Insufficient Distinction Between DNA from Viable and Nonviable *Staphylococcus aureus* Cells in Wipe-Samples by Use of Propidium Monoazide-PCR

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Abstract

Treatment of dead bacteria with propidium monoazide (PMA) prior to DNA extraction has been described to inhibit subsequent amplification of DNA by polymerase chain reaction (PCR) methods. As an enforcement authority involved in the surveillance of compliance with biosafety regulations, the State Laboratory of Basel City swabs surfaces to check for contaminations with microorganisms. To evaluate the PMA-PCR method for its use in distinguishing DNA from living and dead bacteria in swab samples, we used Staphylococcus aureus *as a model organism. In this study PMA treatment was able to reduce amplification of DNA from dead bacteria by a factor of 1,000. However, PMA also entered living* S. aureus *to some extent, resulting in a reduction of DNA amplification by a factor of 10. When cells were spotted on a dry surface and collected again by wiping, cells were still viable on agar plates, but a distinction between DNA from living or dead bacteria was no longer possible using the PMA-PCR method. The process of drying and wiping seemed to interfere with the membrane of* S. aureus *resulting in diffusion of PMA into still viable cells. The authors conclude that this method offers a limited capacity to distinguish between living and dead* S. aureus *in liquid culture but does not allow determining the amount of living* S. aureus *cells in wipe-samples.*

Introduction

As an enforcement authority, the State Laboratory of Basel City, Switzerland carries out surveillances of laboratories subjected to the "Swiss Containment Ordinance" (The Federal Authorities of the Swiss Confederation, 1999). We sample for microbial contaminations by swabbing laboratory surfaces and by measuring the amount of organism-specific gene-copies in these samples (Schmidlin et al., 2010). The presence of living bacteria is determined by measuring the increase of gene-copies after cultivation of the sample. However, this method allows detection only of the presence and not measurement of the initial amount of viable bacteria in the wipe-sample. Further, not all microorganisms can be cultivated, and alternative methods to determine living organisms like agar plates are not applicable. Therefore, having a simple method at hand to distinguish between DNA from viable and nonviable microorganisms is desirable.

Combined PMA-treatment and PCR has been described for the gram-positive *Listeria monocytogenes* (Pan & Breidt, 2007; Wagner et al., 2008), *Clostridium perfringens* (Wagner et al., 2008), *Bacillus subtilis* spores (Rawsthorne et al., 2009) and the gram-negative *Salmonella enterica* (Wagner et al., 2008), *Bacteroidales fragilis* (Bae & Wuertz, 2009), and *Enterobacter sakazakii* (Cawthorn & Witthuhn, 2008) as an easy and effective way to distinguish between living and dead bacteria. Due to the increased membrane permeability of dead cells, PMA diffuses into dead cells where it intercalates with DNA. Upon light activation, PMA covalently binds to DNA and inhibits subsequent PCR amplification of this DNA (Hixon et al., 1975).

The utility of this method for routine sampling was tested with the gram-positive *S. aureus,* a frequently handled pathogen in diagnostic laboratories and one of the most common causes of community- and hospital-acquired infections. After evaluation of the optimal reaction conditions, the capability of this method to distinguish between dead and living bacteria was tested in liquid cultures and in swab samples from surfaces.

Materials and Methods

Reagents

PMA™ (Biotium, Inc., Hayward, CA) was kept at -20°C in a 2.5 mM stock solution in 20% dimethyl sulfoxide (DMSO).

Cells

S. aureus (ATCC25923) cells were grown in tryptone soy broth (TSB) (Oxoid, Pratteln, Switzerland) or on blood agar (BA) plates (Columbia agar with sheep blood, Oxoid) under aerobic conditions at 37°C. Overnight cultures were diluted as indicated. Heat inactivation was performed by incubation of the bacteria for 30 minutes at 80°C.

Swabbing

S. aureus cells were placed on an empty plastic Petri dish and air-dried for 4 hours. The dishes were then wiped with a TSB-moistened cotton swab. The samples were placed in TSB growth medium and kept on ice until aliquots for DNA extraction were taken.

PMA Treatment

Unless indicated otherwise, cells were treated with a final PMA concentration of 50 µM on ice for 5 minutes followed by exposure to a light source (300W at approximately 20 cm distance) for 2.5 minutes.

DNA Extraction

DNA was extracted using QIAamp® DNA Mini Kit with the QIAcube™ (both Qiagen, Basel, Switzerland) according to the manufacturer's instructions.

