Confocal Laser Scanning Microscopy Is Appropriate to Detect Viability of *Enterococcus faecalis* in Infected Dentin

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Abstract

The purpose of this study was to explore the potential of confocal laser scanning microscopy (CLSM) for in situ identification of live and dead *Enterococcus faecalis* in infected dentin. Eight cylindrical dentin specimens were infected with *Enterococcus faecalis* in BHI for 21 days. After the experimental period, the specimens were stained with fluorescein diacetate (FDA) and propidium iodide (PI) or acridine orange (0.01%) and analyzed by CLSM. Two noninfected dentin specimens were used as negative controls. CLSM analysis shows that the discrimination between viable (green) and dead (red) bacteria in infected dentinal tubules could be observed after staining with FDA/PI. Acridine orange was able to show metabolic activity of the *E. faecalis* cells inside the dentinal tubules showed by its red fluorescence. The viability of bacteria in infected dentin can be determined in situ by CLSM. FDA/PI and acridine orange are useful for this technique. *(J Endod 2008;34:1198–1201)*

Key Words

Confocal laser scanning microscopy, dentin, *Enterococcus faecalis*, viability

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Supported by FAPESP (2007/01838-7).

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0099-2399/$0 - see front matter

doi:10.1016/j.joen.2008.07.001

Bacterial invasion of the dentin caused by caries or fracture results in inflammation, necrosis of the dental pulp, root canal infection, and apical periodontitis (1). Procedures such as histologic sections, scanning electron microscope observation, and microbiological analyses at different levels of the root canal can be routinely done. The bacteria's ability to penetrate into the dentin can be determined through microbiological analyses by the number of colony-forming units (CFUs) (2, 3), the number of tubules infected in the histologic sample (4), or the presence of bacteria in the root canal walls (5).

Each one of these methods has its advantages and disadvantages. Microbiological sampling techniques can estimate the number of colony-forming units of cultivable bacteria and the quantitative analysis of the dentin infection can be done. However, this method does not give clear information about the spatial distribution of bacteria inside the dentin. Histologic sections show the distribution of the bacteria in infected dentin but do not give information about the viability of the bacteria. Both techniques are often used to determine the antibacterial activity of dental materials and for clinical research.

Transmission electron microscope has been used for visualization of infected tubules in carious lesions and in root canal infection on clinical specimens (6, 7), providing high resolution images of the bacteria inside dentinal tubules. However, this technique spends time and requires multiple steps for specimen preparation.

In the last years efforts have been done to show viability of bacteria using fluorescent labels on infected dentin (8–13). However, the distribution of live and dead bacteria inside the dentinal tubules of unprocessed specimens in a high optical resolution which allows the differentiation of the bacterial cells is not described.

Confocal laser scanning microscopy (CLSM) allows getting a series of optical sections as thin as 0.3 μm of intact undisturbed biological samples. A CLSM analysis is used commonly with vital staining techniques to determine the viability profile, architecture, and spatial distribution in microbial biofilms (14).

The aim of this study was to explore the potential of CLSM for in situ identification of live and dead bacteria inside infected dentinal tubules and to describe the distribution and vitality of the bacteria *Enterococcus faecalis* in infected dentin of bovine origin.

Materials and Methods

Specimen Preparation

Ten noncarious bovine teeth were selected and stored in 5% formalin buffer. A root segment with a length of about 7 to 8 mm was prepared by sectioning the root tip and after the crown was removed at 2 to 3 mm below the cementoenamel junction (9). Each root canal was enlarged to a size of a Gates Glidden bur #5 (1.30 mm) under 1% sodium hypochlorite irrigation. The smear layer was removed using an ultrasonic bath of 17% EDTA for 4 minutes. One specimen was visualized by standard electron microscopy (Jeol JSM T220; Jeol, Tokyo, Japan) to confirm the ability of this technique in removing the smear layer. The teeth were washed with sterile water for 10 minutes and stored in sterile water for 1 week (15) to remove any residual chemical compound. The specimens were sterilized by autoclave for 20 minutes at 121°C. Then, sterility was checked by incubating of each specimen in 5 mL of brain heart infusion (BHI) broth at 37°C for 24 hours.

