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Analysis of viable vs. dead *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* using selective quantitative real-time PCR with propidium monoazide

M. C. Sánchez, M. J. Marín, E. Figuero, A. Llama-Palacios, D. Herrera, M. Sanz
ETEP (Aetiology and Therapy of Periodontal Diseases) Research Group, University Complutense of Madrid, Madrid, Spain

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Background and Objectives: One of the major disadvantages of DNA-based microbial diagnostics is their inability to differentiate DNA between viable and dead microorganisms, which could be important when studying etiologically relevant pathogens. The aim of this investigation was to optimize a method for the selective detection and quantification of only viable *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* cells by combining quantitative real-time polymerase chain reaction (qPCR) and propidium monoazide (PMA).

Material and Methods: Three different concentrations of PMA (10, 50 or 100 μM) were added to suspensions of 10^6 (CFU)/mL of viable/dead *A. actinomycetemcomitans* and *P. gingivalis* cells. After DNA isolation, qPCR was carried out using specific primers and probes for the tested bacteria. PMA was further tested with different mixtures containing varying ratios of viable and dead cells. The efficacy of PMA to detect viable/dead cells was tested by analysis of variance.

Results: For these specific bacterial pathogens, 100 μM PMA resulted in a significant reduction of qPCR amplification with dead cells (10^6 CFU/mL), while with viable cells no significant inhibition was detected. PMA was also effective in detecting selectively viable cells by qPCR detection, when mixtures of varying ratios of viable and dead bacteria were used.

Conclusions: This study demonstrated the efficiency of PMA for differentiating viable and dead *A. actinomycetemcomitans* and *P. gingivalis* cells. This method of PMA-qPCR may be useful for monitoring new antimicrobial strategies and for assessing the pathogenic potential of *A. actinomycetemcomitans* and *P. gingivalis* in different oral conditions when using molecular diagnostic methods.

Dr Mariano Sanz, Section of Periodontology, Faculty of Odontology, Plaza Ramón y Cajal s/n – Ciudad Universitaria, 28040 Madrid, Spain
Tel: +(34) 913941901
Fax: +(34) 913941910
e-mail: marianosanz@odon.ucm.es

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The oral cavity of healthy individuals contains hundreds of different bacterial, viral and fungal species, which usually attach to hard surfaces and form matrix-embedded biofilms. Within this oral microbiota, varying environmental conditions contribute to the species composition of the different biofilms encountered. In healthy individuals, oral biofilms are typically comprised of gram-positive facultative anaerobes, but in periodontal diseases, such as gingivitis and chronic periodontitis, subgingival biofilms increase in volume and in complexity with higher proportions of gram-negative species and well-recognized pathogens (e.g. *Porphyromonas gingivalis*, *Campylobacter rectus*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*) (1). These complex biofilms induce a local chronic inflammatory response that might lead to the destruction of the tooth-supporting tissues, which is the characteristic pathology in chronic periodontitis.

Owing to the significant association between the presence of bacterial pathogens *P. gingivalis* and *A. ggregatibacter actinomycetemcomitans* and the etiology and progression of periodontitis, their identification using both cultivation and molecular identification methods has been the target of many investigations. Although the detection and identification of bacterial species in subgingival plaque samples using cultural techniques has been the traditional gold standard diagnostic method, the advent of molecular techniques has improved extensively our knowledge on the microbial composition of subgingival plaque samples.

Cultural techniques have disadvantages, being expensive and laborious intensive procedures that require relatively long times for the growth of colonies, what limits the number of samples that can be evaluated. Moreover, the viable plate count method is highly dependent on the different growth media used and the likely microbial interactions occurring during growth (2). Its main advantage, however, is the maintenance of bacterial viability throughout the diagnostic process, from sampling to plate counting, which assures the metabolic and reproductive activity of isolates.

Culture-independent molecular methods for identification and quantification of subgingival pathogens have been developed during recent years (3–8). Among the available alternatives, quantitative real-time polymerase chain reaction (qPCR) represents a rapid method not only able to detect, but also to accurately quantify pathogenic bacteria in oral samples (9–11). The attractiveness of PCR-based techniques includes their enhanced specificity and sensitivity over traditional culture techniques, as well as their ability to obtain results more rapidly (12). In spite of these advantages, a major disadvantage of PCR is that it detects DNA from both viable and dead bacterial cells, due to the relatively long persistence of DNA after cell death and, therefore, DNA-based diagnostics may overestimate the number of live cells. In fact, DNA derived from cells destroyed by heat, disinfectants or antibiotics may serve as a template for PCR amplification from days up to 3 wks following their loss of viability (13–16).

