Notes & Tips

Sodium chloride affects propidium monoazide action to distinguish viable cells

Valdir C. Barth Jr., Fernanda Cattani, Carlos A.S. Ferreira, Sílvia D. de Oliveira *

Laboratório de Imunologia e Microbiologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre/RS CEP 90619-900, Brazil

ARTICLE INFO

ABSTRACT

Propidium monoazide (PMA) is a DNA-intercalating agent used to selectively detect DNA from viable cells by polymerase chain reaction (PCR). Here, we report that high concentrations (>5%) of sodium chloride (NaCl) prevents PMA from inhibiting DNA amplification from dead cells. Moreover, *Halobacterium salinarum* was unable to maintain cell integrity in solutions containing less than 15% NaCl, indicating that extreme halophilic microorganisms may not resist the concentration range in which PMA fully acts. We conclude that NaCl, but not pH, directly affects the efficiency of PMA treatment, limiting its use for cell viability assessment of halophiles and in hypersaline samples.

A rapid method for estimating viable cells is crucial in several situations, such as food contamination and microbial ecology studies. The adoption of culture-based estimation methods is time-consuming and limited to the portion of biodiversity that is culturable in the laboratory and also is not able to account for cells in a latent state called "viable but nonculturable cells" [1,2]. Therefore, different approaches using molecular biology, such as polymerase chain reaction (PCR),[1] have been used to indirectly detect and quantify microorganisms in environmental, clinical, food, and water samples [3–6]. Nevertheless, PCR also detects DNA from nonviable cells that may persist for days after death, overestimating the actual viable cell number in the sample [7]. To overcome this limitation, a pretreatment with DNA–ligand dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA), has recently been used as a promising way to selectively detect viable cells [8,9]. After nonspecifically intercalating into DNA molecules, these ligands covalently bind to DNA by photoactivation, inhibiting its amplification in a subsequent PCR assay. Both compounds have little ability of crossing intact membranes, which prevents them from entering viable cells, thereby only targeting DNA from membrane-compromised cells. However, due to its higher charge, PMA is less membrane permeant than EMA, making the former much more selective than the latter [10]. This property has led PMA to be used for assessing cell viability in various microbial species, such as *Enterobacter sakazakii*, *Helicobacter pylori*, *Escherichia coli*, *Enterobacter aerogenes*, *Alcaligenes faecalis*, and *Staphylococcus* spp. [11–14].

Still, little is known about how features of the medium/environment could influence PMA's mode of action. Food and environmental samples may have a broad range of pHs and high sodium chloride (NaCl) concentration (e.g., brined foods, hypersaline lakes), which could decrease the performance of dead cell inhibition through PMA. Because there is no description of PMA being applied to halophiles, hypersaline environments, or a variation of pH, this study aimed to evaluate the effect of NaCl and pH on PMA action to determine cell viability using the halophilic archaeum *Halobacterium salinarum*.

H. salinarum (ATCC 19700) was cultured under agitation (180 rpm) as described previously [15]. A 48-h (late exponential phase) culture was stored in 20% glycerol at −20 °C for further use. To evaluate PMA treatment in a halophile, aliquots of 200 μl of *H. salinarum* culture from the late exponential phase, containing approximately 10^8 colony-forming units per milliliter (CFU/ml), were submitted to two inactivating processes: cells were either heated to 100 °C for 15 min or harvested and resuspended in 500 μl of 70% isopropanol for 15 min. After contact with isopropanol, cells were resuspended in the previous sterile medium or TE buffer (10 mM Tris and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). To confirm posttreatment nonviability, 100 μl was spread on an agar surface and incubated at 37 °C for a period of 10 days. Afterward, PMA (Biotium, Hayward, CA, USA) was added to the samples in increasing concentrations, ranging from 10 to 100 μg/ml. The sample tubes were protected from light for 5 min, with regular homogenization, and exposed to a 500-W halogen light for 5 min at a distance of 15 cm from the light source. The samples were laid on ice to avoid excessive heating. After centrifugation, they

* Corresponding author. Fax: +55 5133203568.
E-mail address: silviadias@pucrs.br (S.D. de Oliveira).

[1] *Abbreviations used:* PCR, polymerase chain reaction; EMA, ethidium monoazide; PMA, propidium monoazide; NaCl, sodium chloride; CFU/ml, colony-forming units per milliliter.

were resuspended in TE buffer and submitted to DNA extraction using guanidine isothiocyanate as described elsewhere [16].

