International Journal of Biological Research, 5 (2) (2017) 59-62

International Journal of Biological Research

Website: www.sciencepubco.com/index.php/IJBR
doi: 10.14419/ijbr.v5i2.8561
Research paper



Determination of viability in foodborne bacteria with inter-calating dyes: ethidium monoazide (EMA) and propidium monoazide (PMA)

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Abstract

The ability to distinguish between living and dead cells is considered to be very important for biological researches. It is an important problem that the technology used up to day does not allow the quantitative differentiation of specific cells in a mixed cell community. Determination of whether the microorganisms present in the foods are in a viable form is an important phenomenon in determining the disease-forming potential.
It is a fact that DNA, which is found in cells that lose their viability, can maintain its activity for a long time. Discrimination of live-dead cell occurs when the intercalating dye is covalently bound to DNA that is cleaved in the dead cell where membrane integrity is impaired. The formation of the covalent bond is activated by photoactivation. Inter-collating dyes only affect dead cells that are damaged by cell wall or membrane integrity. Due to the covalent binding of the inter-collating dye, DNA amplification cannot occur in PCR and other molecular techniques based on PCR.
Among the non-permeable stains, it is accepted that PI is the most commonly used. PMA is identical to PI and additionally contains azide groups. Azide groups allow PMA to cross-covalently bond with DNA in bright light. Another inter-collating dyes with an azide group is ethidium mono azide (EMA).The The PMA molecule provides a higher selectivity on discrimination of live-dead cells by virtue of its' higher charge when compared to EMA. Many researchers have combined EMA and PMA with PCR, Real-time PCR and LAMP in order to differentiate the live population of bacterial, viral, fungal and parasitic food-borne pathogens because they are claimed to be more successful in complex samples than in fluorescence based techniques.

Keywords: *Ethidium Monoazide (EMA); Foodborne Bacterial Pathogens; Intercalating Dyes; Propidium Monoazide (PMA); Viability.*

1. Introduction

The ability to distinguish between living and dead cells is considered very important for biological researches. It is an important problem that the technology used up to day does not allow the quantitative differentiation of specific cells in a mixed cell community. Determination of whether the microorganisms present in the foods are in a viable form is an important phenomenon in determining the disease-forming potential.
It is known that there are many molecules with the ability to chelate DNA and RNA bases. Among them, covalently attached, molecules are considered to be more advantageous than non-covalent molecules. Covalent binding molecules provide selective attachment to the nucleic acid. The binding sites of the covalently linked molecules and the changes in the region to which they are attached can be detected by sequencing methods. Psoralen and its derivatives from these molecules are described as substances that are photo chemically linked to nucleic acids and are considered useful in the studies of nucleic acid structure. Azide derivatives are also other group of molecules with similar properties. (Vladescu 2007, Biebricher 2015).
One of the earliest studies of ethidium bromide in azide groups belongs to Hixon et al (1975). Besides this, Bolton and Kearns (1978) observed for the first time that ethidium bromide is covalently linked photochemically to nucleic acids, while at the same time stimulating DNA repair mechanisms in human lymphocytes.
DNA-chelating dyes to have the property of changing the structure and mechanical properties of DNA. This structural change can complicate the enzymatic reactions in DNA. The complexity in this way is considered to be necessary to the functioning of chelating agents even though it is an unwanted condition in biochemical events. (Vladescu 2007, Biebricher 2015).
Discrimination of live-dead cell occurs when the membrane integrity of the inter-collating dyes is covalently bound to the DNA that is cleaved in the dead cell. The formation of covalent bond is occurred by photoactivation. Inter-collating dyes only react in dead cells that are damaged by cell wall or membrane integrity. Due to the covalent binding of the inter-collating dye, DNA amplification cannot occur and as a result of this. Detection is impossible in PCR and other molecular techniques based on PCR(Rudi et al 2005).

2. Ethidium monoazide (EMA)

Bolton and Kearns (1978) stated that the covalent binding of Ethidium Monoazide (EMA) to nucleic acids is a binding exactly the same as ethidium. Researchers have for the first time suggested that the EMA molecule can bind to nucleic acids up to 75% by the action of high-wavelength light. Besides this, they

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declared that the fluorescence of binding of EMA and ethidium to nucleic acids has the same environmental sensitivity indicates that this property of EMA can also be used as fluorescence photoaffinity markers of nucleic acids.

