Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies

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SUMMARY

DNA-based methodology for the identification and detection of specific bacteria in dental plaque offers advantages over culturing techniques. One drawback of current molecular techniques like real-time quantitative polymerase chain reaction (RT-QPCR) is that they are not able to distinquish between live or dead bacteria. To overcome this problem an assay was assessed to discriminate between viable or dead bacteria using DNA intercalating substances, propidium monoazide (PMA) and ethidium monoazide (EMA) in combination with RT-QPCR. The assay was tested on oral pathogens: Streptococcus mutans, Prevotella intermedia and Aggregatibacter actinomycetemcomitans. To determine the effectiveness of EMA and PMA, different concentrations (from 5 to 100 μ g ml⁻¹) of the substances were added to viable or heat-killed suspensions of both organisms (ranging from 10⁸ to 10⁴ colonyforming units ml⁻¹). Afterwards, PMA was tested on mixtures of varying ratios of viable and dead cells. After DNA extraction, RT-QPCR was performed using species-specific primers. Both compounds inhibited PCR amplification from dead cells. The EMA treatment resulted in the largest signal decrease but EMA also inhibited DNA amplification from viable cells. For this reason,

PMA was selected for use in further experiments. It was shown to be efficient in allowing selective PCR detection of only viable cells in mixtures containing both viable and dead cells. The amount of amplified DNA corresponded to the percentage of viable cells in the sample. The developed assay will potentially be useful for assessing bacterial loads remaining after disinfection protocols without interference by nonviable bacteria.

INTRODUCTION

Oral microbiology has been implicated with pathologies such as tooth decay, endodontic infections, gingivitis and periodontitis for a long time (Marsh & Martin, 1999). To assess current and future treatment protocols directed towards oral pathogens, an accurate quantification of viable bacteria is essential. DNA-based methodologies for the detection, identification and quantification of specific bacteria in dental plaque offers advantages over culturing techniques (Sanz *et al.*, 2004). One drawback of quantitative molecular techniques like real-time quantitative polymerase chain reaction (RT-QPCR) is that they are

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not able to distinguish between live and dead bacteria. Bacterial DNA is only slowly degraded after the loss of bacterial viability, so the remaining DNA of already dead bacteria can still act as template DNA during PCR. Consequently, oral pathogens killed by antimicrobial protocols will still be counted when a PCR-based molecular method is used. This may lead to an underestimation of treatment results. A strategy to circumvent this problem focuses on the presence of rapidly degrading RNA instead of DNA. However, working with RNA is more technically demanding and RNA molecules are merely an indication of bacterial activity and not of abundance.

Membrane integrity has been a well-established characteristic to discriminate between viable and dead bacterial cells. This characteristic is frequently used in the domains of microscopy and flow cytometry with live/dead staining using membrane-impermeable or permeant dyes, like propidium iodide, to discriminate between living and dead cells (Boulos et al., 1999; Wang et al., 2010). Nogva et al. (2003) were the first to introduce a similar concept in RT-QPCR procedures to differentiate between viable and dead cells. Ethidium monoazide (EMA) is a DNA/RNA intercalating substance, which only enters bacterial cells with compromised cell walls and cell membranes. Following photoactivation it irreversibly cross-links to the nucleic acids, by converting the azide group into a highly reactive nitrine radical that can form a covalent link to DNA. DNA covalently bound to EMA cannot be PCR amplified. Hence, when EMA is applied before DNA extraction, only DNA from viable cells can be amplified after DNA extraction. Unbound EMA that is left is simultaneously inactivated by reacting with water molecules in the solution, forming a hydroxylamine that is no longer capable of linking to DNA (Nocker & Camper, 2009). RT-QPCR EMA treatment has been tested on various species including Escherichia coli 0157:H7, Salmonella typhimurium, Listeria monocytogenes and Campylobacter jejuni, with promising results (Nogva et al., 2003; Rudi et al., 2005). However, it was shown that EMA is also capable of penetrating viable cells of certain bacterial species resulting in partial DNA loss from viable bacteria (Flekna et al., 2007; Kobayashi et al., 2009). Therefore, the focus was shifted to an alternative chemical, propidium monoazide (PMA), which does not penetrate cells with an intact cell membrane. PMA has already been used

successfully not only for selective staining of a wide variety of dead cell types but also as a more selective alternative for EMA in vitality QPCR. Research on PMA-driven inhibition of dead cell DNA amplification has been mostly focused on environmental samples (Flekna *et al.*, 2007; Nocker *et al.*, 2007; Wagner *et al.*, 2008; Lin *et al.*, 2010) or respiratory samples (Rogers *et al.*, 2008) with successful results.

