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Bacterial localization and viability assessment in human *ex vivo* dentinal tubules by fluorescence confocal laser scanning microscopy

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Abstract

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Aim To develop a convenient method for the localization and quantification of live and dead bacteria in human *ex vivo* mineralized dentinal tubules.

Methodology The roots from human single-rooted teeth (n = 12) were infected with *Enterococcus faecalis* V583 and either treated with calcium hydroxide paste or left untreated; six control roots were uninoculated and untreated. Following further incubation, roots were stained with fluorescent DNA-binding reagents, washed thoroughly, sectioned and examined by confocal laser scanning microscopy. Computer-assisted determinations of fluorescence (bacterial viability) were compared statistically.

Results Bacteria were distributed in the tubules

throughout the length of the roots but tubule penetration distance was slightly reduced in the apical sections. There was no significant difference in bacterial tubule penetration between roots from different teeth and small standard deviations indicated reproducibility appropriate for experimental application. Following treatment with calcium hydroxide paste, live and dead bacteria were readily distinguishable by contrasting green and red fluorescence. Bacterial viability determinations amongst roots treated in the same way were not significantly different, and the small standard deviation is commensurate with experimental application.

Conclusions Fluorescent viability staining is a convenient, accurate and reproducible method for localizing and quantifying live and dead bacteria in human *ex vivo* mineralized dentinal tubules.

Keywords: bacterial viability, CLSM, *Enterococcus faecalis*, Live/Dead stain.

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Introduction

The aim of root canal treatment is to disinfect the root canal system through chemo-mechanical preparation, with or without interappointment medication, followed by the placement of a well-condensed root filling and coronal restoration. The frequent isolation of bacteria from teeth with failed root canal treatments indicates that some organisms are able to tolerate the conditions imposed by disinfecting agents and that the effectiveness of many endodontic antimicrobial treatments is limited (Engström 1964, Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, Hancock *et al.* 2001, Pinheiro *et al.* 2003). This may be attributed to bacterial invasion of the dentinal tubules, in which the organisms are protected from intracanal irrigants and medicaments (Shovelton 1959, 1964, Chirnside 1961, Akpata & Blechman 1982, Love 1996, Love *et al.* 1997).

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The technique most often used for both *in vivo* and *ex vivo* assessment of endodontic bacterial viability involves progressive circumferential reaming of the canal followed by culturing the recovered filings either on bacteriological agar or in liquid medium (Ørstavik & Haapasalo 1990, Heling & Chandler 1996, Perez *et al.* 1996, Peters *et al.* 2000). This method is labour intensive and possibly of questionable accuracy. Conventional microbiological staining methods have been applied to dentinal sections to examine the distribution of bacteria within the tubules but give no information on bacterial viability (Chirnside 1958, Shovelton 1964, Love 1996).

The nucleic acid-binding fluors, SYTO[®]9 and propidium iodide (PI), have been widely applied in environmental studies, food microbiology (Mason et al. 1994, Haugland 2002) and dental research (Hope & Wilson 2003, 2006, Chávez de Paz et al. 2007), including endodontic investigations (Weiger et al. 2002, Nagayoshi et al. 2004, Zapata et al. 2008). These reagents are marketed as the Live/Dead stain (Baclight[™]; Invitrogen Corporation, Carlsbad, CA, USA), as they differentiate between viable and non-viable bacteria. SYTO[®]9 penetrates intact biological membranes, whereas PI penetrates only bacteria with compromised plasma membranes and quenches the SYTO[®]9 fluorescence on binding the nucleic acid. Thus, simultaneous application of the stains generates red-fluorescing dead bacteria and green-fluorescing live bacteria, and these can be visualized by fluorescence microscopy (Auty et al. 2001). Weiger et al. (2002) applied the Live/Dead stain to circumpulpal filings from an endodontic infection model (using extracted human teeth) but did not attempt to visualize the bacteria directly within the tubules. Nagayoshi et al. (2004) used the Live/Dead stain to visualize bacteria within the tubules in a demineralized bovine dentine infection model. Demineralization prior to infection was necessary to facilitate subsequent microtome sectioning for conventional fluorescence microscopy, and the researchers did not attempt to measure the survival of the tubular bacteria. Zapata et al. (2008) refined the approach by combining fluorescent viability staining with confocal laser scanning microscopy (CLSM) to visualize bacteria infecting bovine dentinal tubules. CLSM not only reduces the need for demineralization and microtome sectioning of the dentine but also greatly improves the resolution of the images (Scivetti et al. 2007). Again, however, these investigators did not quantify in situ bacterial viability.

