Possibilities of Gutta-Percha–centered Infection in Endodontically Treated Teeth: An *In Vitro* Study

Saji George, BSc, MSc, PhD, Bettina Basrani, DDS, PhD, and Anil Kishen, BDS, MDS, PhD

Abstract

Introduction: Implanted biomaterials could provide surface for bacterial adherence and formation of biofilm, eventually leading to biomaterial-centered infections (BCIs). In this *in vitro* study, we examined the biofilm-forming capacity of *Enterococcus faecalis* on gutta-percha (GP) points under different nutrient status and surface conditioning with saliva and serum. **Methods:** GP points (n = 420) coated with different root canal sealers (Sealapex or Roth) were conditioned with saliva or serum for 2-, 4-, and 12-week intervals and subsequently were incubated with *E. faecalis* for 2 weeks under nutrient-rich and nutrient-deprived environments. The biofilm-forming capacity and the nature of biofilm formed on GP were assessed by using a viable cell assay and scanning electron microscopy (SEM). **Results:** *E. faecalis* produced biofilms on GP points (with and without root canal sealers) under both nutrient-rich and nutrient-deprived environments after conditioning with saliva or serum. SEM reflected that the biofilms formed under nutrient-rich conditions were regular, whereas the biofilms formed under nutrient-deprived conditions were irregular and scanty. Under nutrient-deprived conditions, longer conditioning periods in saliva or serum were required for bacterial adherence. **Conclusions:** Under the conditions of this study, saliva and serum conditioning of GP points plays an important role in the biofilm formation under tough environmental conditions. *(J Endod 2010;36:1241–1244)*

Key Words

E. faecalis, endodontic biofilm, environmental conditions, gutta-percha, surface conditioning

From the Discipline of Endodontics, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada.

Dr George's current address: Division of Nanomedicine, Department of Medicine, University of California, Los Angeles, California.

Address requests for reprints to Dr Anil Kishen, BDS, MDS, PhD, Associate Professor, Discipline of Endodontics, Faculty of Dentistry, 124 Edward St, Toronto, Ontario, Canada M5G 1G6. E-mail address: anil.kishen@dentistry.utoronto.ca.

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It is understood that bacteria adherent to a biomaterial or tissue surface and growing as a biofilm are the major cause of biomaterial-centered infections (BCIs) (1). BCIs are unique in a way that the opportunistic bacteria choose to colonize the surface of the material and simultaneously affect the adjacent tissues. Bacteria existing in a biofilm are resistant to the detrimental effects of localized or systemic antimicrobials (2). Hence, biofilm infections are rarely resolved by the host defense mechanisms even under medication (3). The slow growth of bacteria and the heterogeneity of the microorganisms in the biofilm structure are considered to contribute to their high antimicrobial resistance (4).

Enterococcus faecalis accounts for approximately 80% of the enterococcal infections, and treatment of this type of infections is usually difficult because of its high resistance to antibiotics (5). Interestingly, *E. faecalis* is the most prominent and occasionally the only bacterial species to be isolated from root canals of treatment-failed teeth (6). Bacteriologic investigations have demonstrated *E. faecalis* in 30%–89% of teeth with postendodontic treatment infection, mostly as monoculture (7–9). These findings highlight the ability of *E. faecalis* to survive harsh environmental conditions prevailing in the root canals of endodontically treated teeth with gutta-percha (GP) and sealer (10, 11). It is also suggested that *E. faecalis* could survive endodontic irrigant (12) by resisting high concentrations of intracanal medicaments and wide variations in pH (13). However, the complete mechanism by which it defies both chemical and biologic agents is still unknown.