Several authors have attempted to detect only viable cells by identifying mRNA using reverse transcriptase PCR (17,18). The reproducibility in determining viable and dead cell counts accurately with this technique is hampered, however, by the use of mRNA as the target (19). Other currently applied microbial diagnostic methods able to discern between viable and dead cell methods are based on microscopy and flow cytometry using BacLight^{TM}(Invitrogen, Life Technologies Ltd, Paisley, UK) staining kits (20–24), although their capability of identifying viable cells has a narrower detection range than culturing (14).

In the last few years, a novel alternative has been developed based on the presence of membrane integrity as the criterion for distinguishing between viable and irreversibly damaged cells. Live cells with intact membranes have the ability to exclude DNA binding dyes that easily penetrate dead or membrane-compromised cells. This DNA-based methodology combines the use of a live/dead discriminating dye, propidium monoazide (PMA) with the speed and sensitivity of qPCR. It has been tested with a diversity of bacterial species, being able to differentiate between viable and dead cells (2,19,25–30). The possible influence of PMA on the qPCR signal has also been thoroughly investigated (10,15,31,32).

PMA is a DNA-intercalating dye with the azide group allowing covalent binding to DNA between the bases, with little or no sequence preference, when exposed to bright visible light (maximum absorbance at 460 nm). PMA is impermeable to intact cell membranes, and thus, can only react with DNA from dead cells or those with compromised membrane integrity. Upon penetrating compromised cell membranes, photolysis of PMA using a bright visible light produces a nitrene that forms a covalent link with DNA and other related molecules (15). Photo-induced PMA cross-linking will therefore, inhibit PCR amplification of DNA from dead cells, while not affecting the DNA from viable cell. Compared to unbound DNA, PMA-bound DNA leads to a reduction in qPCR signal, which reduces the qPCR bacterial cell counts.

Owing to the intrinsic characteristics of each microorganism and the different requirements of the assay conditions (e.g. time and temperature of incubation with DNA-intercalating dyes, such as PMA or etidium monoazide), time to exposure to light source and amplification efficiency of the qPCR assay, this methodology may produce heterogeneous results. Moreover, insufficient differentiation of live and dead cells has been reported with various bacteria when using this methodology (e.g. *Campylobacter jejuni* and *Listeria monocytogenes*) (33). It is, therefore, necessary to test this methodology with each target pathogen to ensure a successful differentiation between viable and dead DNA cells. It is the goal of this investigation to test the use of PMA in conjunction with qPCR

in the detection and quantification of the main, recognized periodontal pathogens in the subgingival microbiota, *A. actinomycetemcomitans* and *P. gingivalis*.

Material and methods

Bacterial strains and culture conditions

Standard reference strains of *A. actinomycetemcomitans* DSMZ 8324 and *P. gingivalis* ATCC 33277 were used in this investigation. The selected bacteria were grown on blood agar plates (Blood Agar Oxoid no. 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg/mL hemin (Sigma, St Louis, MO, USA) and 1.0 mg/mL menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% H_2, 10% CO_2 and balance N_2) at 37°C for 24–72 h. Growth kinetic was calculated by generating growth curves of each bacteria in triplicate. Bacterial colonies were collected from the cultured blood agar plates and transferred to 12 mL of modified brain–heart infusion medium, and anaerobically incubated at 37°C for 24–48 h. This protein-enriched medium contains brain–heart infusion (Becton, Dickinson and Company; Becton, Dickinson Co., Franklin Lakes, NJ, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/mL hemin (Sigma), 1.0 mg/mL menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). After incubation, the bacteria were harvested in the late exponential growth phase and added to fresh modified brain–heart infusion medium to begin the growth curve at the lag phase [0.05 of optical density (OD) at 550 nm using a spectrophotometer (Shimadzu, Kyoto, Japan)]. At specified time intervals, measurements of the OD at 550 nm were made and 100 μL aliquots were taken. These aliquots were diluted serially in phosphate-buffered saline (PBS; pH 7.2; 0.1 mol/L), plated in duplicate on supplemented blood agar plates, and incubated in anaerobic conditions at 37°C for 4–7 d. After incubation, the counts of the total number of colony-forming units per milliliter (CFU/mL) of samples were calculated. Bacterial colonies were collected from the cultured blood agar plates and transferred to 12 mL of modified brain–heart infusion medium, and anaerobically incubated at 37°C for 24–48 h. At the exponential growth phase, the concentration of grown bacteria in the obtained suspension was determined by OD at 550 nm and adjusted to concentrations of 10^6 CFU/mL by serial dilutions if necessary in the same growth medium. The concentration was confirmed by plating 100 μL aliquots diluted serially in PBS in duplicate on supplemented blood agar plates, incubated in anaerobic conditions at 37°C for 4–7 d. After incubation, the counts of the total number of CFU/mL were carried out.