Ethidium monoazide (EMA) was first used by Nogva et al. (2003) to distinguish dead and living cells. The researchers used *E. coli*, *Salmonella* spp., and *L. monocytogenes* as the model microorganisms, and they obtained the dead and live cell suspensions with heat treatment and certain disinfectants at certain doses (96% ethanol, 70% isopropanol, 500 ppm benzalkonium chloride).Rudi Rudi et al. (2005) suggested that EMA-PCR was more successful in larger areas and complex specimens when compared to fluorescent-based standard techniques used to distinguish dead and live microorganisms of C. jejuni.

In later times, many investigators (Regan et al 2003, Rudi et al 2005, Flekna et al 2007, Lu et al 2009, Wang et al 2009, Kobayashi et al 2009, Soejima et al 2011, Wang et al 2012, Wang & Levin 2006, Liu et al 2012) combined the use of EMA with methods such as PCR, Realtime PCR, and LAMP to detect many bacterial (Table 1), viral, fungal and parasitic agents.

3. Propidium monoazide (PMA)

Propidium Monoazide (PMA) is a molecule formed by the chemical modification of Propidium Iodide (PI). The molecule is formed by the addition of an azide group at the phenothiazine ring. The azide groups under this condition are cross-linked under bright light. The similarity to PI provides the PMA with the selectivity of not to bind to live cells.

The photo induced binding of membrane integrity disrupted cells results in the DNA forming an insoluble compound and disassociating with cell debris during extraction procedures. Since DNA in living cells remains intact, DNA-based molecular methods produce a positive reaction (Nocker 2006).

The PMA is exposed to bright light after penetration and is cross-covalently bonded to damaged DNA. (Fig 1). These binding results in loss of amplification, resulting in DNA becoming an insoluble compound and giving a negative results in genomic DNA extraction. In this way, the PMA application allows a highly selective discrimination in a population of dead and living cells (Nocker et al 2006).

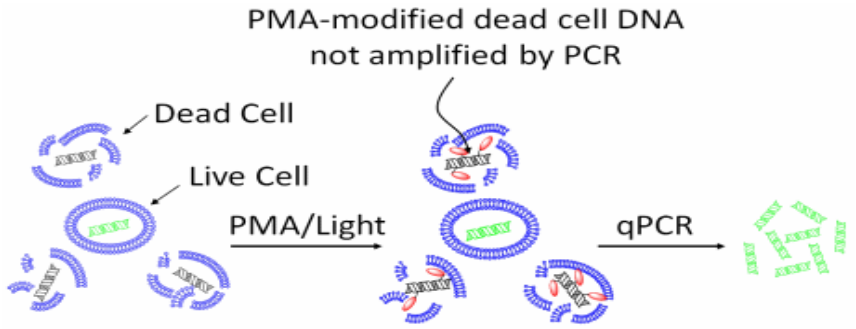


Fig. 1: Inhibition of PCR Amplification in PMA Applied Dead Cells (Nocker Et Al 2006). This Picture is from the Courtesy of Biotium, Inc.

PMA was first demonstrated in *E. coli* 0157:H7, *L. monocytogenes*, *Micrococcus luteus*, *Mycobacterium avium*, *Pseudomonas syringe*, *Salmonella* Typhimurium, *Serratia marcescens*, *S. aureus* and *Streptococcus scombrines* cells by Nocker et al (2006) suggesting that it provides superior selectivity in cells with impaired membrane integrity with high penetration.

In recent years, there have been many studies of PMA used for the determination of viability of food-borne microorganisms from intercalating dyes(Garcia-Cayuela et al 2009, Rawsthorne et al 2009, Josefsen et al 2010, Kralik et al 2010, Taskin et al 2010, Chen et al 2011, Lovdal et al 2011, Elizaquivel et al 2012, Fujimoto & Watanabe 2013, Gensberger et al 2013, Zhang et al 2014, Banihashemi et al 2015).

Garcia-Cayuela et al (2009) studied the quantitative evaluation of live cells by PMA-qPCR in mixed cultures of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei* subsp. *casei*, *Streptococcus thermophilus*) and *Bifidobacterium lactis* in fermented dairy products. In the study decrease, of live microorganism was observed with PMA-qPCR and classical cultural method in fermented milk stored at 4 ° C.The results of the two methods were found similar and linear correlations were observed (0.995).The The researchers declared that the PMA-real-time PCR method was more advantageous in terms of yielding in as little as three hours than the classic cultural methodology that requires a three-day period to obtain results.

