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RESEARCH NOTE

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Improved Detection of γ-Irradiated *Vibrio vulnificus* after Heat and Cold Shock Treatment by Using Ethidium Monoazide Real-time PCR

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Abstract Gamma (γ)-irradiation can be used to control pathogens such as *Vibrio vulnificus* in seafood. The effects of irradiation on microbial cell populations (%) have been studied in order to develop detection methods for irradiated foods. The method used in this study was ethidium bromide monoazide (EMA) real-time polymerase chain reaction (PCR), using *V. vulnificus* specific primer, EMA, and SYBR® Green to discriminate between γ-irradiated and non-irradiated cells. Confocal microscope examination showed that γ-irradiation damaged portions of the cell membrane, allowing EMA to penetrate cells of irradidated *V. vulnificus*. γ-Irradiation at 1.08 KGy resulted in log reduction (−1.15±0.13 log reduction) in genomic targets derived from EMA real-time PCR. The combination cold/heat shock resulted in the highest (−1.74±0.1 log reduction) discrimination of dead irradiated *V. vulnificus* by EMA real-time PCR.

Keywords: γ-irradiation, ethidium bromide monoazide, real-time polymerase chain reaction (PCR), *Vibrio vulnificus*, cold and heat shock

Introduction

Vibrio vulnificus is a human pathogen that is common to the estuarine environment (1,2). It also has been detected in seafood, such as, clam, fish, etc. Disease is usually associated with such underlying conditions as cirrhosis, diabetes, and hemochromatosis (3). In order to reduce the numbers of pathogens and spoilage microorganisms, decontamination of processed foods is necessary. Attempts have been made to eliminate or reduce the incidence of pathogens by various treatments, such as, the use of food grade chemicals, salts, antibiotics, heat, low temperature and, recently, γ-irradiation (4). Irradiation could make a significant contribution to the reduction of food-borne disease caused by pathogens. γ-Irradiation processing is a physical treatment of food with high-energy ionizing radiation, which causes damage to the membrane and DNA of the cells (5,6). It can be used to prolong the shelf life of food products and to reduce health hazards through destruction of bacteria. Several detection methods have been developed to detect bacteria in irradiated foods. A microbiological method based on the use of the direct equifluorescent filter technique (DEFT) and the conventional aerobic plate count (APC) have been used for the detection of bacteria in irradiated food (7). The DNA comet assay has also been used as a detection method for irradiated foods (5,6). However, these methods are time consuming and are not radiation specific (8). Recently, ethidium bromide monoazide (EMA) real-time polymerase chain reaction (PCR) techniques have significantly improved detection of bacterial pathogens in seafood (9,10). EMA has been used as a DNA binding dye to differentiate viable and dead cells. EMA penetrates only membrane-damaged cells with a compromised membrane/cell wall system; therefore, only DNA from viable cells can be detected. In our approach, specific primers, based on the open-reading frame of the *vvh* gene, conjugated with the fluorescent SYBR® Green dye were used for EMA real-time PCR. *V. vulnificus* was the target organism, while γ-irradiation was the killing treatment. We reported optimal concentration of EMA for discriminating γ-irradiated *V. vulnificus* by using EMA real-time PCR (11).

The purpose of this study was to determine the effectiveness of 2 factors (Na^+ concentration, incubation temperature) for improving discrimination between γ-irradiated and non-irradiated *V. vulnificus* by EMA real-time PCR.

Materials and Methods

Cultivation and harvesting of *V. vulnificus* The strain used in this study was *Vibrio vulnificus* 4062 supplied by C. Amaro and was originally isolated from an European eel. All media were from Difco (Franklin Lakes, NJ, USA). The culture was preserved at 20°C in cooked meat medium supplemented with 1% NaCl. Prior to experimentation, preserved cultures were plated on tryptic soy agar containing 0.25% glucose (TSA^+). One loop of growth was inoculated from the agar plate into a tube containing 10 mL of TSB^+ followed by incubation overnight at 32°C with rotary agitation (148 rpm). The next day 100 μL from the overnight culture was transferred to 7 mL of PNC [5% peptone, 1% NaCl, and 0.08% cellobiose, pH 8.0] in a tube to enhance growth (2). The culture was incubated at 37°C for 3 hr with rotary agitation (260 rpm) until mid-exponential growth phase. Cells were then adjusted to an absorbance of 0.2 with APB [1% peptone and 1% NaCl, pH 8.0] at 600 nm in 1-cm path cuvets. Finally, cell preparations of 1×10^6 CFU/mL were made by the addition of an appropriate amount of APB for each experiment. At the same time, the number of CFU were determined by spread plating 0.1 mL of the cell

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preparation onto triplicate plates of TSA^+ followed by incubation at 37°C for 24 hr.