The aim of this study was to prove the effectiveness of both EMA and PMA treatments in combination with RT-QPCR on *Prevotella intermedia*, *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*, three pathogenic bacteria implicated in different oral pathologies. The results showed that the combination of EMA or PMA with RT-QPCR could also be a means of distinguishing viable from dead bacteria in oral bacterial samples, thereby facilitating the assessment of antimicrobial protocols against pathogenic oral bacteria. To our knowledge, this methodology has never been explored in the dental field and it could be an added value for scientists working in oral microbial research.

METHODS

Strains and culture conditions

Prevotella intermedia ATCC 25611, Streptococcus mutans ATCC 25175, and Aggregatibacter actinomycetemcomitans ATCC 43718 were used as model organisms, so that both gram-positive and gram-negative bacteria were represented. Bacterial strains were grown on blood agar plates (Oxoid, Basingstoke, UK) supplemented with 5 μ g ml⁻¹ hemin (Sigma Chemical Co, St. Louis, MO), 1 µg ml⁻¹ menadione and 5% sterile horse blood (Biotrading, Keerbergen, Belgium). Bacteria were collected from blood agar plates and transferred to 10 ml brain-heart Infusion broth (Difco Laboratories, Detroit, MI). Prevotella intermedia was incubated overnight at 37°C in an anaerobic atmosphere, A. actinomycetemcomitans and S. mutans were both incubated overnight at 37°C in a 5% CO2 environment. The bacterial concentration was adjusted by measuring optical density at 600 nm to obtain bacterial suspensions with concentrations ranging from 1×10^8 to 1×10^3 colonyforming units (CFU) mI^{-1} .

Stress conditions

A heat-killing protocol was followed to kill cells before EMA or PMA treatment (Talaro, 2007). To determine the most effective heat-killing protocol, bacterial suspensions underwent heat treatment at different temperatures and for different periods of time. Absence of viability was checked by microbial culturing. Both *A. actinomycetemcomitans* and *P. intermedia* suspensions were killed after 15 min in a heating block at 95°C. For *S. mutans*, viability was lost after 30 min at 95°C.

Mixtures of viable and dead cells

Next to heat-killed solutions of the selected bacteria, mixtures were made consisting of heat-killed cells and viable cells of the same bacterium. Mixtures were prepared with equal volumes of each cell suspension using different concentrations of viable bacteria $(10^3-10^8 \text{ CFU ml}^{-1})$ or dead bacteria $(10^3-10^8 \text{ CFU ml}^{-1})$. A mixture containing only viable cells was used as the positive control.

EMA/PMA cross-linking

The PMA and EMA were purchased from Biotium (Hayward, CA). Both compounds were dissolved in 20% dimethylsulfoxide to produce stock concentrations of 1 mg ml⁻¹, down to 50 μ g ml⁻¹. These were stored at -20° C in the dark. Solutions of cross-linkers (10 μ l) were added to 90- μ l culture aliquots to obtain final concentrations of the compounds ranging from 5 to 100 μ g ml⁻¹. Following a 5-min incubation in the dark, samples were exposed for 10 min to a 650 W halogen light source placed 20 cm above the samples. The samples were kept on ice during this period, to avoid excess heating. Both EMA and PMA were handled as potential carcinogens.

