of culture media was replaced every third day. After this period, the samples were removed from the culture plates and rinsed three times in the phosphate buffer saline to remove non-adherent cells from these samples. The biofilm-forming capacity of Enterococcus faecalis on gutta percha points was examined using viable cell assay.

Viable Cell Assay

Fourteen samples, from each group, were transferred individually into the test tubes with 10 ml of phosphate buffer saline and agitated vigorously using a vortex shaker. 10uL of inoculum, from this suspension, was serial diluted and consequently, plated on previously prepared agar plates using a micropipette. Manual counting of the colony- forming units (CFUs), was performed after 72 hours of incubation (37oC) to determine the total viable cells adhering to samples. Log values were recorded. CFU counting (expressed in log10 cells per unit GP) provides a relative assessment of bacteria in biofilm formed on samples.

Similar procedure was carried out for the samples which were conditioned for thirty days.

The mean Log values were calculated & statistically analyzed using ANOVA followed by Duncan Multiple Range test. $P < 0.05$ was considered statistically significant.

Scanning electron microscope examination was done to assess biofilm formation.

Results

GP specimens of all the groups showed biofilm formation. There was a statistically significant difference ($p<0.05$) between the groups with respect to sealer, conditioning medium and duration. (Table1).

Comparison of means of samples coated with or without sealers showed mean CFU count was maximum (6.45+0.01) with GP without sealer, whereas GP with Sealapex exhibited the least value (4.01+0.03). Comparison of means of samples conditioned with saliva or serum had significant difference ($p < 0.01$) and while ranking them, GP without sealer conditioned by saliva had maximum mean CFU count (6.54+0.01) whereas GP Sealapex conditioned by serum showed minimum (3.82 +0.03) biofilm formation. While ranking according to the duration of conditioning, control saliva 15 days group had maximum mean CFU (6.54 + 0.01) and GP Sealapex serum 15 days group exhibited least (3.71+ 0.05) biofilm formation. (Table 2) (Figure1).

SEM showed E.faecalis biofilm on specimens. The biofilm formed was mostly regular and monolayered. (Figure 2)

Table 1: Comparison of mean Log values of CFU obtained by viable cell assay between groups using ANOVA.

*differ significantly ($p<0.05$).

Sample Sealer (n=56)	Mean CFU	Conditioning Medium (n=28)	Mean CFU	Duration Mean CFU	
				15 days (n=14)	30 days (n=14)
GP No Sealer	6.45±0.01	Saliva	6.54 ± 0.01	6.54 ± 0.01	6.54± 0.02
		Serum	6.35 ± 0.01	6.33 ± 0.01	6.37 ± 0.01
GP Sealapex	4.01±0.03	Saliva	4.19 ± 0.02	4.10 ± 0.02	4.27 ± 0.01
		Serum	3.82 ±0.03	3.71 ± 0.05	3.92 ± 0.02
GP Tubliseal	5.27±0.07	Saliva	5.78 ± 0.02	5.79 ± 0.02	5.76 ± 0.02
		Serum	4.76 ± 0.04	4.79 ± 0.04	4.73 ± 0.05
F value (1%)	14.84**	62.06 **		7.00 **	

Table 2: Duncan Multiple Range Test for comparison and Ranking of samples.

Ranked Order			
Groups	Duration Mean CFU	Conditioning medium Mean CFU	Sealer Mean CFU
Control saliva 15 days	6.54 + 0.01[a]		
Control saliva 30 days	6.54 + 0.02[a]	6.54 + 0.01[a]	
Control serum 30 days	6.37+0.01[b]		
Control serum 15 days	6.33+ 0.01[c]	6.35 + 0.01[b]	6.45+0.01[a]
GP Tubliseal saliva 15 days	5.79+ 0.02[d]		
GP Tubliseal saliva 30 days	5.76+ 0.02[d]	5.78 + 0.02[c]	
GP Tubliseal serum 15 days	4.79+ 0.04[e]		
GP Tubliseal serum 30 days	4.73+ 0.05[f]	4.76 + 0.04[d]	5.27+0.07[b]
GP Sealapex saliva 30 days	4.27+ 0.01[g]		
GP Sealapex saliva 15 days	4.10+ 0.02[h]	4.19 + 0.02[e]	
GP Sealapex serum 30 days	3.92+ 0.02[i]		
GP Sealapex serum 15 days	3.71+ 0.05[j]	3.82 +0.03[f]	4.01+0.03[c]

* Means with the same superscript does not differ significantly ($p<0.01$).