γ-Irradiation The radiation source was a Cs-157 package irradiator (Gammator 50 Irradiator; Radiation Machinery Corporation, Parsippany, NJ, USA), which produced a dose rate of 0.209 KGy/hr. The dose rate was established using a dosimeter. The temperature during radiation was room temperature. Samples (1×10^6 CFU/mL) were prepared in triplicate tubes for irradiation treatment. The samples were placed in uniform parts of the radiation field and arranged to minimize any differences in the radiation dose. For all experiments a dose level of 1.08 KGy was applied to cell suspensions containing 1×10^6 CFU/mL in a 1% saline solution and suspensions tested immediately after irradiation.

Heat treatment of *V. vulnificus* Microcentrifuge tubes (1.5-mL) containing 1.0 mL of cell suspensions (1×10^6 CFU/mL) in a saline solution were heated at 100°C in a water bath for 5 min. The heat treated tubes were allowed to cool to room temperature and the absence of viable cells determined by smearing 0.3 mL in triplicate onto TSA+ followed by incubation at 37°C for 24 hr.

Confocal laser scanning microscopy EMA was used to stain the *V. vulnificus* for confocal microscopy study. The cells (1×10^6 CFU/mL) were stained with EMA in the dark following γ-irradiation, non-irradiation, heat killed, and a combination of γ-irradiation and cold/heat shock, then samples were subjected to a halogen lamp (500 W) for photolysis. After the cells were collected by centrifugation at 10,000×g for 5 min, and suspended with 10 μL of saline solution, cells were (1×10^8 CFU/mL) mounted on microscope slides. The images were obtained with an immersion oil 60× objective and a 10× eye piece by an inverted light microscope connected to the confocal microscope. Fluorescent images were observed under a confocal microscope (Nikon, Tokyo, Japan). A 543 nm emission detection bandwidth were used to visualize EMA. Original EZ-C1 (Ver 3.3) software was used for image acquisition and processing.

Osmotic effectiveness of NaCl on irradiated *V. vulnificus* by EMA real-time PCR Viable cell suspensions containing 1×10^6 CFU/mL derived from PNC broth culture were transferred to 3 different microcentrifuge tubes. The cells from duplicate microcentrifuge tubes were then gently mixed with varying concentrations of NaCl solutions (1, 3, and 4%) from stock solutions.

The first cell suspension (1 mL, 1% saline) was treated with irradiation for 5 hr (1.08 KGy). The second suspension (1 mL) was used for the control without γ-irradiation. Aliquots of 0.2 mL were removed from both the irradiated and control tubes to new tubes, then 2 μL of EMA stock solution (0.05 μg/μL) was added to each tube. The tubes were then placed in the dark at room temperature for 5 min to allow the EMA to penetrate the dead cells and to bind to the DNA (10). The tubes were then set into crushed ice, with their lids off, and exposed to light from a halogen lamp (500 W) at a distance of 15 cm for 10 min to photolyse the EMA. The EMA was handled as a carcinogen. After photolysis, 200 μL of dH_2O was added to each tube followed by 400 μL of 2× TZ solution and the lysis procedure as described below was followed. A 4 mL lysed solution (1×10^3 CFU) was used for the real-time PCR.

EMA real-time PCR discrimination of γ-irradiated cells by cold and heat shock treatment Cold and heat shock were used in this experiment. After irradiation (1.08 KGy), the first sample was treated using cold shock, the second sample was treated with heat shock, and the last sample was treated with a combination of cold and heat shock. After irradiation, the first and second samples (cells containing 1×10^6 CFU/mL in 1% saline) were immediately exposed to 0°C for 15 min for cold shock and 40°C for 10 min for heat shock. The third sample was subjected to 0°C for 15 min for cold shock, 37°C for 15 min, and 40°C for 10 min for heat shock. The samples were treated with EMA as described above before being subjected to cell lysis and the real-time PCR.