DNA extraction and QPCR

The DNA was extracted from bacterial samples using a DNeasy Tissue kit (Qiagen Ltd., VenIo, the Netherlands) in accordance with the manufacturer's instructions. The amounts and quality of extracted DNA were estimated by electrophoresis through 1% agarose gel. A QPCR assay was performed with a CFX96 Real-Time System (BioRad, Hercules, CA). The Tagman 5' nuclease assay PCR method was used for detection and guantification of bacterial DNA. Primers and probes were targeted against the 16S rRNA gene for both P. intermedia [forward (F): 5'-CGGTCTGTTAAGCGTGTTGTG-3', reverse (R): 5'-CACCATGAATTCCGCATACG-3', probe: 5'-TGG CGGACTTGAGTGCACGC-3'] and A. actinomycetemcomitans (F: 5'-GAACCTTACCTACTCTTGACA TCCGAA-3', R: 5'-TGCAGCACCTGTCTCAAAGC-3', Probe: 5'-AGAACTCAGAGATGGGTTTGTGCCTTA GGG-3'). For S. mutans (F: 5'-GCCTACAGCTCAGA GATGCTATTCT-3', R: 5'-GCCATACACCACTCAT GAATTGA-3', Probe: 5'-TGGAAATGACGGTCGCC GTTATGAA-3') the construction of primers and probe was based upon the glucosyltransferase B (gtfB) gene. Taqman reactions contained 12.5 µl Mastermix (Eurogentec, Seraing, Belgium), 4.5 µl sterile H₂O, 1 μ l of each primer and probe and 5 μ l template DNA. Primers and probes were used at different concentrations depending on the organism. Assay conditions for all primer/probe sets consisted of an initial 2 min at 50°C, followed by a denaturation step for 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Quantification was based on a plasmid standard curve.

RESULTS

Effectiveness of EMA and PMA on heat-killed bacterial suspensions

Different concentrations of EMA and PMA (5-100 µg ml⁻¹) were tested on heat-killed suspensions containing 5×10^8 CFU ml⁻¹ of the selected microorganisms. The positive control was a heat-killed suspension of the different bacteria not subjected to treatment. Experiments were repeated three times, as was the RT-QPCR analysis. Figure 1 shows that EMA and PMA both inhibited PCR amplification from dead cells. For P. intermedia and S. mutans an average signal reduction of 3.8 log was achieved when EMA was used, whereas for A. actinomycetemcomitans this was a 3.3 log reduction. The signal reduction was even larger for P. intermedia when PMA was used (5 log reduction), but for S. mutans no difference could be observed between the two compounds. For A. actinomycetemcomitans results



Figure 1 Effect of ethidium monoazide (EMA) and propidium monoazide (PMA) on heat-killed bacterial suspensions: heat-killed bacterial mixes containing 5×10^8 colony-forming units (CFU) ml⁻¹ *Streptococcus mutans, Prevotella intermedia* or *Aggregatibacter ac-tinomycetemcomitans* were subjected to increasing concentrations of EMA (A) or PMA (B). The effects are shown as the log conversion of the number of bacteria per millilitre, the bars indicate the mean values of three independent experiments, and the error bars indicate standard deviations.

indicated a lower effect of PMA corresponding to a reduction of only 2 log.

The largest signal reductions were observed when working with the highest concentrations of the compounds. For both *S. mutans* and *A. actinomycetem-comitans* the difference in effect between 50 and 100 μ g ml⁻¹ of both compounds was small, whereas results for *P. intermedia* illustrated an additional 1–2 log decrease when employing 100 μ g ml⁻¹ EMA or PMA. Because of the larger decreases seen with *S. mutans* and *P. intermedia*, these two bacteria were chosen as the model organisms for subsequent experiments (Fig. 1).

Effect of bacterial concentration

To test whether the compounds had the same effect on different concentrations of heat-killed bacteria, bacterial suspensions were made of S. mutans and *P. intermedia* of 5×10^8 , 5×10^6 and $5 \times$ 10⁴ CFU ml⁻¹. Results of the RT-QPCR were compared with the positive control that did not receive EMA or PMA treatment. For S. mutans both concentrations of the compounds gave similar outcomes. The effects of EMA were slightly better than those of PMA with an average signal reduction of 3 log in comparison with 2.5 log for PMA. However, when the bacterial concentration was 5×10^4 CFU ml⁻¹, the signal reduction only reached 1-2 log. The latter was also observed for heat-killed cells of P. intermedia. For this bacterium the highest concentration gave the highest signal reduction with a log reduction of 4-5 for EMA and 3-4 for PMA. Again, EMA had the best results (Fig. 2).

