Evaluation of the Propidium Monoazide–quantitative Polymerase Chain Reaction Method for the Detection of Viable *Enterococcus faecalis*

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Abstract

Introduction: One limitation of DNA-based molecular assays is their inability to distinguish between live and dead cells. A sample treatment with propidium monoazide (PMA) before DNA amplification has been proposed to overcome this problem. The aim of this *in vitro* study was to test different concentrations of PMA coupled with quantitative polymerase chain reaction (qPCR) for the detection of viable *Enterococcus faecalis*. **Methods:** Viable or heat-killed suspensions of *E. faecalis* (10 colony-forming units/mL) were treated with PMA at 10, 50, and 100 μg/mL before DNA extraction. qPCR was performed using primers complementary for *E. faecalis* 16S ribosomal RNA sequence. PMA was also tested on bacteria suspensions containing different proportions of viable and dead cells. Bacterial suspensions without PMA treatment were used as positive controls. **Results:** The treatment of heat-killed suspensions with PMA at different concentrations significantly reduced the DNA amplification when compared with the group without treatment ($P < .0001$), indicating that DNA from dead cells was not used as templates. The greatest reduction in qPCR amplification of dead cell DNA was found when 100 μg/mL PMA was used ($P < .005$). In mixtures containing live/dead cells, PMA allowed selective detection of viable cells. **Conclusions:** PMA was effective in inhibiting qPCR amplification from the DNA of dead cells, enabling *in vitro* detection and quantification of viable cells of *E. faecalis*. *(J Endod 2016;42:1089–1092)*

Key Words

Endodontic, *Enterococcus faecalis*, propidium monoazide, quantitative polymerase chain reaction

The reduction of the viable bacterial load in root canals is a key element for the success of endodontic treatment (1). Molecular methods targeting nucleic acids are the most preferable choice to assess bacterial reduction after endodontic procedures because of their sensitivity and specificity and because they allow the quantification of endodontic infectious agents (2). However, the major drawback of DNA-based methods is the detection of both viable and nonviable cells (3), especially in the access-limited regions of the teeth where host deoxyribonucleases may not reach the free DNA from dead cells in a short period of time. This fact may lead to an overestimation of bacterial targets after endodontic disinfection protocols.

Strategies based on the metabolic activity and cell membrane integrity may be used for selective detection of viable cells in combination with molecular methods (4). The first approach involves the use of RNA instead of DNA as a template for amplification because RNA reflects the metabolic activity of viable bacteria and is more promptly degradable than DNA. However, RNA-based methods do not permit precise quantification of bacterial load because RNA levels may vary depending on the physiological status of the cell (5). The second approach to detect viable bacteria is based on the selective permeability of intact membranes. To achieve this goal, the sample is treated with propidium monoazide (PMA), which does not cross the cellular membrane of living cells. Once inside the membrane-damaged cell, PMA is a DNA intercalating dye that binds to the bacterial DNA after photoactivation and prevents its amplification. Thus, only the DNA of membrane-intact cells should be detected by polymerase chain reaction (PCR) in PMA-treated samples (6, 7). The use of PMA was recently combined with quantitative PCR (qPCR) to allow quantification of viable bacteria in clinical samples.

For successful application of PMA-qPCR, optimization of experimental conditions is necessary because many factors may influence the results, including the dye concentration and intrinsic characteristics of microorganisms among others (5–7). The optimal dye concentration that efficiently inhibits DNA amplification from the dead cells of oral pathogens has been the subject of recent studies, including gram-positive bacteria such as *Streptococcus* spp and gram-negative bacteria such as *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Veillonella parvula*, and *Fusobacterium nucleatum* (5, 8–10).

Enterococcus faecalis is a commonly detected species in the root canals of teeth with post-treatment apical periodontitis (11–14), and it may remain viable in root canals even after chemomechanical procedures (15). PMA-qPCR assays have also been used in clinical studies searching for viable *E. faecalis* and total bacteria in endodontic infections

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(16, 17). However, PMA concentrations have varied widely in these previous studies, which may have influenced the ability of PMA in detecting viable bacteria in root canal samples. Therefore, the aim of this *in vitro* study was to test different concentrations of PMA coupled with qPCR for the selective detection of viable cells of *E. faecalis*. The null hypothesis was that the PMA concentration does not influence DNA amplification from dead cells of *E. faecalis*.

Materials and Methods

E. faecalis Culture and Killing Conditions

E. faecalis standard reference strain (American Type Culture Collection 29212) was grown on brain-heart infusion (BHI) (Difco, Detroit, MI) agar plates. Bacterial cells were suspended in BHI broth and incubated at 37°C in air for 16 to 18 hours. Forty microliters of the suspension were inoculated into 4 mL BHI broth, and the cells were grown to the midexponential growth phase. The bacterial concentration was determined by measuring optical density at 495 nm and adjusted to concentrations of 10 colony-forming units (CFU)/mL by serial dilutions in BHI broth. The concentration was confirmed by plating aliquots of serially diluted suspensions on BHI agar plates in triplicate. The determination of total CFU/mL was performed after incubation at 37°C in air for 16 to 18 hours.