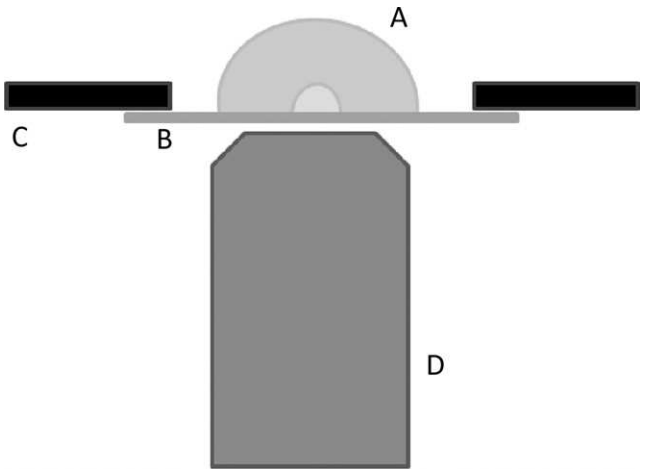

Figure 1. Schematic diagram of the experimental design. (*A*) Dentin segment on (*B*) glass coverslip glued to a (*C*) modified chamber device. (*D*) Objective lens.

Dentin Infection with *E. faecalis*

The strain *E. faecalis* (ATCC 29212) from the American Type Culture Collection was used in this study. To create the bacterial inoculum, isolated colonies (24 hours) of pure cultures of *E. faecalis* grown aerobically on BHI agar plates were suspended in 3.0 mL BHI. The cell suspension was spectrophotometrically adjusted to match the turbidity equivalent to 0.5 McFarland standard. For dentin infection, under laminar flow, eight blocks were transferred individually into 3 mL BHI inoculated with 200 μL of the *E. faecalis* suspension for 21 days. BHI was continuously changed every 48 hours, and the purity of the broth was verified to avoid contamination. After the incubation period (21 days), the root canals of dentin blocks were washed with 1 mL phosphate-buffered saline (PBS) to remove nonadherent bacteria of the root canal walls. Superficial longitudinal grooves were made in the buccal and lingual surface to facilitate the fracture of the specimens. Preventing contamination from the outer surface, the radicular cement was exposed to a gas flame and ultraviolet light for 10 minutes.

The Determination of Bacterial Viability

After the root was fractured, one dentin segment was washed with 100 μL PBS and then stained with 50 μL fluorescein diacetate (FDA; Sigma, St Louis, MO) and 50 μL propidium iodide (PI, Sigma). FDA is a nonfluorescent cell permeable dye that is converted to fluorescein (green) by intracellular esterases produced by metabolically active microorganisms (14, 16–18). PI is a fluorescent molecule impermeable to the cellular membrane and generally excluded from viable cells; thus, live bacterial cells are fluorescent green, whereas dead bacteria with damaged membranes are fluorescent red (19).

The other dentin segment was washed with 100 μL PBS and stained with 50 μL 0.01% acridine orange; such dye has the ability to bind with bacterial RNA-emitting red fluorescence and to bind with bacterial DNA-emitting green fluorescence (20, 21). This information allows analysis of the bacterial metabolism because cells in the log phase emit red fluorescence and those in the stationary phase emit a green fluorescence (11, 19–22). For both techniques, the dentin was incubated with the dyes at room temperature for 10 minutes in a dark environment and then rinsed with PBS for 1 minute. The specimens were immediately analyzed by the CLSM technique. Another two sterile roots were stained with the same protocol and used as the negative control.

CLSM Analysis

The dentin segments were examined on an inverted Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany; Fig. 1). The respective absorption and emission wavelengths were 494/518 nm for fluorescein diacetate and 536/617 nm for PI. For the acridine orange staining, the excitation and emission for RNA was 460 and 650 nm; the excitation and emission for DNA was 500 and 526 nm, respectively. The sequential frame scan mode was used to prevent crosstalk. The mounted specimens were observed using a 40× oil lens and a 63× oil lens with an additional zoom of 3×. The 40× pictures were obtained by using 23 sections of 1-μm step size in a format of 1,024 × 1,024 pixels. The 63× zoom 3 pictures were taken by using 10 sections with a 0.3-μm step size and 1,024 × 1,024 pixels. The images were acquired using the Leica Application Suite-Advanced Fluorescence software (LAS AF, Leica Microsystems GmbH).

Results

To evaluate whether CLSM analysis is an adequate method for in situ identification of live and dead bacteria inside dentinal tubules, first we analyzed the dentinal tubules staining before bacterial infection in vitro. CLSM analysis showed that after staining with acridine orange (Fig. 2*A* and *B*) or fluorescein diacetate (data not shown) noninfected dentinal tubules could be visualized with a predilection of the dyes to label the peritubular dentin.

At 21 days of dentin infection, the teeth were examined with the 40× oil lens, the specimens stained with acridine orange or FDA/PI showed that dentinal tubules of all the studied teeth were infected with *E. faecalis* (Fig. 2*C*). Infected tubules were clearly differentiated from noninfected ones by its fluorescence. However, morphologic identification of viable and nonviable bacteria was difficult at this magnification. No colonization or weak colonization of the root canal walls could be seen in some specimens. Bacterial penetration in dentinal tubules was variable between 100 μm and 400 μm (Fig. 2*C*).