Effect of EMA and PMA on viable cells

Although the above outlined results illustrated that EMA treatment resulted in the largest signal decrease, it has been reported that EMA can also penetrate viable cells of certain bacteria. Therefore, the effects of both EMA and PMA treatment on viable cultures of P. intermedia and S. mutans were tested and compared with the signal reduction for the corresponding heat-killed suspensions and positive controls (Fig. 3). Results for P. intermedia indicated that EMA inhibited DNA amplification from viable cells by 1-2 log, whereas no effect was seen for PMA. For S. mutans, a small log reduction of 0.5-1.0 log on amplification of viable cells was demonstrated for PMA, and for EMA this reduction reached 2 log (Fig. 3). Because of the strong inhibitive effect of EMA on the amplification of viable cells, all subsequent experiments were conducted with PMA at a concentration of 100 μ g ml⁻¹.

Effects of PMA on mixtures of viable and heat-killed cells

To determine the effectiveness of PMA to selectively detect viable cells, in the presence of dead cells, various mixtures comprising viable and dead cells were evaluated (Table 1). For the treatment to be effective in preventing DNA amplification from dead cells, it G. Loozen et al.



Figure 2 Effect of ethidium monoazide (EMA) and propidium monoazide (PMA) on different concentrations of heat-killed bacterial suspensions: Heat-killed bacterial mixes of *Streptococcus mutans* (A) or *Prevotella intermedia* (B) were subjected to 50 or 100 μ g ml⁻¹ EMA or PMA. The effects are shown as the log conversion of the number of bacteria per millilitre, the bars indicate the mean values of three independent experiments, and the error bars indicate the standard deviations.

would be expected that the amount of amplified DNA would correspond to the percentage of viable cells in the sample. Table 1 demonstrates a linear relationship between the cycle threshold (C_T) value and the number of viable cells, as long as the ratio of dead cells to viable cells was <4 and the concentration of heat-killed bacteria did not reach 1 × 10⁸ CFU ml⁻¹.

Influence of amplicon size

Because of these promising results for *S. mutans* and *P. intermedia*, the question was raised as to why the results gained with *A. actinomycetemcomitans*

(Fig. 1) were less effective. We hypothesized that because the signal decrease observed after EMA or PMA treatment is partly the result of PCR inhibition, the amplicon size might be important for achieving better results. For both *S. mutans* and *P. intermedia*, a fragment of more than 120 base pairs (bp) was generated after RT-QPCR, whereas the amplicon size for *A. actinomycetemcomitans* was only 82 bp. Therefore the differences in signal reduction might be associated with fragment length.

Accordingly, *A. actinomycetemcomitans* samples treated with various concentrations of PMA underwent RT-QPCR with two different sets of primers, amplifying fragments of different lengths. Results showed that an additional signal decrease of 1 log occurred for *A. actinomycetemcomitans* when primers amplifying a larger fragment of the genome were used (Fig. 4).

DISCUSSION

In agreement with previous reports introducing the application of EMA/PMA in combination with RT-QPCR to differentiate between viable and dead bacterial cells, the efficacy of these DNA-intercalating dyes in selecting against DNA from dead cells was confirmed. Both compounds demonstrated their ability to inhibit DNA amplification from dead cells (Figs 1 and 2). However, both EMA and PMA also decreased DNA amplification from viable cells. For PMA this effect was small and could only be demonstrated for *S. mutans* (Fig. 3).