Rawsthorne et al (2009) used PMA as an intercalator in their study aimed at molecular detection of *Bacillus subtilis* spores in vivo and inactive form. The researchers found that the results of PMA-qPCR and classical method, which identify the survivors of microorganisms that applied thermal inactivation stress, were consistent and statistically different from real-time PCR results without PMA.

Chen et al (2011) used PMA and LAMP methods in combination to determine the number of live *Salmonella* spp cells. In experiments in which melon, spinach and tomato were experimentally contaminated, inactivation of the microorganism was confirmed by PMA-LAMP method in suspensions containing microorganisms inactivated by heat treatment at concentrations up to 10^8 CFU / ml. The researchers found that PMA-LAMP had similar results with PMA-qPCR, besides these 100 times more sensitive than PMA-PCR.

In another study Elizaquivel et al (2012) wanted to detect live *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* cells in a mixed suspension. Researchers have primarily identified optimal concentrations of PMA and reagent D compounds which they have combined with qPCR. In the study, both reagents were found to provide a similar decrease in the number of pathogens detected, but in the rest of the study only PMA was applied to these microorganisms as reagent D was found toxic to the *Salmonella* and *L. monocytogenes* cells. The researchers have determined the optimal PMA concentration to be used for viability determination of inoculated microorganisms in spinach and mixed salad as 50 μM, and they claimed that PMA-qPCR was an appropriate method for determining the viable microorganism counts in fresh vegetables.

Zhang et al (2014) used PMA to identify live cells in a multiplex PCR (mPCR) method for the detection of emetic and non-emetic *Bacillus cereus* groups. Limit of Detection (LOD) was determined as $4.0x10^2$ KOB / ml in cultures containing dead microorganisms without PMA, $7.5x10^0$ CFU / ml in cultures containing viable microorganisms and $7.5x10^1$ CFU / ml in live cultures treated with PMA. The LOD value of experimentally contaminated samples was determined as $1.0x10^3$ CFU / g.

Banihashemi et al (2015) compared qPCR and PMA-qPCR methods in order to determine the viability of enteric pathogens in a river used as the drinking water source in Canada's Southern Ontario region. In the study conducted with *Salmonella enterica*, thermophilic *Campylobacter* spp., *E. coli* O157: H7 and *Arcobacter butzleri* microorganisms, the ratio of dead microorganisms detected in river water was found to be very low and the difference between living and total numbers was found to

be only about 0.5 log CFU / ml. The researchers expressed PMA-qPCR as a reliable method for the quantitative determination of live microorganisms in surface waters.

4. Comparison of ethidium monoazide (EMA) with propidium monoazide (PMA)

In the first study of PMA has been applied to distinguish cell viability by Nocker et al (2006), have suggested that PMA is more advantageous because of its higher selectivity compared to EMA. The researchers attributed this to the penetration of EMA into living cells in some bacterial species. Although transport pumps bring EMA out of the cell with the help of metabolic activity in living cells, it still causes a large amount of DNA loss by the action of residual EMA. Because PMA has a higher load than the EMA (Fig 1), it does not pass through the intact cell membrane, which is considered to be an inhibitory effect of DNA loss (Nocker et al 2006). Nocker and Camper (2006) have stated that EMA, which they have tested in *E. coli* O157: H7, causes loss of nearly 60 % of living cells.

Among the non-permeable dyes, it is considered that PI is the most commonly used. PMA, in addition to being identical to PI, it additionally contains azide groups. Azide groups also allow the PMA to cross-covalently bond with DNA in bright light. The PMA molecule has a higher load when compared to EMA (Fig. 2) and has the advantage of being able to stain dead cells with higher selectivity (Nocker 2006).

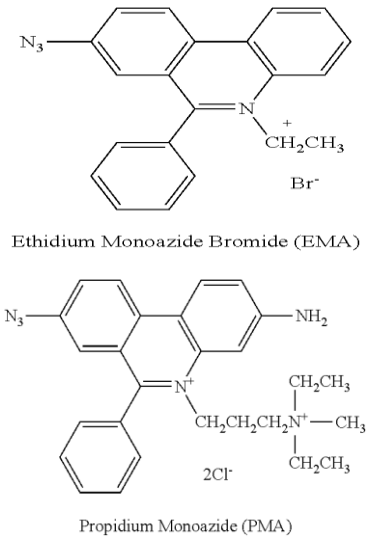


Fig. 2: Molecular Formulas of EMA and PMA (Myron Et Al 2012).