Inactivation of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*

Two hundred and fifty microliter aliquots of a 10^6 CFU/mL cell suspension of *A. actinomycetemcomitans* and *P. gingivalis* were inactivated by exposure to 70% isopropanol for 10 min. Then, the isopropanol was removed by centrifugation at 9000 ***g*** for 5 min before resuspension in 250 μL of PBS. Three different mixed bacterial cultures prepared on separate days were performed ($n = 3$). The absence of viable cells was confirmed by culturing an aliquot of the treated cells on supplemented blood agar plates for 72 h at 37°C in anaerobic conditions.

Propidium monoazide treatment in viable/dead isolates

PMA (Biotium Inc., Hayword, CA, USA) was added at final concentrations of 10, 50 or 100 μM to sample tubes containing 250 μL of either viable or isopropanol-killed cells (all derived from a 10^6 CFU/mL cell suspension). Following an incubation period of 10 min at 4°C in the dark, the samples were subjected to photo-induced cross-linking of PMA by light exposing for 20 min using a 550 W halogen light source, placed 20 cm above the samples. The sample tubes were laid horizontally on ice during this period to avoid excessive heating. After photo-induced cross-linking, the cells were centrifuged at 9000 ***g*** for 3 min before DNA isolation. To control for any influence on the bacteria viability of the process alone (incubation at 4°C and exposure to light source), 250 μL of viable and isopropanol-killed cells (all derived from a 10^6 CFU/mL cell suspension) subjected to the same process, but without exposure to PMA, were used as control samples. Three different experiments prepared on separate days were performed ($n = 3$).

Propidium monoazide treatment in mixed viable/dead isolates

After the isopropanol killing of a 10^6 CFU/mL cell suspension of *A. actinomycetemcomitans* and *P. gingivalis* as described, dead cells were mixed with viable cells (10^6 CFU/mL) in defined proportions. The mixtures were prepared where viable cells represented 100%, 75%, 50%, 25% or 0% of the total bacterial cell concentration. PMA treatments were carried out in triplicate on 250 μL of the cell mixtures using 100 μM PMA, 10 min of incubation in the dark at 4°C and then exposed to the light for 20 min. As controls, the same mixtures with decreasing proportions of viable cells, but without any PMA treatment, were assessed. Then, all samples were subject to genomic DNA isolation following the protocol described afterwards.

DNA isolation and quantitative PCR

The DNA was isolated from all PMA-treated samples and from controls using a commercial kit (ATP Genomic DNA Mini Kit®; ATP Biotech, Taipei, Taiwan), following the manufacturer's instructions. The hydrolysis probes 5′ nuclease assay PCR method was used for detecting and quantifying bacterial DNA. Primers (synthesized by Invitrogen, Carlsbad, CA, USA) and probes (synthesized by Applied Biosystems,

