

# Validation of Biofilm Assays to Assess Antibiofilm Efficacy in Instrumented Root Canals after Syringe Irrigation and Sonic Agitation

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## Abstract

**Introduction:** Different methods to characterize bacterial biofilms have been established, each presenting with distinct advantages and shortcomings. The aim of this study was to validate the ability of microbiological culture, the adenosine-5'-triphosphate (luminescence) assay, and molecular and microscopic methods to assess antibiofilm efficacy. **Methods:** Thirty-nine extracted single-rooted teeth were selected. *Enterococcus faecalis* biofilms were grown for 21 days and randomly distributed into 3 groups. All canals were instrumented (F3 ProTaper Universal; Dentsply Sirona, Johnson City, TN) and irrigated (ProRinse needles, Dentsply Sirona) as follows: group 1, sodium hypochlorite and EDTA irrigation; group 2, supplemented with sonic agitation of NaOCl, and group 3, sterile distilled water irrigation. Bacteriologic samples were collected before (S1) and after canal preparation (S2) and subjected to quantification by culture methods, quantitative reverse transcriptase real-time PCR (qRT-PCR), and luminescence assay. The biofilm structure and bacterial cell viability were evaluated under scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Data were subjected to statistical analysis to determine the statistical significance ( $P < .05$ ). **Results:** S1 samples showed approximately 8-log colony-forming-units of bacteria using both culture and qRT-PCR. The reduction in bacterial populations and relative luminescence was highly significant in the S2 samples from groups 1 and 2 ( $P < .001$ ). SEM and CLSM showed well-matured root canal biofilms in the pretreatment samples that were reduced after treatment. Irrigation with NaOCl combined with sonic agitation significantly decreased the percentage of live cells ( $P < .05$ ) but was not able to eliminate the biofilm structure. **Conclusions:** This study highlighted the maximum reduction of microbes after instrumentation-syringe irrigation. Although supplementary sonic agitation reduced the

root canal biofilm further, it did not completely eliminate the biofilm from a single root canal model. The merits of combining microbiological and molecular quantification methods with CLSM for the comprehensive assessment of antibiofilm efficacy in root canals were emphasized. (*J Endod* 2017; ■:1–7)

## Key Words

Biofilm, culture, detection techniques, microscopy, polymerase chain reaction

A biofilm is a complex biological community consisting of bacterial colonies and extracellular polysaccharides (EPSs), giving it the characteristics of a multicellular system. Microbiological assays that effectually characterize biofilm bacteria have been a challenging subject in endodontic microbiology (1); yet, this is a cardinal step used routinely for the estimation of microbial biomass while assessing the efficacy of different antimicrobial irrigants/irrigation strategies in endodontics. Current biofilm assays characterize features such as the number/type of resident microbes, vitality (dead/living cells) of resident microbes, thickness, structure (homogeneous, irregular, dense, or porous), topography, and biomass or EPS levels (1, 2). Apparently, every assay provides distinctly different information, and, therefore, depending on a sole method for biofilm characterization would be inadequate.

Biofilm evaluation is an essential step for assessing antibacterial efficacy in root canal disinfection (2–4). Direct enumerations of colony-forming units (CFUs) using culture-based methods have been a gold standard in bacterial detection/quantification. However, culture methods have been suspected because of their inability to detect viable but nonculturable bacteria (4, 5) and the need for a prolonged incubation time with multistep culture techniques (2). This approach is further marred by the limitations of sampling from root canals (6). The specificity of culture medium, transport medium, toxicity of by-products, and metabolic dependency in multispecies biofilm could further amplify the limitations of cultivation methods (5). Accordingly, microscopic,

## Significance

This study highlighted the maximum reduction of microbes after instrumentation-syringe irrigation. Supplementary sonic agitation reduced the biofilm contents further but did not completely eliminate the biofilm in the tested single-canal models. The benefit of combining microbiological and molecular quantification methods with microscopy for the comprehensive assessment of antibiofilm efficacy in root canals was emphasized.

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## Basic Research—Technology

biochemical, and molecular techniques have been advocated to reliably estimate bacterial biofilms.

Polymerase chain reaction (PCR) is a highly sensitive molecular method used for microbial detection in root canals (5, 7). A real-time PCR (also known as quantitative PCR [qPCR]) is used to amplify and simultaneously quantify a targeted DNA molecule, enabling both detection and quantification of the specific DNA sequences (4, 8). Real-time PCR is a distinct technique that uses reverse transcription to quantify messenger/noncoding RNA. Quantitative reverse transcriptase real-time PCR (qRT-PCR) effectively quantified the number of RNA transcripts of specific genes from bacteria growing in biofilms. qRT-PCR is a sensitive method to quantify gene expression from biofilms in which only a small amount of the sample is available (8). qPCR is widely used in endodontics to assess the degree of disinfection achieved after root cleaning with topical antimicrobials as well as shaping (9–11).