To test the effect of EMA/PMA on amplification of viable cells, overnight cultures of the bacteria were used. It is well accepted that overnight cultures also contain fractions of dead cells. Based on this fact, the log decrease in amplification of viable bacteria that was observed could be attributed to the effect of the compounds on the dead cell fraction. With this in mind, for *P. intermedia*. EMA seemed to be the most effective compound, as the observed log decrease was not observed for PMA (Figs 1 and 2). This is in line with previous studies by Chang et al. (2010) and Chen & Chang (2010), working with Legionella pneumophila, that pointed to EMA as a superior compound. They attributed the lesser effects of PMA to the lower penetration efficiency of PMA compared with EMA for heat-killed cells. However, PMA should be considered as the superior compound for the oral

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Figure 3 Effect of ethidium monoazide (EMA) and propidium monoazide (PMA) on viable cells: Heat-killed and viable bacterial suspensions of *Prevotella intermedia* and *Streptococcus mutans* were subjected to 100 μ g ml⁻¹ PMA (A,C) or EMA (B,D). A comparison was made with positive controls that did not receive PMA or EMA treatment. The effects are shown as the log conversion of the number of bacteria per millilitre, the bars indicate the mean values of three independent experiments, and the error bars indicate the standard deviations.

Dead cell density (log ₁₀ CFU ml ⁻¹)	$C_{\rm T}$ value with a viable cell density of				
	8 log ₁₀ CFU ml ⁻¹	$6 \log_{10} \text{CFU mI}^{-1}$	$4 \log_{10} \text{CFU ml}^{-1}$	$3 \log_{10} \text{CFU ml}^{-1}$	R^2
Prevotella intermedia					
0	18.90 ± 0.67	23.06 ± 0.54	28.97 ± 0.04	30.66 ± 0.31	0.9929
3	18.73 ± 0.03	22.69 ± 0.30	28.99 ± 0.15	30.25 ± 0.91	0.9937
4	18.76 ± 0.54	23.09 ± 0.01	30.06 ± 0.14	32.70 ± 0.78	0.9893
6	18.44 ± 0.32	23.11 ± 0.15	28.47 ± 0.06	28.96 ± 0.30	0.9791
8	18.07 ± 0.57	22.77 ± 0.07	25.79 ± 0.27	27.58 ± 0.31	0.9896
Streptococcus mutans					
0	19.42 ± 0.41	27.53 ± 0.26	33.07 ± 0.92	35.54 ± 0.49	0.9878
3	19.85 ± 0.33	27.54 ± 0.42	32.09 ± 0.15	34.08 ± 0.20	0.9813
4	20.15 ± 0.78	27.81 ± 0.45	33.13 ± 0.44	34.97 ± 0.99	0.9832
6	20.07 ± 0.65	27.79 ± 0.21	31.59 ± 0.43	34.79 ± 0.57	0.9784
8	19.43 ± 0.65	24.68 ± 0.24	27.60 ± 0.68	29.05 ± 0.75	0.9761

Table 1 Propidium monoazide (PMA) assays on varying ratios of viable and dead cells: Mixtures of viable and dead cells of *Prevotella intermedia* and *Streptococcus mutans* were subjected to treatment with 100 μ g ml⁻¹ PMA

Results are displayed as average $C_{\rm T}$ values of three experiments, standard deviations and linear regression coefficients are indicated.

bacteria tested here, based on the results gained for *S. mutans* (Fig. 3C,D). First, a log decrease was seen in PCR amplification levels from viable cells after treatment with PMA. This decrease could be attributed to the effect of PMA on the fraction of dead cells present, and therefore the efficiency of PMA on heat-killed cells can be assured. This effect was not

noticeable for *P. intermedia*. This is presumably because the fraction of dead cells in overnight cultures of *S. mutans* is much higher than the fraction of dead cells in a similar culture of *P. intermedia*.

Second, the log decrease seen after EMA treatment of viable cells was much greater and cannot be attributed solely to the presence of dead cells. It



Figure 4 Influence of amplicon size on effectiveness of propidium monoazide (PMA) treatment: After treatment of a heat-killed suspension of *Aggregatibacter actinomycetemcomitans* $[5 \times 10^8$ colony-forming units ml⁻¹] with different concentrations of PMA, reverse transcription quantitative polymerase chain reaction was performed with two different primer sets amplifying fragments with different lengths, 82 base pairs (bp) (A) and 200 bp (B). The effects are shown as the log conversion of the number of bacteria per millilitre, the bars indicate the mean values of three independent experiments, and the error bars indicate the standard deviations.

seems that EMA had an effect on amplification of viable cells.