To verify the influence of NaCl concentration and pH on PMA treatment, different solutions containing 0, 5, 10, 15, and 20% (w/v) NaCl and pHs 4.0 and 10.0 were prepared in distilled water. An aliquot of 200 μl of each solution was artificially contaminated with either *H. salinarum* genomic DNA or *H. salinarum* heat-inactivated cells, corresponding to a final concentration of 90 μg/ml (estimated by a spectrophotometer) or 10^7 CFU/ml, respectively, incubated at room temperature for 30 min, and submitted to the PMA treatment at a final concentration of 10 μg/ml. The experiments were performed in triplicate. In addition, *H. salinarum* survival was estimated under different osmotic pressure conditions by exposing 500 μl of a late exponential culture to increasing NaCl concentrations (0–20%) for 20 min in triplicate. Afterward, each sample was diluted 10-fold in saline solution (25% NaCl), and 100 μl of each dilution was spread on solid medium surface and incubated for 10 days at 37 °C. Statistical analyses of the resulting CFU/ml were performed using analysis of variance (ANOVA), proceeded by Dunnett's test, comparing the experimental groups with a control group containing 25% NaCl. The significance level (α) was set to 0.01.

The PCR mix was prepared in a final volume of 25 μl to a final concentration of 2 mM $MgCl_2$, 1× *Taq* buffer (10 mM Tris–HCl [pH 8.8 at 25 °C], 50 mM KCl, and 0.08% [v/v] Nonidet P40), 0.8 μM of forward primer (Arch349F: 5′-GYGCASCAGKCGM-GAAW-3′) and reverse primer (Arch806R: 5′-GGACTACVSGGG-TATCTAAT-3′) targeting 16S archaeal ribosomal RNA (rRNA) gene [17], 200 μM of each deoxynucleotide, and 1.5 U of *Taq* DNA polymerase. PCR underwent an initial denaturing step at 95 °C for 5 min, 35 cycles comprising a denaturing step at 94 °C for 30 s, an annealing step at 56 °C for 45 s, and an extension step at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Primers anneal preferentially to Archaea 16S ribosomal DNA (rDNA) sequences, resulting in a 420-bp fragment for *H. salinarum*.

Both isopropanol and heat treatments resulted in the absence of colony formation after 10 days of incubation, indicating complete nonviability of *H. salinarum* cells. Still, even the highest tested concentration of PMA (100 μg/ml) was unable to completely suppress detection of DNA from the inactivated samples in PCR when the PMA protocol was applied in the cultivation medium containing 25% NaCl. Nevertheless, resuspending these same inactivated samples in low-salt solutions (e.g., TE buffer) prior to PMA treatment resulted in no amplification, even in the lowest PMA concentration tested (10 μg/ml). For that reason, the same protocol was applied in different concentrations of NaCl to verify whether this salt was responsible for the treatment failure. As expected, a negative correlation between concentration of NaCl and PMA inhibition property could be observed. Concentrations of 5% NaCl and above seemed to limit the inhibition caused by PMA to dead cells' DNA. The same outcome was verified when PMA was used directly on genomic DNA in the presence of NaCl at concentrations of 5, 10, 15, and 20% (Fig. 1). On the other hand, very different concentrations of hydrogen ions (pHs 4.0 and 10.0) did not affect PMA action.

Moreover, the culture showed direct dependence on high amounts of NaCl to maintain its full integrity. It was observed that there was a significant ($P < 0.01$) reduction of 1 log in cell counts when the concentration was dropped from 25 to 15% NaCl, becoming entirely nonculturable at 5% NaCl (Table 1). The concentration in which *H. salinarum* culture started to lose viability was below the maximum concentration allowed to use PMA; consequently, we were unable to test PMA toxicity in a viable culture of this microorganism. PMA has already been used in combination with a variety of samples to exclude DNA from membrane-compromised cells. Some studies, however, report the inability to completely remove dead cells' DNA from PCR detection, even though
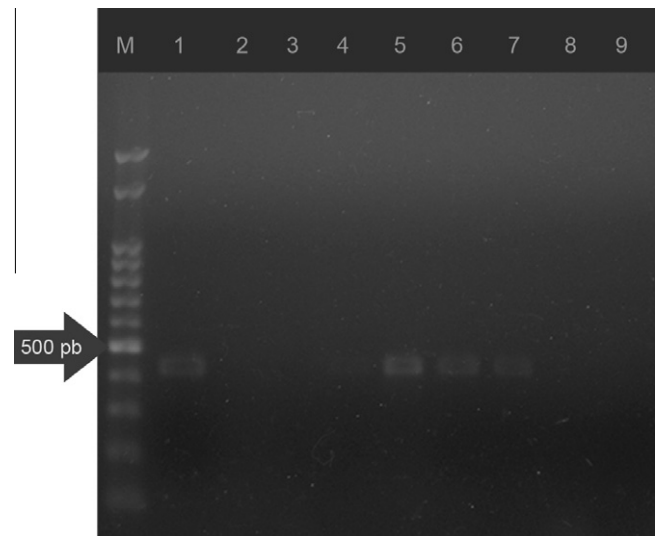