The acridine orange dye showed the presence of RNA (red fluorescence) in the infected dentinal tubules showing metabolic activity of the bacterial cells (Fig. 3). Microorganisms inside the dentinal tubules could be well identified by its coccoidal structure using the 63× oil lens with an additional zoom of 3.

The FDA/PI (Fig. 4) staining technique showed difference in the viability of the bacteria. A single dentinal tubule presenting both viable and nonviable bacteria was the most frequent finding, whereas the presence of single tubules with nonviable bacteria was the less frequent finding. It was evident that the diameter of the dentinal tubules could contain a great number of bacterial cells.

Discussion

Dentin infection has particular significance for the study of dental caries and apical periodontitis (23). Microbiological, histologic, and

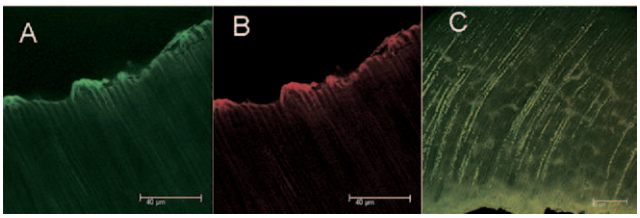

Figure 2. Acridine orange–negative control showing absence of bacteria in the dentinal tubules, (*A*) 526 nm and (*B*) 650 nm emission. (*C*) Twenty-one day–infected dentin stained with FDA/PI; infected tubules can be differentiated by their fluorescence.

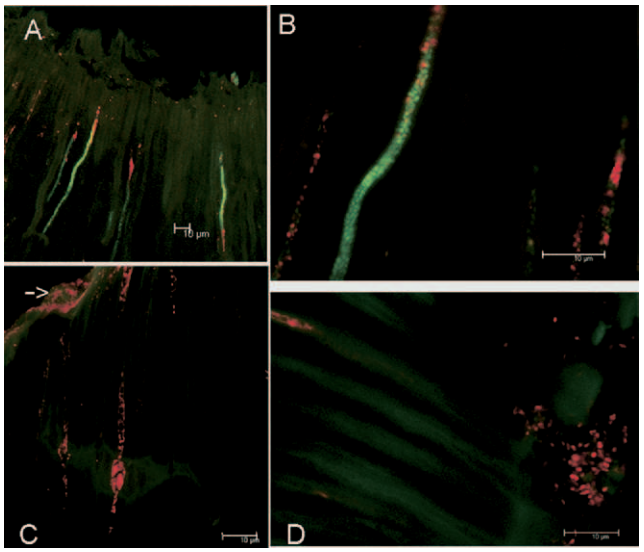

Figure 3. CLSM of 21 day–infected dentin stained with acridine orange. (*A*) Marked green and red fluorescence are seen inside the infected dentinal tubules. The magnification of some tubules shows red fluorescence indicating the presence of RNA, which is a signal of bacterial metabolic activity. Fluorescent green cells represent the presence of DNA. (*B*) Microorganisms inside the dentinal tubules are well distinguished by its coccoidal structure. (*C*) Bacterial cells (red) in the root canal wall (arrow) penetrating dentinal tubules. A bacterial colony in the root canal wall is seen in (*D*) red fluorescent cells predominating in this structure and in an adjacent dentinal tubule indicating metabolic activity of the bacterial cells. All the bars represent 10 μm.

transmission electron microscopy techniques have been used to study the presence of bacteria inside the dentinal tubules (7).

Since the work of Haapasalo and Ørstavik (3), the bovine dentin block model and the bacterial strain *E. faecalis* are commonly used as a reference to test endodontic medications (24, 25). Bovine dentin has been shown to be similar to human dentin in structure, composition, and diameter of tubules (26). Additionally, the strain *E. faecalis* has shown ability to infect dentinal tubules (3), to tolerate high pH levels (27), and to survive in obturated root canals (28). Also, it is the most prevalent bacterial strain in endodontic cases with persistent endodontic lesions as shown by culture- or molecular methods–based studies (29, 30).

The use of fluorescent probes in order to determine bacterial viability in microbiological research on dentistry has increased in the last years (8, 10, 14, 16). Most previous analysis (2, 9) showed that the presence of viable and dead bacteria in dentin samples could be determined through staining a root dentin pellet and using appropriate fluorescent probes. Previous studies (31) showed that CLSM is able to detect bacteria in carious lesions samples by the use of the inmunofluorescence technique or to detect the vitality of specific bacteria colonizing the root canal walls (8, 11–13). However, all the mentioned studies failed to show spatial distribution and viability of the bacteria inside dentinal tubules.