Bacterial biofilm is cited as the primary cause of chronic and recurrent endodontic infections (14). It has been shown that *E. faecalis* formed biofilm structures on root canal dentin in nutrient-rich and nutrient-deprived conditions under both aerobic and anaerobic environments (15, 16). But there is a gap in knowledge on the biofilm formation in endodontic biomaterials; therefore, investigations on the biofilm-forming capacity of *E. faecalis* on GP and the factors governing it would help to understand the conditions that favor the survival of *E. faecalis* in postendodontic environment. The objective of this *in vitro* study was 2-fold: (1) to examine the biofilm-forming capacity of *E. faecalis* on GP points under different environmental conditions (nutrient-rich and nutrient-deprived) and (2) to understand the role of different conditioning fluids (saliva and serum) and duration of conditioning on the biofilm-forming capacity of *E. faecalis* on GP.

Materials and Methods

Four hundred twenty GP points, size 40 and taper 0.40 (Dentsply Maillefer, Ballaigues, Switzerland), were selected for the study. GP points were disinfected by placing them in 5.25% sodium hypochlorite for 1 minute, followed by rinsing in sterile distilled water and drying in a biohazard cabinet. Plastic straws of 30-mm length and 3.5-mm diameter were selected to simulate root canal lumen. The straws were disinfected with 70% of ethanol for 5 minutes and dried in the biohazard cabinet. Two root canal sealers, Roth sealer (Type 801 Elite; Roth International Ltd, Chicago, IL) (zinc oxide–eugenol sealer) and Sealapex (Sybron Kerr, Orange, CA) (calcium hydroxide sealer), were used in this study.

The zinc oxide–eugenol and calcium hydroxide sealers were mixed as per the manufacturer's instructions and were coated on the inner walls of the straw and the surface of the GP points (210 samples per group). The straw stub with the GP sealer was left undisturbed for 8 days at 37°C. After 8 days, the sealer-coated

GP points were removed gently from the straw stubs and subjected to liquid conditioning with saliva or serum for simulating clinical conditions. Whole saliva collected from caries-free subject was filter-sterilized and was used as a conditioning fluid to simulate orthograde fluid leakage, and serum was used to simulate retrograde fluid leakage. Fetal bovine serum (HyClone; Thermo Scientific, Logan, UT) stored in sterile centrifuge tubes was used for conditioning the GP surface with serum. The GP points were divided into 12 groups on the basis of the combinations of root canal sealers and conditioning liquids used.

GP points from both the groups (zinc oxide sealer and calcium hydroxide sealer) were equally divided and incubated in saliva or serum in a sterile glass container at 37 °C (n = 105 per group). The conditioning of GP points was carried out for 2-week, 1-month, and 3-month intervals. After the specific time intervals, 5 GP points were removed from each group to check the possibilities of contamination, and 30 GP points from each group were removed and incubated with *E. faecalis*. *E. faecalis* (ATCC 29212) strains maintained in culture plates at 4 °C were used in this study. The cells were activated by inoculating a single colony of *E. faecalis* in 50 mL All Culture (AC) medium at 37 °C for 24 hours in an orbital incubator. Fresh culture for inoculation containing 10^8 cells/mL (1 OD at 600 nm, UV-VISIBLE Spectrophotometer; Shimadzu, Kyoto, Japan) was prepared in either AC medium (simulating nutrient-rich condition) or phosphate-buffered saline (PBS) (simulating nutrient-deprived condition) by adding cells from the overnight grown culture. The GP points were incubated with *E. faecalis* in separate wells of a 24-well microwell plate under nutrient-rich and nutrient-deprived conditions in anaerobic environment at 37 °C for a period of 2 weeks. To remove the dead cells and to continuously replenish medium, the culture media were replaced every second day. After this period, the GP points were removed from the microwell plates and rinsed 3 times in PBS to remove nonadherent cells from the GP.