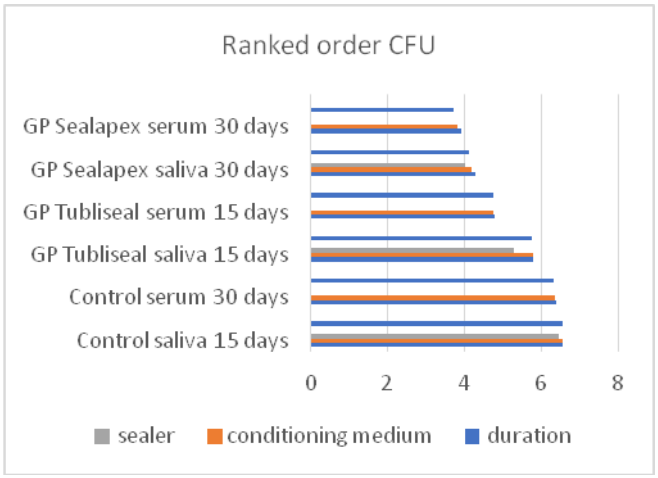

Fig 1: Graph showing ranked order of groups.

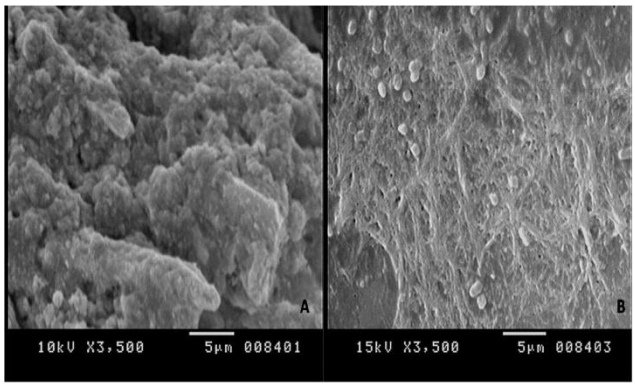

Figure 2- A- Scanning Electron Microscope Image at 3500x magnification of Gutta Percha Point coated with Sealapex sealer, conditioned with serum for 15 days. B- Scanning Electron Microscope Image at 3500x magnification of Gutta Percha Point coated with Sealapex sealer, conditioned with saliva for 15 days.

Discussion

The three major components involved in biofilm formation are bacterial cells, a solid surface, and a fluid medium. Bacteria can form biofilms on any surface that is bathed in a nutrient containing fluid. E. faecalis is one of the most efficient organisms, which enters and recovers from the viable but nonculturable (VBNC) state and follows a survival strategy which is adopted by bacteria when exposed to environmental stress. These bacteria may be encountered either as a mono-infection or mixed with

one or more species. Therefore, it is one of the few bacteria to be isolated as a monoculture from the root canal. [4,5,11] Both specific and non-specific interactions play important roles in the ability of the bacteria to adhere to the biomaterial surface.[12,13,14]

In the present study, role of saliva and serum conditioning on biofilm formation was examined on the rationale that saliva through coronal leakage and serum via apical retrograde fluid movement can coat GP placed within the root canal. Saliva and serum provide a constant source of nutrient for development of biofilm. When GP points are exposed to saliva or serum, an organic monolayer becomes adsorbed to the surface of the exposed material. This monolayer, mostly consisting of polysaccaharides and glycoproteins, provides an excellent anchor for planktonic cells to attach to surface of GP[15].

Recontamination of the root canal system by coronal leakage; sealer dissolution by saliva; percolation of saliva in the interface between sealer and root canal walls (particularly if a smear layer is present) and/or between sealer, and gutta-percha should be cogitated.[16,17] Coronal exposure of the root canal obturation to saliva for a relatively short period of time (30 days or more) might be considered an indication for retreatment [18,19].