Nagayoshi et al. (10) showed that bacterial viability in dentinal tubules could be checked by using conventional fluorescence microscopy, but the high background haze originated from the out-of-focus planes decreased the contrast and made it impossible to distinguish between individual cells, stained dentin, and artifacts. Besides, conventional fluorescence microscopy required previous demineralization of the dentin samples creating an artificial condition. For these reasons, conventional fluorescence microscopy is not the best method to visualize the bacterial distribution in dentinal tubules.

In this context, better methodologies for the identification of bacterial viability in dentin are needed. The CLSM analysis used in this study has advantages over the conventional fluorescence microscopy to visualize bacteria in dentinal tubules such as better image resolution and the ability to eliminate scattered or out-of-focus light and to show individual bacterial cells inside dentinal tubules. In fact, our results confirm the ability of *E. faecalis* to infect dentin (3, 4, 15) and substantial improvements to show that the vitality of the bacteria in dentinal tubules was notorious when compared with conventional fluorescence and previous CLSM studies.

To our knowledge, we describe for the first time the visualization of live and dead as well as metabolically active bacterial cells in single dentinal tubules using confocal microscopy. It becomes evident that bacterial invasion of dentin by *E. faecalis* is an active process mediated by cell division. Additionally, the capacity to analyze thicker specimens with minimal preparation provides examination under near environmental conditions. This method gave information about the severity of the dentin infection, and the vitality of bacteria in the dentinal tubules could be determined in an effective way at the cellular level.

Despite the great advances of polymerase chain reaction (PCR) technology to detect culture-difficult species and uncultivable microorganisms (30, 32), PCR-based methods cannot distinguish between DNA from viable or dead cells (33, 34). This fact is important during the characterization of the microflora that survives the endodontic treatment. In situ detection of bacterial DNA and RNA by acridine orange and

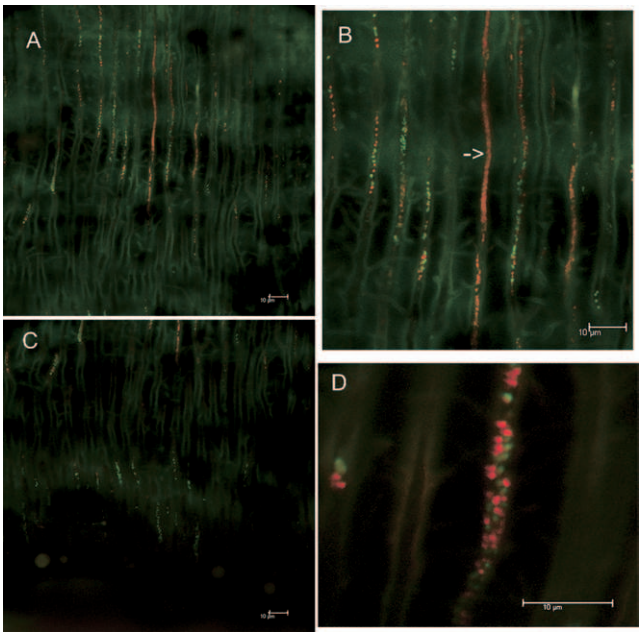

Figure 4. CLSM of 21 day–infected dentin stained with fluorescein diacetate/propidium iodide. *A* and *C* represent two consecutive microscope fields approximately 350 μm inside the radicular dentin from the root canal wall. (*C*) Vital bacteria (green) are seen in dentinal tubules near the root canal wall. However, both vital (green) and dead (red) bacteria are more numerous in the second microscopic field (*A*) approximately 250 μm inside the radicular dentin. A magnification of this area is seen in *B*. All dentinal tubules are infected. A single tubule with a great quantity of dead cells (red) is visible (arrow). Such dead cells are not differentiated by its cellular structure that is amorphous. Discrimination between vital (green) and dead (red) bacteria inside the other dentinal tubules are clearly differentiated. Numerous branches in the radicular dentin are observed. High-resolution 63× oil lens with additional zoom 3 (*D*) showing living coccoidal cells (green) together with red dead bacterial cells inside a single dentinal tubule. All the bars represent 10 μm.

determination of viability by the FDA/PI technique using confocal microscopy could be promising in elucidating the significance of the residual bacteria in uncultivable state or detectable only by PCR, especially after the use of sodium hypochlorite that efficiently destroys bacterial DNA (33, 35).

CLSM technique can be useful as a complement to the established microbiological, histologic, standard electron microscopy, and PCR-based techniques for the identification of viable bacteria (eg, during the in vitro evaluation of dental materials that would be in contact with infected dentin and for the study of the metabolic activity of bacterial cells during the process of dentin infection).

Conclusions

The present study has shown that the viability of bacteria in infected dentin can be determined in situ in an effective way by CLSM. FDA/PI and acridine orange are useful for this technique and have the potential for endodontic and cariology research.

Acknowledgments

The authors thank André da Silva and Edimauro de Andrade for their expert technical assistance.

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