In this study, Live/Dead staining was applied to the human *ex vivo* dentinal tubule infection model to assess

the capacity to differentially image and quantify viable and non-viable bacteria within mineralized tissue by CLSM.

Materials and methods

Preparation of teeth

Approval to collect single-rooted teeth, extracted for orthodontic reasons, was obtained from the University of Otago Ethics Committee. Teeth with intact crowns and vital pulps were collected and stored in 70% ethanol (Goodis et al. 1993). Using a hand curette, soft tissues and bone fragments were removed from the root surfaces. Following the protocol of Haapasalo & Ørstavik (1987), cementum was removed with Sof-lex[™] discs (3M ESPE, Seefeld, Germany). Teeth were decoronated at the cemento-enamel junction using a rotary diamond saw with water irrigation (Gillings-Hamco, Rochester, NY, USA). Canal patency was confirmed with a size 10 file when the tip was visible at the apical foramen. Eighteen roots with patent canals were selected and stored in saline (0.9% NaCl; Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia).

All roots were soaked in 2% sodium hypochlorite (NaOCl) for 2 h to remove any organic matter on the tooth, followed by a saline rinse for 10 min. The roots were prepared using Protaper[™] instruments (Dentsply, Tulsa, OK, USA) according to manufacturers' recommendations with F2 as the final file. The working length was calculated as 1 mm short of the length of the patency file tip visible at the apical foramen. Each file was lubricated with RC Prep™ (Premier Products Company, Plymouth Meeting, PA, USA), and the canal was irrigated with 2% NaOCl. The apical 2 mm of each root was removed with the rotary diamond saw. The roots were placed in 17% ethylene diaminetetraacetic acid with cetrimide solution (Endosure EDTA-C; Gunz Dental Pty Ltd, Rosebery, NSW, Australia) for 30 min to remove the smear layer. This was followed by washing in NaOCl (30 min) to disinfect and dissolve any remaining organic matter from within the canal. All roots were then washed (30 min) and stored in saline.

Bacterial isolates and growth conditions

Enterococcus faecalis V583 (provided by Prof G. Cook, University of Otago) was maintained by cultivation at 37 °C on blood agar [brain–heart infusion agar supplemented with 5% (v/v) defibrinated horse blood] under anaerobic conditions [5% (v/v) CO_2 , 10% (v/v) H_2 in N_2]. Bacteria were prepared for experiments by inoculating brain–heart infusion containing 0.5% yeast extract (BHY) (Difco Laboratories, Detroit, MI, USA) and incubating for 18 h at 37 °C.

Bacterial infection/disinfection of dentinal tubules

Six prepared roots were placed in each of three test tubes containing BHY (50 mL) and sterilized by autoclaving (121 °C for 20 min). Two tubes were subsequently inoculated with 500 µL of the overnight E. faecalis culture, whilst one tube remained uninoculated to serve as the control for background staining. All roots were incubated at 37 °C under aerobic conditions without agitation, with daily transfer to sterile BHY. After 7 days, all roots were rinsed in saline, and calcium hydroxide [Ca(OH)₂] paste (Dental Therapeutics AB, Saltsjö-Boo, Sweden) was placed in the canals of six inoculated roots. The paste was placed from the coronal end until excess extruded out the apical end, and both ends of the root canal were then sealed with wax (White Utility Wax Rods Round, Kerr Corporation, Orange, CA, USA). Thus, six roots remained uninoculated, six were inoculated but otherwise untreated, and six were inoculated and treated with Ca(OH)₂. All 18 roots were then incubated in BHY for a further 7 days at 37 °C.