TaqMan® Quantitative Real-time PCR

A specific fragment of the 23S rRNA gene from *S. aureus* (Ludwig & Schleifer, 2000) was amplified using the following conditions: 100 nM primers, 100 nM probe, 1xTaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), and 5 µl DNA were mixed in a 25 µl reaction. DNA was amplified by an initial heating step of 20s at 95°C followed by 45 cycles of 3s at 95°C and 30s at 60°C. Amplification and analysis were performed on the StepOne™Plus System (Applied Biosystems). For quantification, PCR products of the respective sequences were cloned into pGEM-T Vector using the pGEM®-T System II (Promega, Wallisellen, Switzerland), and standard dilutions of plasmids with known gene-copies were amplified. The limit of quantification (LOQ) was determined at 10 copies per PCR reaction ($2x10^3$ copies per ml).

Results

PMA Treatment

To check the effect of PMA on amplification of DNA from *S. aureus* cells, the authors treated living or dead (heat inactivated) cells with PMA or vehicle control. After DNA extraction, the amount of *S. aureus* 23S rRNA gene-copies was measured by quantitative real-time PCR. PMA treatment of the dead cells reduced PCR amplification of DNA significantly but not completely (Figure 1, lanes 5 to 8). Concentrations higher than 50 µM did not increase the effect. On the other hand, amplification of DNA from living cells was already reduced by a concentration of 10 µM PMA (Figure 1, lanes 1 to 4). Treatment of cells with any of the tested PMA concentrations did not affect the number of colony forming units (CFUs) counted on agar plates (data not shown). For additional experiments the authors used a PMA concentration of 50 µM, similar to published experiments with other organisms (Cawthorn & Witthuhn, 2008; Pan & Breidt, 2007; Wagner et al., 2008). These conditions resulted in a 14-fold reduction of amplification of DNA from living bacteria and a 1,616-fold reduction of DNA from dead bacteria (mean values of four independent experiments with a standard deviation of 8 and 759, respectively). Altering the incubation time of the cells on ice with PMA or the light exposure time did not improve the selectivity of the method for dead bacteria.

Detection of DNA from Living Bacteria in the Presence of DNA from Dead Bacteria or Free DNA

A serial dilution of living cells mixed with a constant amount of dead cells was used to simulate the mixed population presumably present in environmental samples. Under these conditions living cells could be identified in the presence of a 100-fold excess of dead bacteria (Figure 2, lanes 1 to 3). However, with a lower ratio of living to dead cells, this method was not sensitive enough to provide accurate information about the presence of living cells in the sample (compare lanes 4 and 5).

The efficiency of PMA treatment to inhibit amplification of free DNA was similar to the efficiency obtained with mixtures of living and dead cells. Again, a serial dilution of living cells was mixed with constant concentrations of, this time, free plasmid DNA containing the 23S rRNA sequence. A maximal reduction of the amplification of free DNA by a factor of 2,000 was achieved (Figure 3, compare light and dark grey bars in lane 5). Again, amplification of DNA coming from living bacteria was slightly reduced (Figure 3, lanes 6 and 7). If the free DNA was present in an excess of less than 200-fold, amplification was sufficiently impaired by the PMA treatment to reveal the presence of living microorganisms (dark grey bars lanes 6 to 10). However, a higher excess of free DNA did cover living bacteria in the mixture (lanes 3 to 5).

Wipe Test

To test whether this method is applicable to swab samples from laboratory surfaces, the authors simulated the process of sampling by spreading heat-inactivated and/or untreated *S. aureus* cells in a Petri dish, letting the liquid dry for 4 hours before wiping the "contaminated" area with a moist cotton swab according to routine procedure. The sample cells were treated with PMA, and 23S rRNA gene-copies were measured in the DNA extracts as described above. Surprisingly, PMA treatment similarly reduced amplification of DNA from dead cells as from living cells (Figure 4), although the process of drying and wiping did not affect the viability of the cells (confirmed by CFU counting, data not shown). A distinction between heat-inactivated and living cells was not possible any longer after wiping.

Discussion

In this study PMA treatment did reduce amplification of DNA extracted from dead *S. aureus* cells by a factor of approximately 1,000. This corresponds to a ΔC_t-value of 10 in the quantitative real-time PCR reaction. A reduction of the C_t-value of 8 with DNA from dead *L. monocytogenes* has been described (Pan & Breidt, 2007). Other publications (Cawthorn & Witthuhn, 2008; Pan & Breidt, 2007; Wagner et al., 2008) report similar efficiencies of the PMA treatment. Therefore, a complete inhibition of PCR amplification of DNA from dead cells could not be expected.