Carlsbad, CA, USA) were targeted against 16S rRNA genes for both *A. actinomycetemcomitans* [forward (F): 5′-GAA CCT TAC CTA CTC TTG ACA TCC GAA-3′ reverse (R): 5′-TGC AGC ACC TGT CTC AAA GC-3′, probe: 5′-AGA ACT CAG AGA TGG GTT TGT GCC TTA GGG-3′ (34); Amplicon size: 80 bp] and *P. gingivalis* [(F): 5′-GCGCTCAA CGTTCAGCC-3′. (R): 5′-CACGAAT TCCGCCTGC-3′, Probe: 5′-CAC-TGAACTCAAGCCCGGCAGTTTC AA-3′ (35); Amplicon size: 67 bp]. PCR amplification was performed in a total reaction mixture volume of 20 μL. The reaction mixtures contained 10 μL of 2× TaqMan master mixture (Taqman Gene Expression Master Mix®; Applied Biosystems), optimal concentrations of primers and probe (300, 300 and 100 nM for *A. actinomycetemcomitans*, and 300, 300 and 300 nM, for *P. gingivalis*), and 5 μL of DNA from samples. The negative control used was 5 μL of sterile water [no template control (NTC)]. The samples were subjected to an initial amplification cycle of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Quantitation was achieved through the use of calibration curves in each run. Purified genomic DNA of both bacteria, *P. gingivalis* strain ATCC 33277 and *A. actinomycetemcomitans* strain DSMZ 8324, was obtained from 10^9 CFU/mL axenic suspensions following the described protocol of DNA isolation. CFU/mL was determined by OD at 550 nm based on the growth curves generated and confirmed by plated 100 μL aliquot, diluted serially in PBS in duplicate on supplemented blood agar plates, incubated in anaerobic conditions at 37°C for 4–7 d. After incubation, counts of the total number of CFU/mL were carried out. Purified DNAs were measured by using the Nanodrop® ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) to determine their purity and concentration. Serial dilutions of purified DNA, with a range 10^9-10 CFU/mL correspondence, were prepared as stock solutions and conserved at −20°C. The samples were analyzed with a FASTA HT 7900 thermocycler (Applied Biosystems). The plates used in the study were transparent MicroAmp® Fast Optical 96-Well reaction Plate (0.1 mL) (Applied Biosystems), sealed by MicroAmpTM Optical Adhesive Film (Applied Biosystems).

To verify analytical specificity, homologies of the selected primers and the probe with unrelated sequences were checked by a search with the BLAST program from the National Center for Biotechnology Information. Additionally, qPCR was performed to confirm the amplicon size obtained. The band position of the PCR products was in accordance with the expected amplicon length. The repeatability of the assay was ensured with the same sample analyzed twice in the same assay. The sensitivity (expressed as the limit of detection, LOD) was based on the calibration curves and the NTCs values obtained.

Data analyses

The Cq [quantification cycle, previously known as threshold cycle (Ct)] values and correlations with CFU/mL (based on calibration curves in each run) were automatically generated through the SDS 2.3 SOFTWARE® (Applied Biosystems). Data were expressed as mean and standard deviation (SD). All assays presented act in accordance with the premise of calibration curves with slope range 3.3–3.5 cycles/log decade, $r^2 = 0.99$ and efficiency range 1.9–2.0.

Kolmogorov–Smirnov goodness-of-fit tests were computed for each variable to evaluate the normality of the distribution. Analysis of variance (ANOVA) and post hoc testing with Bonferroni's correction for multiple comparisons were used. Results were considered statistically significant at $p < 0.05$. A software package (IBM® SPSS® STATISTICS 19.0; IBM Corporation, Armonk, NY, USA) was used for all data analysis.

Results

Effects of propidium monoazide on dead cells

Table 1 depicts the effectiveness of PMA on *A. actinomycetemcomitans* and *P. gingivalis* suspensions of dead cells, comparing the qPCR results after using different concentrations of PMA (10, 50 and 100 μM) with the positive controls, without PMA exposure. The Cq values of dead cells treated with PMA gradually increased with the increased concentration of PMA used, which indicates a reduction in the amplification of DNA derived from cells killed by isopropanol, and therefore, a reduction in the amounts of *A. actinomycetemcomitans* and *P. gingivalis*. For both bacterial species, the mean Cq values at 100 μM PMA fell below the LOD. These results showed that 100 μM PMA was effective in preventing qPCR amplification of the target sequence from the dead cell suspension for both bacterial species. For *P. gingivalis*, similar results were obtained at 50 μM PMA.