Staining protocol

The fluorescent stains SYTO[®]9 and PI were diluted in saline to give final concentrations of 10 and 60 μ mol L⁻¹, respectively. The wax seal was removed from the roots receiving Ca(OH)₂, and all the roots were washed with saline. The roots were then transferred to tubes containing the prepared stain, mixed thoroughly and maintained at room temperature in the dark for 30 min. The staining solution was replaced with saline

and the preparations agitated to remove excess stain. The washing step was repeated twice more, and the roots were blotted dry.

Embedding and processing

A schematic diagram of the embedding, sectioning and microscopic examination is shown in Fig. 1. After adhering the coronal ends of the roots to an acetate sheet with cyanoacrylate, the stained roots were placed in a 2-mL disposable spectroscopic cuvette (LP Italiana SPA, Milano, Italy). The cuvette was filled with methyl methacrylate (Vertex[™] Self Curing; Vertex-Dental BV, Zeist, Netherlands) and pressurized to two bar for 20 min to cure. The preparation was mounted on a CATSI specimen holder (Struers A/S, Ballerup, Denmark), and five evenly distributed transverse sections (1 mm thick) were cut using an Accutom-50 (Struers A/S) (Fig. 1). The sections were placed in sterile saline and stored in the dark at 4 °C.

Microscopy

Sections were examined by CLSM (510 META NLO, Axiovert 200; Carl Zeiss Ltd, Jena, Germany) with illumination by a Krypton/Argon laser (488 nm). A 477/543-nm double dichroic mirror was used as an excitation beam splitter and a 545-nm short-pass filter divided green and red fluorescence between the photo-multipliers. A 505- to 550-nm band-pass filter was used to visualize SYTO[®]9 and a 650-nm long-pass filter for PI.

Image analysis

Fluorescence images were analysed with Amira 5.0 (Visage Imaging Inc., Andover, MA, USA), and image stacks were viewed with LSM Image Browser (Carl Zeiss



Figure 1 Schematic illustrating the sectioning of roots and confocal laser scanning microscopy (CLSM) visualization of bacteria. Roots were sectioned from the coronal to the apical (a) to create five sections 1 mm thick (b). Four optical slices were imaged by CLSM (c).

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Ltd). The initial stacks, comprising both green and red fluorescence, were split into individual component colour channels and saved as grev-scale images. The split images were re-opened in Amira 5.0, and each stack was calibrated to define voxel size. For each grevscale image, fluorescence was adjusted ('thresholded') such that signals of intensity less than 20% were regarded as background (Yang et al. 2000, Hope & Wilson 2006). Using the drawing tool, the area of dentine visible in the stack was outlined to exclude the lumen and contents from the calculations. The quantification tool was used to determine the number of voxels in each optical slice, and the data were exported to a spreadsheet (Excel 2008 V12.2.5; Microsoft Corporation, Redmond, WA, USA). The total fluorescence in each optical slice was obtained by adding the number of voxels calculated from the grey-scale images of the respective red and green channels. Bacterial survival was then expressed as the proportion (%) of green voxels. Four optical slices, each 118.16 µm in depth, were taken per section. Initial experiments using live and dead *E. faecalis* cells prepared on glass slides, stained and digitally imaged determined that the

fluorescent intensity of grey-scale-adjusted individual

green bacteria was essentially equivalent (99.6% ±

33.5) to that of red bacteria.

Descriptive statistics and analyses of variance were calculated with Prism 3.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Visualization of bacteria in dental hard tissue

Green and red fluorescent bacteria were visible within the tubules of infected root sections when examined by CLSM (Fig. 2) but not evident in uninfected control roots (images appeared blank and are therefore not shown). Bacterial penetration of the dentinal tubules reached an average maximum distance of 653 µm from the lumen (Fig. 3). Lateral distribution of bacteria was generally uneven within individual root sections (Fig. 2), but there was no significant difference in bacterial penetration distance between equivalent sections from different teeth (Fig. 3). However, bacterial penetration distances varied significantly between sections within the same root, with greater penetration in the more coronal sections (Fig. 3). The standard deviations of the penetration distances throughout the length of the roots were not more than 35% of the mean for each root (Fig. 3). Measurement of maximum bacterial penetration distance was comparable between



Figure 2 Confocal laser scanning microscopy images of *Enterococcus faecalis*-infected dentinal tubules following treatment with $Ca(OH)_2$ and without treatment (control). Columns (a) and (b) show the same sections examined with 505- to 550-nm and 650-nm band-pass filters, respectively, to visualize dead (red fluorescent) and live (green fluorescent) bacteria. Column (c) shows composite images superimposing red and green fluorescence. L, dentinal lumen; W, root canal wall; D, dentine.