These results are in accordance with results from previous studies using EMA (Nocker et al., 2006; Flekna et al., 2007; Wagner et al., 2008; Yáñez et al., 2011). EMA is excluded by viable cells by means of efflux pumps and these pumps can have different substrate specificities according to the specific microorganism. Some bacteria can therefore better exclude EMA than others (Kobayashi et al., 2009). In this way EMA cannot be trusted to solely enter bacteria with decreased membrane integrity. This property may, however, be exploited in the future with regard to cell activity. EMA can also enter dead bacteria that are metabolically inactive and have an intact membrane, whereas PMA will only enter cells that are membrane-compromised. In this regard one can potentially distinguish between the physiological states of bacteria, using PMA as a marker for intact cells and EMA as a marker for active cells.

One possible reason why PMA does not seem to affect viable cells is the higher charge of the PMA molecule and the greater impermeability of the compound through the intact cell membrane (Nocker *et al.*, 2006). Both *P. intermedia* and *S. mutans* responded well to treatment with PMA. The highest concentrations of the compound demonstrated the strongest inhibition of dead cell DNA amplification. The signal decrease observed for *P. intermedia* was superior to the reduction seen for *S. mutans.* When a higher concentration (100 μ g ml⁻¹) of the compound was used, an additional signal decrease was observed for *P. intermedia.* For *S. mutans* no difference in signal reduction was seen between the different concentrations of PMA. Optimal assay conditions such as concentrations of the compounds and light exposure time might depend upon the targeted bacterial species. This may explain why *P. intermedia* had a better response towards the compounds.

When working with mixtures of viable and dead cells in different ratios a linear relationship was illustrated between the $C_{\rm T}$ value and the number of viable cells, $C_{\rm T}$ values decrease with increasing number of viable bacteria. This was also demonstrated by Pan & Breidt (2007) who observed a loss in linearity when the number of viable cells was $<1 \times 10^3$ CFU ml⁻¹. From the results of both studies it can be concluded that the minimum number of DNA copies that are available for RT-QPCR analysis and the number of dead cells in a mixture of viable and dead cells are two factors limiting the range of the assay. For the assay to work efficiently, viable cells should be present in numbers >1 \times 10³ CFU ml⁻¹, and the ratio of dead cells to viable cells should not be >4. For oral microbiology, this limitation should not pose a major problem because the range in which the assay is effective is in accordance with the concentrations of viable bacteria found in clinical samples.

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One way to optimize the PMA assay is to take into account the length of the DNA fragment that will be amplified. As was shown for *A. actinomycetemcomitans*, RT-QPCR targeting a longer DNA fragment (200 bp) resulted in an additional log decrease compared with RT-QPCR amplifying a shorter DNA fragment (82 bp). Soejima *et al.* (2008) demonstrated that high concentrations of EMA led to breaks of double-stranded DNA. So the results seen after RT-QPCR amplification of a longer DNA fragment may be explained by the increase in cleavage sites for PMA in a larger amplicon (Soejima *et al.*, 2007). Hence for a sensitive live/dead RT-QPCR quantification, choice of primers and amplicon length is crucial.

One issue concerning the PMA RT-QPCR concept is that the principle is based on membrane integrity as a viability criterion. In a model described by Nocker & Camper (2009) three viability criteria were dismetabolic cussed: culturability, activity and membrane integrity. In this model, cells that were alive passed all three criteria. The problem lies, however, with intact cells that have already lost detectable respiration and metabolic activity. The speed by which cells lose metabolic activity and membrane integrity depends on the causative agent of cell death. For assessing certain treatment applications it would be desirable to also exclude metabolically inactive cells. In this case PMA treatment could be supplemented with a molecular viability assay based on biological activity. Alternatively, an assay could be set up using EMA in combination with PMA, because EMA is also able to enter membrane-intact bacterial cells.

In summary, this study showed that EMA and PMA are able to inhibit DNA amplification from dead cells. However, as EMA also exerted an effect on amplification of viable cells only PMA could be used to discriminate between the three strains of live or dead oral bacteria. In future the assay needs to be further assessed for a number of different oral bacteria, and its applicability in complex mixed cultures needs to be proven. Furthermore validation studies on clinical samples should be carried out.

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