Fig.1. Amplification of genomic DNA from *H. salinarum* after PMA treatment in the presence of pH 4.0, pH 10.0, and different concentrations of NaCl. M: 100-bp DNA ladder; lane 1: positive control (no PMA treatment); lane 2: negative control; lanes 3 to 7: 0, 5, 10, 15, and 20% NaCl, respectively; lane 8: pH 4.0; lane 9: pH 10.0.

Table 1
Effect of NaCl concentration on *H. salinarum* survival.

NaCl concentration (%)	Cell count[a] (log CFU ml^{-1})
0	0[*]
5	0[*]
10	1.93 ± 0.10[*]
15	7.44 ± 0.11[*]
20	8.35 ± 0.11
Control	8.56 ± 0.11

[a] Means of triplicates ± standard deviations.
[*] Statistically different from control group ($P < 0.01$).

cell death is observed by the loss of cultivability in the adequate culture medium or by fluorescence microscopy [18]. Such observations indicate different factors involved in PMA activation and binding other than the membrane integrity. Only few other proposed parameters have been described to determine the general outcome of this technique, such as target length [13,19], inactivation process [20,21], and sample granularity [22], and only the latter considers an intrinsic property of the sample. So far, to our knowledge, it has not been reported whether PMA is applicable to high-salt concentration samples, such as hypersaline lakes, sea water, brined foods, and marine fish samples, or to controlled acidic and alkaline samples. Although pH did not considerably affect amplification yield, NaCl was found to have a direct impact in blocking the amplification of DNA from dead cells. A recent report showed negative effects of high concentrations of NaCl (>4%) and acidic pH (<2.0) on the differentiation of dead cells using EMA in *E. coli* [23]. The pH range tested in this study did not include highly acidic conditions such as pH 2.0, but it showed no difference in the milder acidic pH 4.0. On the other hand, the results observed using 5% NaCl–PMA are in agreement with those reported using 4% NaCl–EMA. Shi et al. [23] proposed that the high osmotic pressure caused by NaCl could dehydrate the cell periphery, altering cell membrane permeability and thereby preventing EMA from accessing its target, even in membrane-compromised cells. However, as could be seen in this study, PMA action seemed to be attenuated even when used directly on extracted DNA, showing little or no influence of the cell membrane on the result. It has been known that, in those conditions, interaction of sodium ions with phos-

phate groups and conformational changes (e.g., supercoiling and transition from B to A and Z forms [24–26]) may occur, possibly lowering PMA affinity to intercalate itself into the bases, thereby failing to mark those molecules. Although no in-depth analysis of PMA efficacy on different DNA structures and topologies has been published, some studies suggest that ethidium bromide (a structurally similar compound) possesses low affinity for Z-DNA formed in ionic environments in vitro [27,28]. Depending on the sample and microorganism under study (e.g., halotolerants), the problem could easily be bypassed by partially or completely removing NaCl. On the other hand, that may be a major concern when studying hypersaline environments and halophilic microorganisms because that procedure could provoke cell lysis by osmotic stress, resulting in loss of viability. Such an effect could be observed in this study using the halophile *H. salinarum*, which presented a strong dependence on high (>15%) NaCl concentration in the medium to survive—much higher than the maximum possible concentration for successful PMA use. Accordingly, previous studies have reported, by other plating-free methods, the inability of this microorganism to replicate in NaCl concentrations lower than 13% (130 g L^{-1}) [29]. This NaCl dependency possibly could be explained by the high prevalence of acidic amino acid residues in its cell wall, making necessary Na$^+$ ions to stabilize its negative charge, avoiding membrane disruption and subsequent cell lysis [29]. Such a metabolic need of halophilic microorganisms sets a limitation in PMA treatment, and ignoring the interference of NaCl on this technique would lead to a considerable overestimation of the viable cell number in the sample. Therefore, we conclude that PMA treatment is not suitable for assessing microbial viability in cases involving halophiles and hypersaline environmental samples because lowering the salt concentration could induce halophilic microorganisms to undergo cell lysis, resulting in an underestimation of the viable microbial community.

Acknowledgment

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