Human saliva contains proline-rich proteins that aggregate together to form salivary micelle-like globules (SMGs). These globules from saliva get adsorbed to the clean surface to form acquired pellicle, which acts as a ‘‘foundation’’ for the future multilayered biofilm. Regardless of the obturation technique or filling material employed, entire recontamination of the root canal can occur after a short period of microbial challenge.[7,20] Percolation of serum may occur due to apical retrograde fluid movement as a result of chewing forces, which can provide an ideal environment for any surviving bacterial species to grow and cause persistent endodontic infections.[10,21]

In the excessive root filling cases with periapical lesions, gutta-percha points, could play a role in the initiation of infection[22]. Serum is essential for the attachment of bacterial cells and that the interaction between bacterial cells and the gutta-percha point surface is influenced by serum concentration. In the presence of high concentrations of serum, E. faecalis cells colonized in thick biofilms on the gutta-percha point surfaces suggesting that serum-rich environments favour an increase in the density of attached cells and the formation of an extracellular matrix. Serum provides a variety of proteins and glycoproteins. When exposed to high concentrations of serum, gutta-percha point surfaces are thought to become coated with the serum pellicle, and it is possible that proteins and glycoproteins in the serum pellicle serve as receptors that are recognized by specific bacterial species. The ability to bind to serum proteins might be advantageous for these bacteria in establishing single-species bio-films on gutta-percha points. It is likely that serum increases the surface hydrophobicity of planktonic bacteria, and that this elevated hydrophobicity promotes bacterial adherence.

In over-filled teeth, because the extruded gutta-percha points in the over-extended area are wet, the sealer around the apical foramen might not solidify and will therefore, soon dissolve through contact with serum-like fluid. Residual bacteria in root-filled teeth will colonize the space where the sealer melts on the gutta-percha surfaces, and consequently, they might form extra-radicular biofilms via the extruding gutta-percha points.[23]

A bacterial leakage study demonstrated higher leakage percentage with tubliseal than with Ca (OH)2 based sealers. Endodontic sealers containing calcium hydroxide present satisfactory physico-chemical properties when compared with zinc oxide eugenol sealers commonly used in endodontics.[13,24] E.faecalis can form biofilm and

survive all alkaline pHs (7.3 to 12.3). The biofilm formation decreased with increasing alkalinity (pH).[11,17]

Result of the present study shows similar finding. GP coated with Sealapex, was associated with a reduction in the number of bacteria in biofilm. The Least mean CFU count was observed on samples of GP coated with Sealapex and conditioned with serum for 15 days. This may be due to increased alkalinity (pH) of Sealapex as compared to Tubliseal. Sealapex contains calcium hydroxide, barium sulfate, zinc oxide, zinc stearate, titanium dioxide, plasficizers, Kerr resins, and methyl salicylate.

Current study shows the formation of E.faecalis biofilm on saliva and serum conditioned specimens of GP points incubated for 14 days in a nutrient-rich medium which supports the recurrence of infections as mentioned by other authors.[11,16]

In the present study, E.faecalis biofilm was formed on all specimens of GP points. This finding emphasizes the relevance of disinfecting the GP points, achieving proper coronal seal and confining GP to root canal space to prevent contamination by tissue fluid & formation of biofilm, and complete removal of obturating material in retreatment cases.

Further research is necessary on multispecies biofilm and clinical isolates to understand better, the relevance of these observations.

Conclusion

Within the limitations of this study, it was concluded that *Enterococcus faecalis* biofilm formed on gutta-percha obturating material with or without sealer conditioned in saliva or serum, under nutrient-rich environmental conditions. GP with Sealapex sealer, conditioned in serum, exhibited least E faecalis biofilm. Maximum biofilm formed on GP without sealer conditioned in saliva. Biofilm formation is affected by conditioning medium, duration, and the type of sealer employed.

References

1. Di Rosa R, Creti R, Venditti M, et al. Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of Enterococcus faecalis and Enterococcus faecium. FEMS Microbiol Lett. 2006;256(1):145-150.

2. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998;85(1):86-93.

3. Sijpkens YW, Buurke EJ, Ulrich C, van Asselt GJ. Enterococcus faecalis colonisation and endocarditis in five intensive care patients as late sequelae of selective decontamination. Intensive Care Med. 1995;21(3):231-234.

4. Batra M, Kaur R. Microbiology of endodontic faliure cases. Guident. 2015:60-64.

5. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: Its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006;32(2):93-98.

6. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284(5418):1318-1322.

7. Haapasalo M, Endal U, Zandi H, Coil JM. Eradication of endodontic infection by instrumentation and irrigation solutions. Endod Top. 2005;10(1):77-102.

8. Basrani B, Haapasalo M. Update on endodontic irrigating solutions. Endod Top. 2012;27:74-102.

9. Saunders WP, Saunders EM. Coronal leakage as a cause of failure in root- canal therapy: a review. Endod Dent Traumatol 1994; 10: 105-108.

10. A. Kishen. Periapical biomechanics and the role of cyclic biting force in apical retrograde fluid movement. Int Endod J 2005;38:597-603.

11. Hegde V. Enterococcus faecalis; clinical significance and treatment considerations. Endodontology. 2009:48-54.

12. Ebert J, Roggendorf MJ, Frank K, Petschelt A. Antimicrobial activity of various “active” gutta-percha points against Enterococcus faecalis in simulated root canals. Int Endod J. 2008;41(3):249-257.

13. Jhamb A, Chaurasia VR, Masamatti VKS, Agarwal JH, Tiwari S, Nair D. In vitro evaluation of antimicrobial activity of different Gutta-percha points and calcium hydroxide pastes. J Int Soc Prev Community Dent. 2014;4(2):92-95.

14. Guerreiro-Tanomaru JM, De Faria-Júnior NB, Duarte MAH, Ordinola-Zapata R, Graeff MSZ, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod. 2013;39(3):346-350.

15. Bowden GH, Li YH. Nutritional influences in biofilm development. Adv Dent Res 1997;11: 81-99.

16. George S, Basrani B, Kishen A. Possibilities of gutta-percha-centered infection in endodontically treated teeth: An in vitro study. J Endod. 2010;36(7):1241-1244.

17. Varghese AM, Joshua JA, Shetty D, Damda A. Evaluation of Surface Changes on Gutta-Percha Points Treated with Four Different Disinfectants At Two Different Time Intervals - A SEM Study. IOSR Journal of Dental and Medical Sciences (IOSR-JDMS) 2018;17(3):30-37.

18. Siqueira JF Jr., Rocas IN, Lopes HP, Uzeda M. Coronal leakage of two root canal sealers containing calcium hydroxide after exposure to human saliva. J Endod 1999; 25:14-16.

19. Torabinejad M, Ung B, Kettering JD. In vitro bacterial penetration of coronally unsealed endodontically treated teeth. J Endod 1991; 16:566-69.

20. Ingle JI., Bakland LK, Baumgartener JC. Endodontics. PMPH-USA, St. Louis 6th edn, 2008:268-85.

21. Schirrmeister JF, Liebenow AL, Pelz K, et al. New Bacterial Compositions in Root-filled Teeth with Periradicular Lesions. J Endod. 2009;35(2):169-174.

22.Yuichiro Noiri, Atsushi Ehara, Takashi Kawahara, Naoki Takemura and Shigeyuki Ebisu. Participation of Bacterial Biofilms in Refractory and Chronic Periapical Periodontitis. J Endod 2002;28(10) :679-83.

23. Valera MC, Camargo CHR, Carvalho AS, Gama ERP. In vitro evaluation of apical microleakage using different root-end filling materials. J Appl Oral Sci. 2006;14(1):49-52.

24. Rezende GC, Massunari L, Queiroz IO de A, et al. Antimicrobial action of calcium hydroxide-based endodontic sealers after setting, against E. faecalis biofilm. Braz Oral Res. 2016;30(1). DOI: 10.1590/1807-3107BOR-2016.vol30.0038