Non-significant differences in Cq values were observed for *A. actinomycetemcomitans* when comparing isopropanol-killed cells treated with 10 μM PMA to the positive control (1.2, SD = 1.4; $p = 1.00$). The treatment of isopropanol-killed *A. actinomycetemcomitans* cells with 50 or 100 μM PMA resulted in Cq values below the detection limit [29.0 (SD = 0.1)]. In the case of *P. gingivalis*, statistically significant differences in Cq values were observed when comparing isopropanol-killed cells treated with 10 μM PMA to the positive control (4.5, SD = 0.7; $p < 0.01$) and the treatment with 50 or 100 μM PMA resulted in Cq values below the detection limit [32.1 (SD = 0.1)].

Cq values were used to determine the correlation between the numbers of CFU/mL obtained from qPCR assays. Figure 1 illustrates the effect of different concentrations of PMA on the log CFU/mL in the isopropanol-killed bacterial suspension. Compared to the positive control, statistically significant differences in the number of CFU/mL were observed for *A. actinomycetemcomitans* and *P. gingivalis*, when dead cells were treated with 50 and 100 μM ($p < 0.05$). For both bacterial species, no statistically significant differences were found when the three PMA

Table 1. Effect of increasing propidium monoazide (PMA) concentration on viable and isopropanol-killed *A. actinomycetemcomitans* and *P. gingivalis* cells. The Cq values indicate the mean values of three independent experiments with the standard deviations (SD)

	Cq values [mean value (SD)]							
	A. actinomycetemcomitans				*P. gingivalis*			
	Non-PMA	10 μM PMA	50 μM PMA	100 μM PMA	Non-PMA	10 μM PMA	50 μM PMA	100 μM PMA
Viable cells	22.1 (0.2)	22.6 (0.3)	23.0 (0.2)	23.2 (0.2)	22.8 (0.7)	22.0 (0.5)	22.4 (0.4)	22.7 (0.5)
Dead cells	23.8 (0.7)	28.0 (3.9)	29.2 (2.8)[a]	30.8 (0.8)[a]	26.5 (1.6)	31.4 (1.1)	32.2 (0.7)[a]	32.8 (0.8)[a]
Limit of detection	29.0 (0.1)	29.0 (0.1)	29.0 (0.1)	29.0 (0.1)	32.1 (0.4)	32.1 (0.4)	32.1 (0.4)	32.1 (0.4)
No template control	34.1 (0.4)	34.1 (0.4)	34.1 (0.4)	34.1 (0.4)	35.8 (1.6)	35.8 (1.6)	35.8 (1.6)	35.8 (1.6)

[a]Value below detection limit.

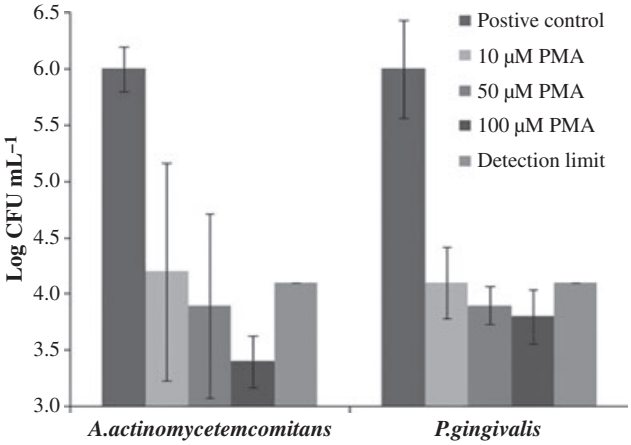

Fig. 1. Effect of propidium monoazide (PMA) on isopropanol-killed bacterial suspension: dead bacterial suspension, containing 10^6 colony forming units (CFU)/mL for *A. actinomycetemcomitans* and *P. gingivalis*, were subjected to increasing concentrations of PMA. The effects are shown as the log conversion of CFU/mL. The bars indicate mean values of three independent experiments, and the error bars indicate standard deviations.

concentrations used to treat dead cells were compared ($p = 1.00$).