Figure 3 Maximum tubule penetration distance of *Enterococcus faecalis* in *ex vivo* root infection model. (a) Comparison of bacterial penetration between roots (five sections/root; mean \pm SD from six roots). Overall mean (from 30 sections) was $653 \pm 131 \mu$ m. The greatest standard deviation for an individual root was 35.2% of the corresponding mean. (b) Comparison of penetration distance within roots. Five vertical sections (mean \pm SD), approximately 1 mm apart, from each of six teeth. Mean penetration distances of sections 1 and 2 were significantly greater than those of sections 4 and 5 (P < 0.05, repeated-measures ANOVA). The maximum standard deviation was 20.5% of the corresponding mean.

roots, with the largest mean for an individual root exceeding the overall mean (for all roots) by only 15% and the lowest individual mean only 13% less than the overall mean (Fig. 3). The standard deviation determined from maximum penetration distances of all roots was 20% of the mean distance. Background fluorescence (in the absence of bacteria) was occasionally observed within the canal lumen but was minimal within the tubules and did not obscure or otherwise interfere with the fluorescent signal generated by bacteria. The DNA-bound fluors were stable to the extent that stained sections viewed by CLSM 3 months after preparation generated fluorescence comparable to that when originally examined (data not shown).

Quantification of bacterial viability

By comparing green and red fluorescent voxels throughout roots, there was no significant difference in bacterial survival between roots that had been treated in the same way (Table 1). As would be expected, $Ca(OH)_2$ -treated roots contained significantly fewer viable bacteria than did untreated roots in all sections (Fig. 4). Mean bacterial survival derived from multiple sections of untreated roots ranged from 83.36% to 96.24% and for $Ca(OH)_2$ -treated roots from 28.66% to 50.09% (Table 1). The standard deviation of the overall mean bacterial survival for untreated roots was 13.48% and for $Ca(OH)_2$ -treated roots was 25.5%.

Comparison of bacterial survival at different points within roots indicated that most variability was in the apical sections of $Ca(OH)_2$ -treated roots (Fig. 4). However, mean bacterial survival did not differ significantly throughout the length of roots treated in the same way (Fig. 4). For each section of the root, mean survival was significantly less in the $Ca(OH)_2$ -treated roots (Fig. 4). The greatest standard deviation of mean survival derived from multiple optical sections taken from a single untreated root was 18.31% and that for a $Ca(OH)_2$ -treated root was 30.73% (Table 1).

Discussion

Incubation of sterilized instrumented roots in bacterial culture has been used as a method to introduce bacteria into dentinal tubules for experimental purposes (Haapasalo & Ørstavik 1987, Love 1996, Zapata *et al.* 2008) and was adopted effectively in the current study. The investigation sought to develop a convenient method with which to determine the viability of bacteria in the *ex vivo* infection model by adapting and evaluating a fluorescent bacterial viability staining technique. Fluorescence was evident within the tubules and the proportions of red- and green-fluorescing voxels were quantifiable by standard image analysis software.

Quantification of fluorescence is not without its difficulties and limitations however, the principal problems being standardization of fluorescence between optical slices and achieving consistent balance between the red and green signals. The intensity of fluorescence is dependent on various factors that are impossible to control precisely, including thickness of the sections, depth of the optical slice and the degree of mineralization