Figure 1

Distinction between dead and living cells: Overnight cultures of *S. aureus* were either heat-inactivated (30 minutes, 80°C; lanes 5-8) or left untreated (lanes 1-4) before addition of PMA. The amount of *S. aureus* 23S rRNA gene-copies was measured by quantitative real-time PCR. The grey area represents the limit of quantification (LOQ). A representative experiment is shown.

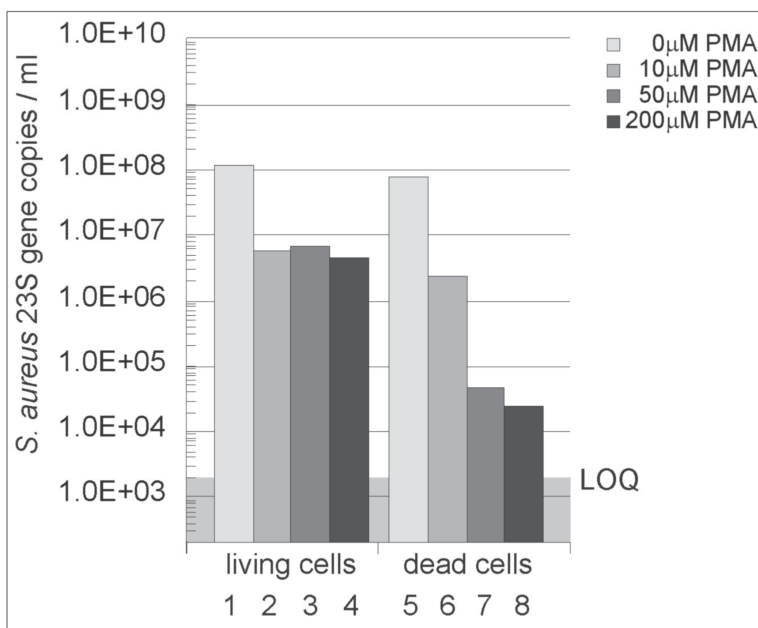


Figure 2

Mixture of dead and living cells: A constant amount of heat-inactivated cells from an *S. aureus* overnight culture were mixed with indicated dilutions of untreated cells. PMA (dark bars) or DMSO (light bars) was added and the amount of *S. aureus* 23S rRNA gene-copies was measured by quantitative real-time PCR. The grey box represents the limit of quantification (LOQ). Error bars represent the standard deviation of two independent experiments.