Effects of propidium monoazide on viable cells

Table 1 shows the effect of PMA on *A. actinomycetemcomitans* and *P. gingivalis* viable cell suspensions, comparing the qPCR results after using different concentrations of PMA (10, 50 and 100 μM) with the corresponding viable cell suspensions without PMA exposure. For both *A. actinomycetemcomitans* and *P. gingivalis*, Cq values of 22.1 (SD = 0.2) and 22.8 (SD = 0.7), respectively, derived from viable cells not treated with PMA, were similar to the obtained Cq values with the same cell suspensions treated with PMA (Table 1). For both bacterial species, no statistically significant differences in Cq values and/or log CFU/mL were observed ($p > 0.05$) when the three PMA concentrations were used with viable cells or when compared with the viable cell suspension not subjected to PMA treatment. No statistically significant differences were found when the three concentrations used to treated isopropanol-killed cells were compared ($p > 0.05$).

Effects of propidium monoazide on mixtures of viable and dead cells

The efficiency of PMA in selectively allowing DNA amplification from viable cells in the presence of dead cells was evaluated. Table 2 depicts the effect of PMA on *A. actinomycetemcomitans* and *P. gingivalis* suspensions where both viable and dead cells were combined. When viable/dead mixed bacterial cells were similarly treated with 100 μM PMA before DNA extraction, Cq values gradually increased from 23.0 (SD = 0.8) to 28.9 (SD = 0.2) for *A. actinomycetemcomitans*, and from 25.3 (SD = 0.8) to 32.7 (SD = 1.0) for *P. gingivalis*, as the percentage of viable cells decreased; therefore, demonstrating a relationship between Cq values and number of viable cells present in a sample. Figure 2 represents a linear relationship between the Cq values and log number of CFU/mL in the viable/dead cell mixtures after the PMA and qPCR reaction for both *A. actinomycetemcomitans* and *P. gingivalis*.

Discussion

The results of the present study have demonstrated that PMA at 100 μM was able to prevent qPCR amplification of DNA from dead cells of *A. actinomycetemcomitans* and *P. gingivalis* and, in the case of *P. gingivalis*, at 50 μM PMA. These results were confirmed when the same PMA treatment was applied to viable cells (no effect observed) or to a combination of viable/dead cells.

Successful qPCR quantification was obtained when tested in viable as well as dead bacterial suspensions of both *A. actinomycetemcomitans* and *P. gingivalis*. Cq values derived from viable and isopropanol-killed positive controls, subjected to the treatment conditions but without the exposure of PMA, indicated that the qPCR technique quantified DNA from viable cells and from dead cells. This amplification of DNA from dead cells may lead to false positive results when using PCR

Table 2. Effect of propidium monoazide (PMA) treatment on defined proportions of viable and isopropanol-killed *A. actinomycetemcomitans* and *P. gingivalis* cell mixtures

	Cq values [mean value (standard deviation (SD)]									
	Ratio of viable/dead cells (%/%)									
	A. actinomycetemcomintans					*P. gingivalis*				
	100 : 0	75 : 25	50 : 50	25 : 75	0 : 100	100 : 0	75 : 25	50 : 50	25 : 75	0 : 100
Viable/dead cells: Non-PMA-treated cells	20.4 (0.4)	21.3 (1.3)	21.2 (0.6)	21.9 (0.3)	22.9 (0.7)	23.8 (0.6)	24.2 (0.9)	24.6 (0.6)	25.4 (0.6)	28.1 (0.8)
Viable/dead cells: PMA-treated cells	23.0 (0.8)	23.9 (0.5)	24.8 (0.9)	25.5 (0.7)	28.9 (0.2)	25.3 (0.8)	26.5 (0.6)	26.5 (0.8)	26.9 (0.7)	32.7 (1.0)
Limit of detection	29.0 (0.1)	29.0 (0.1)	29.0 (0.1)	29.0 (0.1)	29.0 (0.1)	32.1 (0.4)	32.1 (0.4)	32.1 (0.4)	32.1 (0.4)	32.1 (0.4)

Mixtures were prepared with viable cells representing 100%, 75%, 50%, 25% or 0% of the total bacterial cell concentration (10^6 CFU/mL). A final concentration of 100 μM of PMA was used. The Cq values indicate the mean values of three independent experiments with the standard deviations. NTC ranged between 34.1 (SD = 0.4) for *A. actinomycetemcomitans* and 35.8 (SD = 1.6) for *P. gingivalis*.