The biofilm-forming capacity of *E. faecalis* on GP and the nature of biofilm formed were examined by using viable cell assay and scanning electron microscopy (SEM) analysis. For viable cell assay, 10 GP points from each group were transferred individually into an eppendorf tube containing 1 mL of medium (AC in nutrient-rich groups and PBS in nutrient-deprived groups) that was agitated vigorously by using a vortex shaker at 3000 rpm. Subsequently, 10 μL of inoculum from this suspension was serial diluted and plated on previously prepared agar plates. Manual counting of colony-forming units (CFUs) was performed after 24 hours of incubation (37 °C) to determine the total viable cells adhering to GP. CFU counting (expressed in $\log_{10}$ cells per unit GP) provides a relative assessment of bacteria in biofilm formed on GP under each test condition. Furthermore, SEM was carried out on 5 GP points from each group for the qualitative assessment of the extent of biofilm formation.

Results

Generally, under nutrient-rich conditions all GP specimens with and without sealer showed biofilm formation after different periods of conditioning with saliva or serum (Table 1). Compared with 2 weeks of conditioning, the GP points conditioned for 3 months with saliva or serum resulted in a significant increase in number of bacteria per GP point ($P < .05$). Under nutrient-deprived conditions, no viable bacterial cells were detected from any of the GP samples after conditioning in saliva and serum for 2 weeks (Table 2). Longer duration of GP conditioning (3 months) could sustain biofilm formation on GP samples without sealer and Sealapex. However, GP samples coated with Roth sealer showed significantly lower number of bacteria after conditioning for 1 month with saliva/serum (Table 2). There was no significant difference in the number of viable bacteria adhering to GP points conditioned with saliva and those conditioned with serum in any of the groups.

SEM images confirmed the *E. faecalis* biofilms on all GP points tested under nutrient-rich growth medium after conditioning with saliva or serum (Fig. 1). The biofilms formed under these conditions were mostly regular and monolayered. The GP points conditioned with saliva/serum for 2 weeks under nutrient-deprived conditions showed no sign of biofilm formation on any of the groups (Fig. 1). There were signs of bacterial cells adhering to both root canal sealers and GP substrate even in nutrient-deprived condition, when GP points (without sealer and with sealer) were conditioned for more than 1 month in saliva/serum. GP points with Roth sealer showed bacterial adherence and isolated biofilm formation only after 3 months of conditioning with serum/saliva.

Discussion

Factors contributing to the persistence, survival, or re-entry of microbes within the root canal system are primarily responsible for persistent apical periodontitis and endodontic failure (17–19). Nevertheless, coronal leakage through postendodontic restorations and root filling is thought to be a common cause of root canal recontamination (20, 21). Experiments also have highlighted the possibility of apical retrograde fluid movement as a result of chewing forces (22). Although persistent apical periodontitis after endodontic treatment has been a topic of interest in recent times, very few studies have examined the biofilm-forming potential of bacteria on GP points under different clinically relevant conditions.

The role of saliva or serum conditioning on biofilm formation was examined on the rationale that saliva through coronal leakage and/or serum via apical retrograde fluid movement can coat GP placed within the root canal. Saliva and serum provide a constant source of nutrients for the development of biofilms (23). When GP points are exposed to saliva and serum, an organic monolayer becomes adsorbed to the surface of the exposed material, for instance a GP point. This monolayer

TABLE 1. Bacterial Count per GP Point When It Was Conditioned with Saliva/Serum for Different Time Intervals and Kept Incubated with *E. faecalis* in Nutrient-rich Media

Samples		Log of cells/GP points		
Sealer	Conditioning medium	2 weeks	1 month	3 months
GP, no sealer	Saliva	4.89 (0.10)	4.43 (0.234)	4.96 (0.008)
	Serum	4.32 (0.03)	4.05 (0.316)	5.12 (0.03)*
GP + Roth	Saliva	4.65 (0.001)	2.61 (0.407)	4.92 (0.04)*
	Serum	4.63 (0.19)	3.21 (0.086)	4.96 (0.005)
GP + Sealapex	Saliva	4.79 (0.05)	3.31 (0.172)	5.15 (0.04)*
	Serum	2.84 (0.06)	4.27 (0.018)	3.17 (0.06)*

GP, gutta-percha.

*Significant increase ($P < .05$) in the number of bacteria from 2 weeks of conditioning in the respective medium.