Aliquots of the bacterial suspension (10^6 CFU/mL) were heat killed at 95°C for 30 minutes (5). The lack of viability was confirmed by microbial culturing.

Mixtures of Viable and Dead Cells

Viable cell suspensions were mixed with dead cell suspensions in proportions (8) of 100%, 75%, 50%, 25%, or 0% viable cells to the total bacterial cell concentration. The total number of cells in viable–dead cell mixtures was kept constant at 10^6 CFU/mL.

PMA Treatment

PMA treatment was performed as described previously by Loozen et al (5). PMA (Biotium Inc, Hayward, CA) was dissolved in 20% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) to produce stock concentrations of 1 mg/mL, 500 μg/mL, and 100 μg/mL, which were stored at 20°C in the dark. Ten microliters of the PMA solutions were added to 90-μL culture aliquots to obtain final PMA concentrations of 100, 50, and 10 μg/mL. After an incubation period of 5 minutes at 4°C in the dark, samples were exposed to a 650-W halogen light source (GE Lighting, General Electric Co, Cleveland, OH) placed 20 cm above the samples for 10 minutes. The samples tubes were laid horizontally on ice during the light exposure period to avoid excessive heating. Samples without PMA treatment were subjected to the same procedures, except for the PMA addition, and used as controls. Then, all samples were subject to genomic DNA isolation. Experiments were performed in triplicate in 2 independent assays.

DNA Extraction and qPCR

Total nucleic acids were extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Technologies, Madison, WI) following the manufacturer's protocol. Briefly, after centrifugation at 10,000*g* for 10 minutes, supernatants were discarded, and pellets were resuspended in solution containing 450 μL tissue and cell lysis solution and 2 μL proteinase K, which was incubated for 15 minutes at 65°C. After incubation, mixtures were cooled on ice for 5 minutes and added to 225 μL MPC protein precipitation reagent (Epicentre Technologies, Madison, WI). After centrifugation at 10,000*g* for 10 minutes, supernatants were collected and subjected to isopropanol precipitation. Total nucleic acid samples were resuspended in 35 μL Tris-EDTA buffer.

Real-time PCR was performed using 2 μL total nucleic acid suspension as a template and primers complementary for *E. faecalis* 16S ribosomal RNA sequences. Quantitative PCR assays were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) in reactions set up in 96-well plates in a total volume of 22 μL containing 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems), and 100 nmol/L of each primer (5′-CGCTTCTTTCCTCCCGAGT-3′ and 5′-GCCATGCGGCATAAACTG-3′) (18). Deionized water was used instead of template DNA as the negative control. Cycling conditions for qPCR reactions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melting curve analyses were performed from 65°C to 95°C to confirm specificity of the amplified products. Data were analyzed using the Applied Biosystems SDS 7500 software.

The standard curve was built using recombinant plasmids containing the 1500 fragment encoding 16S ribosomal RNA of *E. faecalis* as previously described (15). Plasmid standard dilutions (from 10^7 to 10 DNA copies) and DNA samples were run in triplicate, and the limit of quantification was 10 DNA copies per reaction. The correlation coefficient (r^2), amplification efficiency (E), and y intercept values were 0.99, 95%, and 35.5, respectively.

Data Analyses

The quantification cycle (Cq) values, previously known as cycle threshold values, and the corresponding number of DNA copies were automatically generated through the StepOne Plus software (Applied Biosystems). The mean values for DNA measurements were used to calculate the total number of *E. faecalis* cells per milliliter. Analyses were performed on $\log_{10}$-transformed data for both non–PMA-treated and PMA-treated samples. Analysis of variance and post hoc testing with the Bonferroni correction for multiple comparisons were used. Differences were considered statistically significant when P was $<.05$.

Results

Effects of Different Concentrations of PMA on Heat-killed *E. faecalis* Suspensions

E. faecalis suspensions of dead cells (6 $\log_{10}$ CFU/mL) were treated with different concentrations of PMA (10, 50, or 100 μg/mL) before DNA extraction and amplification by qPCR. Results of the PMA-qPCR were compared with the heat-killed positive control that did not receive PMA treatment. The 3 PMA concentrations significantly reduced the amplification of DNA derived from dead cells ($P < .001$). Compared with the control, the highest PMA concentration (100 μg/mL) resulted in a reduction of DNA amplification of about 3 $\log_{10}$, whereas the lowest PMA concentration (10 μg/mL) resulted in a reduction of about 2 $\log_{10}$ (Fig. 1). Statistically significant differences were found when comparing the 3 PMA concentrations on heat-killed bacterial suspensions ($P < .005$).

Effects of Different Concentrations of PMA on Viable *E. faecalis* Cells

PMA treatment of viable *E. faecalis* cells also resulted in the reduction of DNA amplification (0.8–1.0 $\log_{10}$) when compared with the untreated viable positive controls ($P < .05$) (Fig. 2). No statistically significant differences were found when the 3 PMA concentrations used to treat viable cells were compared ($P > .05$).

Effects of PMA on Mixtures of Viable and Dead Cells

To test the PMA ability to detect viable cells in the presence of dead cells, mixtures of fresh and heat-killed *E. faecalis* suspensions were prepared, maintaining an equal number of cells (6 $\log_{10}$ CFU/mL).