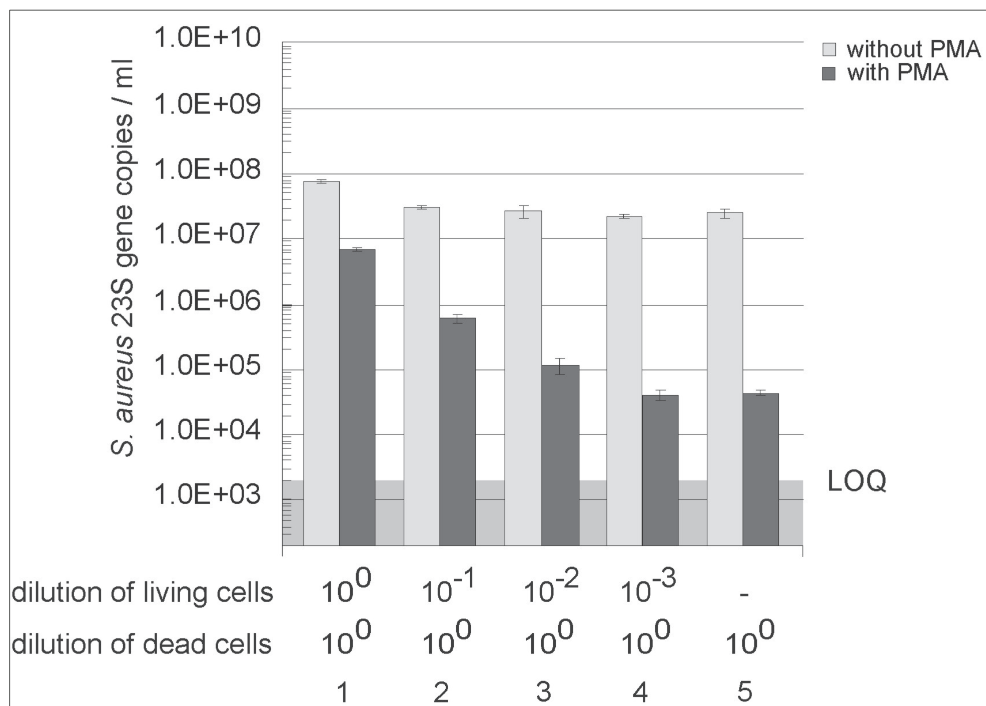
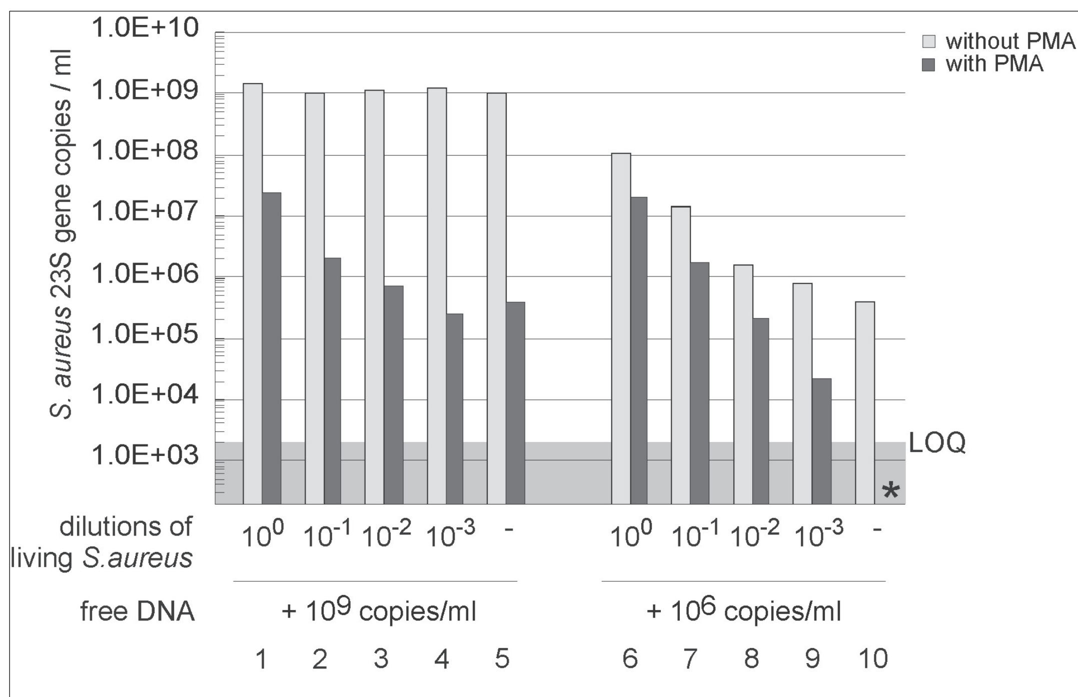


Figure 3

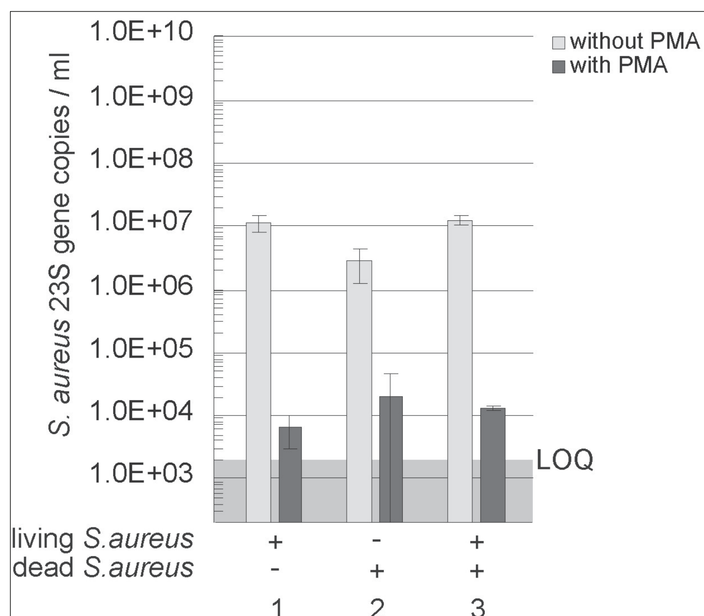
Mixture of free DNA and living cells: Free plasmid DNA containing the 23S rRNA gene sequence and serial dilutions of *S. aureus* overnight cultures were mixed as indicated and treated with PMA (dark bars) or DMSO (light bars). The amount of *S. aureus* 23S rRNA gene-copies was measured by quantitative real-time PCR. The grey box represents the limit of quantification (LOQ).