Scanning electron microscopy (SEM) and transmission electron microscopy have been effective workhorses for the characterization of the biofilm structure (2). The main disadvantage with these techniques is the prerequisite for extensive sample preparations, which could affect the original biofilm morphology. Recently, confocal laser scanning microscopy (CLSM) in combination with specific fluorescence stains that monitor growth and metabolic activities of bacteria has become a versatile tool for biofilm examination (12, 13). However, microscopy offers limited specificity and sensitivity to detect microbes from clinical samples (14). The current study aimed to compare the capabilities of conventional microbiological quantification techniques with microscopic techniques to assess the antibiofilm efficacy after instrumentation-syringe irrigation and supplementary sonic activation of sodium hypochlorite (NaOCl) from an *in vitro* root canal model. This investigation offers information on the degree of microbial/biofilm reduction achieved by root canal instrumentation-syringe irrigation (stage 1) and after sonic agitation of NaOCl (stage 2) and the appropriateness of different biofilm assays to assess the antibiofilm efficacy in root canals.

## Materials and Methods

### Specimen Preparation

Freshly extracted single-rooted teeth without caries and fractures were included in the study after University of Toronto Ethics Board approval. Thirty-nine teeth were selected and autoclaved for sterilization. The teeth were decoronated and standardized to 12-mm-length specimens. Root canals in all the teeth were enlarged with ISO size 20 K-files up to an 11-mm working length (WL). The root canals were irrigated with 6 mL 5.25% NaOCl, 2 mL 17% EDTA, and 2 mL 5.25% NaOCl. NaOCl was neutralized with filter-sterilized 10% sodium thiosulfate. Each of the prepared roots was immersed in 1 mL brain-heart infusion (BHI) broth and autoclaved for sterilization. All the chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St Louis, MO) unless noted otherwise.

### Root Canal Biofilm Formation

Overnight cultures of *Enterococcus faecalis* (ATCC 29212; American Type Culture Collection, Manassas, VA) were grown in BHI broth at 37°C and adjusted to 10<sup>8</sup> CFU/mL (optical density at 600 nm = 1). The prepared root canals were kept in sterile centrifuge tubes, and 0.5 mL bacterial culture was added. One hundred microliters of the culture was added inside the root canal lumen, and the tubes were centrifuged at 1400g, 2000g, 3600g, and 5600g in a sequence for 5 minutes. A fresh solution of bacteria was added between every centrifugation. All tubes with root specimens were then incubated at 37°C for 21 days, changing

the media every 72 hours. Teeth were randomly divided into 2 experimental groups of 12 teeth each according to the irrigation technique used and a control group consisting of 15 teeth. Of the 15 teeth from the control group, 3 samples were processed to evaluate the initial biofilm biomass using confocal microscopy.

The root specimens were removed from the tubes, and the external surfaces were wiped with sterile gauze. The apical foramen was sealed with epoxy resin. An initial microbial sample of the root canal (S1) was taken. Canals were filled with 1 mL sterile phosphate-buffered saline (PBS) solution and gently filed using ISO size 20 K-files. The S1 sample was obtained by the sequential use of 3 paper points placed to the WL. Each paper point remained in the canal for 30 seconds before they were transferred to the tubes containing 4 mL BHI broth for further analysis.

### Treatment Groups

The root canals were sequentially enlarged using ProTaper Universal instruments (Dentsply Sirona, Johnson City, TN) at 300 rpm and 200 g/cm torque (ProMark Endodontic Motor, Dentsply Sirona) up to F3 at the WL. The samples were irrigated with 1 mL NaOCl using a 5-mL syringe and 30-G ProRinse endodontic irrigation probes (Dentsply Sirona) placed in the canal 2 mm short of the WL between successive instruments. After instrumentation, NaOCl was left undisturbed in the canal for 60 seconds, and the final irrigation procedure was performed as follows:

1. Group 1 (syringe irrigation group): 2.5 mL NaOCl, 5 mL EDTA, and 2.5 mL NaOCl.
2. Group 2 (Sonic agitation group): sonic agitation using the EndoActivator (Dentsply Sirona) was applied on the canals filled with EDTA (60 seconds) and NaOCl (30 seconds); a #25/0.04 noncutting polymer tip of the EndoActivator placed 2 mm from the WL was used for each irrigation cycle (15). Overall, 20 mL irrigant was used per canal in both groups for approximately the same time period. After the final irrigation, the root canals were irrigated with 2 mL sodium thiosulfate to inactivate residual NaOCl followed by 2 mL distilled water and dried with sterile paper points.
3. Group 3 (control group): root canals were shaped in the same manner as in group 1 with sterile distilled water used as an irrigant (a total of 20 mL per canal).

The duration for irrigation in all groups was standardized to be approximately 10 minutes. Six specimens from each group were used for microbiological quantification, and the remaining 6 specimens were used for CLSM and SEM.

### Sampling and Processing

Bacteriologic samples collected after canal preparation (S2) were taken using an F4 file ( $n = 6/\text{group}$ ). Canals filled with PBS were enlarged using an F4 file as a hand instrument. The file with dentin shavings was transferred to tubes containing 4 mL BHI broth. A medium-sized paper point was agitated inside the canals, left for 30 seconds to soak up the canal contents, and transferred into the tube along with the file. The BHI broth with the paper points and files was incubated for 4 hours at 37°C to provide an enrichment phase for the bacteria (16). This time duration was chosen based on the bacterial growth in the S2 samples of group 3 to attain an optical density of 0.1.

### Quantification by Culture and PCR

The samples were agitated in a vortex mixer for 1 minute and serially diluted (10-fold) in PBS. Aliquots of 1  $\mu\text{L}$  were plated onto BHI plates and incubated at 37°C for 48 hours. The CFUs grown

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