Table 1	Com	parison	of	bacterial	viab	ility	in	ex	vivo	roots	treated	with	Ca(OH), ai	nd	untreated	roots
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	Root number											
Untreated	1	2	3	4	5	6						
Number of optical slices	23	23	24	23	24	26						
Surviving bacteria (mean %)	86.39	93.68	88.31	87.15	96.24	83.39						
Standard deviation (%)	13.48	9.37	13.33	13.73	4.295	18.31						
Lower 95% confidence interval	80.56	89.63	82.68	81.22	94.43	75.99						
Upper 95% confidence interval	92.22	97.74	93.94	93.09	98.05	90.79						
Overall mean	89.11		SD	13.48								
Ca(OH) ₂ -treated	1	2	3	4	5	6						
Number of optical slices	18	18	17	18	18	17						
Surviving bacteria (mean %)	34.72	47.04	44.68	46	50.09	28.66						
Standard deviation (%)	27.71	18.52	24.42	22.23	24.65	30.73						
Lower 95% confidence interval	20.94	37.83	32.13	34.94	37.83	12.86						
Upper 95% confidence interval	48.5	56.25	57.24	57.05	62.35	44.46						
Overall mean	35.86		SD	25.5								

 $Ca(OH)_2$ -treated roots contained significantly less surviving bacteria than did untreated roots (P < 0.005), but there was no significant difference in survival between roots treated in the same way.



Figure 4 Comparison of bacterial viability at vertical positions within six roots treated with $Ca(OH)_2$ (O) and six untreated roots (\bigcirc). Points are the mean viability for each root for each of five sections (1–5). Mean survival did not differ significantly between sections of roots treated in the same way. $Ca(OH)_2$ -treated roots contained significantly fewer viable bacteria than did untreated roots in all sections (P < 0.001).

of the dentine. Consequently, the CLSM instrument parameters must be adjusted uniquely for each image. These difficulties are not unique to this study and have been discussed in detail previously (Hope & Wilson 2003, Nagayoshi *et al.* 2004). To minimize the problem of slice-to-slice variation, a widely used 'thresholding' approach was adopted (Yang *et al.* 2000). To assess the reliability of this approach, measurements from replicate samples were compared and the data found to be consistent; the associated variability would be acceptable for most experimental applications.

The technique facilitates the determination of the distribution of bacteria both laterally within the tubules and longitudinally throughout the length of the root (a further advantage over conventional culturing methods). Bacterial penetration of the apical tubules was reduced compared with the coronal regions and this may be because of age-related sclerosis (Paqué *et al.* 2006), as well as the reduced number of tubules located at the apex. There was no difference in bacterial penetration between roots, and the relatively small standard deviation derived from the assessment of multiple roots indicated uniformity appropriate for experimental purposes. Note that most studies relying on conventional culturing methods do not report experimental variability.

The lateral distribution of bacteria was confined by the arrangement of the tubules, often resulting in crescent-shaped distribution patterns of bacteria (evident in Fig. 1). In a number of the dentine preparations, more intense accumulations of bacteria were observed in the proximal and distal regions of the tubules, with relatively sparse bacterial growth in the mid-sections. This effect is most apparent in the green-fluorescent images of Fig. 2. The reason for this distribution pattern is not known but tubule anatomy and concentration gradients of either nutrients or toxic products may be involved. Microscopic examination of naturally infected dentine also reveals uneven bacterial distribution within the tubules (Shovelton 1964, Brännström & Nyborg 1971, Tronstad & Langeland 1971, Vojinovic *et al.* 1973, Ricucci & Siqueira 2010), though the effect is not as striking as in the current study, perhaps because naturally infected tubules are less heavily colonized. Alternatively, the effect could be an artefact caused by sample processing but this is less likely as other *ex vivo* root infection models, using alternative techniques, display similar bacterial distribution patterns (Shovelton 1964, Perez *et al.* 1993, Peters *et al.* 2000).

That the bacteria are stained before sectioning of the root provides a further advantage of the fluorescent staining method over culturing. The heat generated by milling the dentine conceivably kills the bacteria, producing misleading results in conventional culturing studies. This becomes irrelevant in the fluorescence method described as the bacteria are irreversibly stained before sectioning. However, it is acknowledged that in both methods, physical disturbances during sample preparation may displace bacteria.

Conclusion

A quantifiable fluorescence microscopy method for the assessment of bacterial viability and distribution within the mineralized tubules of *ex vivo* teeth has been adapted and validated. The technique provides a convenient and reproducible approach for assessing the viability of the bacteria and the extent of bacterial penetration into dentinal tubules. This technique will have application in the comparative assessment of antimicrobial endodontic medicaments.

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