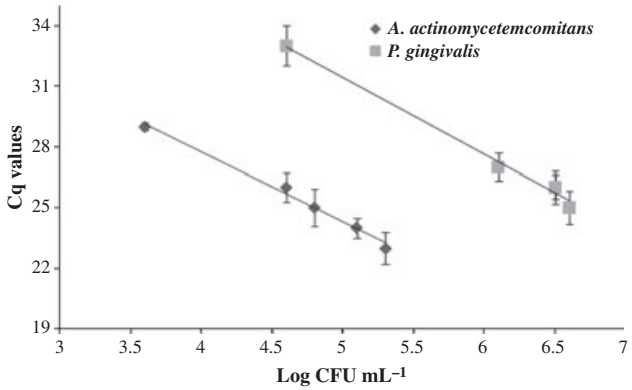

Fig. 2. Relationship between the log colony forming units (CFU)/mL and Cq values from propidium monoazide (PMA) q PCR amplification of *A. actinomycetemcomitans* and *P. gingivalis*. Total number of cells in viable–dead cell mixtures was kept constant at 10^6 CFU/mL. Mixtures were prepared such that viable cells represented 100%, 75%, 50%, 25% or 0% of the total bacterial cell concentration. A final concentration 100 μM of PMA was used. Plotted values are the means and standard deviations derived from three independent assays.

technology and, therefore, the use of molecular methods for detecting and quantifying specific species in mixed bacterial samples must determine whether the DNA amplification results from viable or dead bacteria. This is particularly useful in studies evaluating the pathogenic potential of the target species in relation to the studied pathological process, as well as when determining the efficacy of antimicrobial therapies on biofilms or bacterial communities. Both objectives are important for the specific bacterial species tested in this investigation. *A. actinomycetemcomitans* and *P. gingivalis* are well-recognized periodontal pathogens, strongly associated with periodontitis (36) and, therefore, studies evaluating their etiological role in the initiation and progression of this disease must assure that the number of these species retrieved from subgingival samples represents viable cells. Similarly, studies evaluating the efficacy of different antimicrobial strategies on subgingival biofilms, including targeted strategies against the two mentioned species, must assure that the quantification of bacteria account for differences between viable and dead cells.

Cq values from viable cell suspensions, regardless of PMA treatment, were compared with those obtained from the positive control (dead cells without exposure to PMA). Although Cq values from viable cells suspensions were significantly lower than the obtained from the positive control, only a limited proportion of genomic DNA was destroyed during exposure of *A. actinomycetemcomitans* and *P.gingivalis* cells to isopropanol, providing suitable DNA template for PCR amplification.

In view of the fact that isopropanol can cause destruction of a greater proportion of genomic DNA than other killing methods (16), an alternative method of killing cells, by heat treatment at 95°C for 10 min, was used as the control to ensure that isopropanol did not interfere with the results. For a given Cq value from viable cell suspensions regardless of PMA treatment [25.2 (SD = 0.1) for *A. actinomycetemcomitans* and 24.5 (SD = 0.0) for *P. gingivalis*], the Cq values from positive controls obtained by heat treatment [26.7 (SD = 0.0) for *A. actinomycetemcomitans* and 27.4 (SD = 0.2) for *P. gingivalis*)] demonstrated similar behavior to those obtained by the isopropanol-killing method [26.0 (SD = 0.1) for *A. actinomycetemcomitans* and 26.9 (SD = 0.1) for *P. gingivalis*].

In agreement with previous reports, our results showed that PMA treatment did not significantly inhibit DNA amplification from viable cells, while there was a significant reduction of DNA amplification from dead cells

(2,15,16,19,28,32). For both bacterial species, no statistically significant differences in Cq values and/or log CFU/mL were observed ($p > 0.05$), when the three PMA concentrations were used with viable cells, in comparison with the same cells without PMA treatment. The PMA charge and lack of permeability of this molecule through intact cell membranes are the main reasons for the minimal effect on viable cells (15).