(* = not detected)

Figure 4

Wipe samples: Either heat-inactivated, viable, or mixed *S. aureus* samples were streaked on Petri dishes. After 4 hours the dried samples were wiped and resuspended in culture medium. Then the samples were treated with PMA (dark bars) or DMSO (light bars). The amount of *S. aureus* 23S rRNA gene-copies was measured by quantitative real-time PCR. The grey box represents the limit of quantification (LOQ). The error bars represent the standard deviation of two independent experiments.



Although no effect of PMA on living *L. monocytogenes* has been found (Pan & Breidt, 2007), in this study amplification of DNA from living cells is reduced by a factor of 10 by the applied treatment. This effect cannot be attributed to the toxicity of PMA, as the treatment does not affect the cell count on agar. Further, the values obtained by counting the CFUs correspond to the number of 23S rRNA gene-copies measured by quantitative real-time PCR. A 10-fold reduction, therefore, cannot be explained by elimination of free DNA in the culture. The results suggest that PMA is able to penetrate the cell membrane of living yet stressed *S. aureus* bacteria to some extent. This effect may well be organism-specific.

With the efficiency reported here, populations of living bacteria can be detected in the presence of a 100- to 200-fold excess of dead cells or free DNA in liquid cultures. This experiment shows that with a limit of quantification of the PCR reaction of $2x10^3$ 23S rRNA gene-copies per ml, significant contaminations of living organisms can be detected in a background of less than 200,000 gene-copies from dead bacteria.

However, DNA from living *S. aureus* cells could no longer be distinguished from DNA extracted out of dead cells after wiping, although previous experiments have shown that at least 50% of the cells can be recovered by wiping and that the drying process does not significantly affect the viability of the cells. The process of drying and wiping may disturb the cells' membrane integrity without affecting their viability. In that case PMA would be able to enter cells that still form colonies on BA plates as well as dead cells. An additional cultivation step before PMA treatment might help the cells to recover, however, the initial number of living cells could no longer be determined.

Investigations using ethidium monoazide (EMA), a compound closely related to PMA, also showed that viable cells allow diffusion of EMA into the cell to some extent (Nocker et al., 2006). Most living cells are able to actively pump this compound out of the cell, not allowing EMA to intercalate with DNA. Therefore, it might be possible that PMA is actively exported from living bacteria, whereas dried and growth-arrested bacteria cannot perform this export, leading to an accumulation of PMA in cells that are still viable. However, Nocker and colleagues (Nocker et al., 2006) showed that PMA hardly enters living *E. coli* cells. Therefore, the increased influx of PMA into air-dried living cells in these experiments is most likely due to an increased membrane permeability rather than decreased export capacity.

PMA treatment is based on the fact that membranes of dead cells are more permeable to the compound than those of living cells. In the case of the *S. aureus* membrane, integrity does not seem to be a reliable marker for cell viability. Recently, Kobayashi and colleagues reported similar problems in distinguishing living from dead *S. aureus* using EMA (Kobayashi et al., 2009). Similarly, Flekna and colleagues reported that EMA-PCR is not able to differentiate sufficiently between live and dead *Campylobacter jejuni* and *Listeria monocytogenes* (Flekna et al., 2007). Further, for *S. enterica* PMA's ability to reduce PCR amplification of DNA from dead cells largely depends on the method of disinfection. Cells can be inactivated without disturbing the permeability of the membrane, for example, by short UV light exposure (Nocker et al., 2007). Effects of the environment on cell membrane integrity vary among different organisms. Thus, membrane permeability is a reliable marker for cell viability only for specific organisms under specific conditions.

Thus, the authors have concluded that this method is problematic for determining the amount of living cells in samples from surfaces in laboratories. This conclusion is based on: 1) diffusion of PMA into living cells (especially after wiping); 2) the fact that PMA does not completely eliminate amplification of DNA from dead cells; and 3) the problem that efficiency of the method depends on the organism and means of inactivation. To prevent any misinterpretation, this method should not be used without conducting a carefully controlled study prior to its application.

Acknowledgment

This project was supported by the Swiss Federal Expert Committee for Biosafety (FECB).

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