There are a number of studies using EMA and PMA comparatively (Nam et al 2011, Pan and Breidt 2007, Cawthorn and Witthuhn 2008). (Table 1)

Nam et al (2011) used PMA and EMA comparatively in order to distinguish in the suspension of living and dead Helicobacter pylori cells. Researchers have observed that in experiments performed at a concentration of 1-100 μM, PMA causes maximum dead cell detection and minimal viable cell loss at a concentration of 50 μM., On the other hand, it is indicated that penetration of EMA into both living and dead cells led to degradation of genomic DNA of bacteria. As a result, researchers have suggested that PMA should be used instead of EMA for the viability of *H. pylori* cells. Pan and Breidt (2007) analyzed the sensitivity of PMA-qPCR and EMA-qPCR in the detection of live *L. monocytogenes* cells and found that the ratio of dead cell concentration to live cell was less than 10^4 CFU / ml and the viable cell concentration was more than 10^3 CFU / ml. The researchers declared that the use of PMA for viability detection was more appropriate as a result of their findings. Researchers who achieved the same results in suspensions prepared from bacterial broth cultures and biofilms also found that EMA had a higher rate of penetration to live cells as well as dead cells when compared to PMA. Researchers who have set out here have found that the combination of PMA and qPCR is suitable for use in food, medicine and environmental samples. Researchers who have set out here have found that the combination of PMA and qPCR is suitable for use in food, medicine and environmental samples.

Cawthorn and Witthuhn (2008) applied PMA and EMA at different concentrations (10, 50, 100 μg ml^{-1}) for dead and live cell suspension of *Enterobacter sakazakii*. At 50 and 100 μg ml^{-1} concentrations, PMA completely blocked PCR amplification in dead cells. Only live cells were amplified in live / dead cell suspensions at different concentrations of EMA. Researchers have shown that although EMA does have the same effect on PMA in dead cells, it has also an inhibitory effect on amplification of living cells.

Table 1: PMA and EMA Based Studies in Foodborne Bacterial Pathogens

Bacterial Species	Method	Inter-calating Dye	Reference
C. jejuni, L. monocytogenes	qPCR	EMA	Flekna et al 2007
Salmonella spp.	LAMP	EMA	Lu et al 2009
E. coli O157:H7	qPCR	EMA	Wang et al 2009
S.aureus, S.epidermidis	qPCR	EMA	Kobayashi et al 2009
Enterobacteriaceae	PCR	EMA	Soejimaet al 2011
V. parahaemolyticus	LAMP	EMA	Wang et al 2012
H. pylori	qPCR	EMA/PMA	Nam et al 2011
P. aeruginosa, L.monocytogenes, S. Typhimurium, S. marcescens, E. coli O157:H7	DNA Microarray	PMA	Nocker et al 2009
P. aeruginosa	FISH	EMA	Regan et al 2003
V. vulnificus	qPCR	EMA	Wang and Levin 2006
MRSA	Microfluidic Sistem- PCR	EMA	Liu et al 2012
E. coli	qPCR	PMA	Taskin et al 2010
Bifidobacterium bifidum	qPCR	PMA	Fujimoto and Watanabe 2013
E.sakazakii	PCR	EMA/PMA	Cawthorn and Witthuhn 2008
B.subtilis	qPCR	PMA	Rawsthorne et al 2009
Campylobacter spp.	qPCR	PMA	Josefsen et al 2010
L. innocua	qPCR	PMA	Lovdal et al 2011
MAP	qPCR	PMA	Kralik et al 2010
Salmonellae	LAMP	PMA	Chen et al 2011
E. coli O157: H7, *Salmonella L.monocytogenes*	qPCR	PMA	Elizaquivel et al 2012
B. cereus	mPCR	PMA	Zhang et al 2014
E. coli, P. aeruginosa	qPCR	PMA	Gensberger et al 2013
S. enterica, Campylobacter, E.coli O157:H7, *A.butzleri*	qPCR	PMA	Banihashemi et al 2015

FISH: Fluorescent In Situ Hybridization, MRSA: Methicillin-Resistant S.aureus, MAP: Mycobacterium avium subsp. Paratuberculosis, mPCR: Multiplex PCR

5. Conclusion

When considered within the context of food safety, it should be taken into account that VBNC forms as well as live forms of food borne pathogens are also likely to cause disease. Hereby it is considered that differentiating the VBNC form by the inter-collating dyes may provided a more realistic risk analysis of bacterial pathogens. As a conclusion, it is suggested that these and similar methods for determining viability should be used more widely in food microbiology.

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