The evaluation of the efficacy of PMA combined with the qPCR reaction on mixed (viable/dead) bacterial suspensions, at different proportions, resulted in a linear relationship between Cq values and number of viable cells, demonstrating an increase in Cq values with the decreasing number of viable bacteria. This result further demonstrates that PMA is effective in selectively allowing PCR amplification of DNA from viable cells, also in the presence of DNA from dead cells. These results are consistent with those described in previous studies using the same methodology, when applied in different bacterial species (2,19,25–30). These authors also reported a successful inhibition of qPCR amplification of DNA in the presence of dead cells. There are few reports about this technology being applied to the study of oral bacteria (28,29). Recently, a similar report comparing the effect of two DNA-intercalating dyes (etidium monoazide and PMA) on three oral bacteria, including *A. actinomycetemcomitans* (28), was published. Our investigation provides additional insight into the use of this method for quantifying viable *P. gingivalis* and *A. actinomycetemcomitans*. Their results showed a lesser initial reduction in the qPCR signal derived from dead cells treated with PMA than we have reported in this investigation. This fact was attributed to the amplicon size used (82 bp) (28,32), achieving better results when expanded the amplicon size to 200 bp. Because amplicon sizes larger than 150 bp are not approved for the qPCR technique, in our investigation we have successfully used an amplicon of 80 bp, and hypothesized that the improved results obtained using this methodology with *A. actinomycetemcomitans* might be due to the assay conditions, rather than the size of amplicons. The tight control of temperature, time of incubation, time of light exposure, amplification efficiency of the qPCR assay and quantity of unspecific background DNA in the reaction might be crucial for attaining improved results. Moreover, we have proven in this study that an amplicon of 80 bp was sufficient to demonstrate a positive PMA effect on the dead cells. In addition, we have studied (data not shown) the effect of PMA on purified DNA from *A. actinomycetemcomitans* and *P. gingivalis*, and inhibition of the DNA-qPCR amplification was complete.

To determine optimal assay conditions for allowing the penetration of PMA through membrane-compromised *A. actinomycetemcomitans* and *P. gingivalis* cells, bacterial suspensions were subjected to different temperatures and different periods of cross-linking and light exposure (data not shown). In an initial set of experiments, 100 μM PMA was selected as the final concentration and added to 250 μL culture aliquots, following an incubation period from 1 to 10 min in the dark at 4°C and ambient temperature. After that, samples were subjected to cross-linking of PMA by exposing them to a halogen light source of 550 W placed 20 cm above the samples for 5–20 min. An incubation time of 10 min at 4°C in the dark with PMA and an exposure of 20 min to the light source proved the most effective conditions for these bacteria. A concentration of 10^6 CFU/mL was selected for validating the method, as previous experiments performed using this methodology in a subgingival biofilm model development by our research group (23), used the selected bacteria at this concentration. This methodology will allow us not only to study *A. actinomycetemcomitans* and *P. gingivalis* dynamics within the biofilm, but also to assess the efficacy of different antiseptic compounds on these pathogens within this biofilm model.

The bacterial detection within the range of 10^3–10^4 CFU/mL was limited by contamination from bacterial DNA. This unspecific signal, conceivably conferred by the Taq DNA polymerase and PCR reagents, has been previously reported (19,31). Measures to avoid carryover DNA have been established; for example, separation of pre- and post-PCR laboratory area, use of flow cabin, barrier tips or disposable gloves. None of these methods, however, proved very effective in reducing copy numbers of bacterial ribosomal DNA in the PCR reagents. Although Cq of 40 are theoretically available for the reaction, contamination of reagents with an non-specific DNA background manifest in the NTCs, restricted the sensitivity of the reaction to Cq values between 34.1 (SD = 0.4) for *A. actinomycetemcomitans* and 35.8 (SD = 1.6) for *P. gingivalis* (Table 1). This fact may be responsible for dead cell suspensions demonstrating a quantifiable value using qPCR when combined with PMA. In this investigation, cell death was confirmed in all cases by culture methods, although it is conceivable that in some cases, these quantifiable values from dead cell suspensions were due to an insufficient capacity of isopropanol treatment to kill all the cells, which may have remained viable but not cultivable.

In conclusion, this method of PMA treatment of mixed bacterial suspensions followed by q-PCR analysis, has demonstrated its efficacy for rapid detection and quantification of viable *A. actinomycetemcomitans* and *P. gingivalis*, as well as its efficacy for preventing DNA amplification from the dead cells. The results have demonstrated the potential of this methodology (PMA combined with qPCR) for differentiating viable and dead *A. actinomycetemcomitans* and *P. gingivalis* in oral samples.

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