Lysing of bacterial cells Samples were lysed using TZ solution [2% Triton X-100 and 2.5 mg/mL sodium azide in 0.1 M TrisCl buffer at pH 8.0] previously proven to be effective in lysing bacteria (12). The cell suspensions were mixed with equal volumes of double strength TZ (2×) in 1.5 mL microcentrifuge tubes followed by mixing. The samples were lysed by heating (100°C) the tubes in a boiling water bath for 10 min and then cooled to room temperature. The boiled samples were then centrifuged at 10,000×g for 5 min to pellet cell debris. A 4 μL of the crude supernatant was added directly to the real-time PCR mixture.

Real-time PCR The real-time thermocycler used in this study was a DNA Engine Opticon® 2 system (MJ Research Corp., Waltham, MA, USA). The *Vibrio* primers VF and VR were used to amplify a 205-bp segment between nucleotides 786 and 990 of the open reading frame of the *vvh* gene (13). These primers are specific to conserved *V. vulnificus* genomic sequences not present in other prokaryotes. The forward primer and the reverse primer were 5'-TTC-CAA-CTT-CAA-ACC-GAA-CTA-TGA-3' and 5'-ATT-CCA-GTC-GAT-GCG-AAT-ACG-TTG-3', respectively. The real-time PCR mixture consisted of 12.5 μL of 2× iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μL of 1.0 mM forward primer, 1 μL of 1.0 mM reverse primer, 4 μL of DNA template, and 6.5 μL of Milli-Q water (total 25 mL). Each set of samples included a negative control, in which Milli-Q water was substituted for the DNA sample. The iQ™ SYBR® Green Supermix consisted of 0.4 mM of each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, and 20 nM fluorescein. The real-time PCR was performed with initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 25 sec, primer annealing at 60°C for 25 sec, and primer extension at 72°C for 1 min. The fluorescent product was detected at the last step of each cycle.

Data analysis In the real-time PCR a threshold level of emission above the base line is selected and the point at which the amplification plot crosses the threshold level is

defined as the Ct value (14). The Opticon Monitor 2 software automatically calculated Ct values, which were used to determine the percentage of cell survivors after irradiation treatment. Student's *t*-test was used for statistical analysis. A confidence interval at the 99% level ($p<0.01$) was considered in all cases.

Results and Discussion

Effect of EMA staining on *V. vulnificus* using confocal microscope examination Structural changes of the cell wall/membrane can be expected to facilitate the entrance of chemical compounds following temperature or irradiation stress. EMA penetration of membrane damaged *V. vulnificus* was examined by confocal microscopy following γ-irradiation, combination of γ-irradiation, and cold/heat shock, and heat killed. The use of confocal microscopy showed that cells stained red (white or gray spot in this article) with EMA correlated with the dead cells. The white color mixed with the faint gray resulted from dead cells taking up the EMA dye to saturation. The images obtained from viable controls with EMA showed that the dye did not penetrate nearly all the cell membrane (Fig. 1A). However, EMA uptake was efficient in the cells from heat-killed or a combination of γ-irradiation and cold/heat shock treatment (Fig. 1D, 1C). Furthermore, EMA could partly penetrate γ-irradiated cells (Fig. 1B) although the staining seemed to be more efficient in the case of the combination treatment. The results obtained from the use of EMA treatment in conjunction with confocal examination may show whether cell membranes were damaged.

In our study, γ-irradiation results in damage to the cell membrane, suggesting that the radiolysis of dH_2O generates free radicals (15), and these radicals attack not only DNA but also the membrane in cells. Since exact quantification from using confocal microscope examination is difficult, quantitative discrimination was not attempted in this study.

Effectiveness of discrimination of γ-irradiated *V. vulnificus* after γ-irradiation Different species require different doses of irradiation to reach the same degree of inactivation (15). In this case, the D_{10} value for the inactivation of *V. vulnificus* as determined by plate counts (data not shown) was 0.18 KGy. A standard curve was constructed by plotting the number of genomic targets/real-time PCR versus Ct values (data not shown). The standard curve was used to evaluate the effectiveness of discrimination between γ-irradiated and non-irradiated cells throughout subsequent work in this study.

When *V. vulnificus* was plated on TSA+ after γ-irradiation at 1.08 KGy, there was no growth indicating 0% survival rather than 6.1% survival as indicated by the EMA real-time PCR (data not shown). The irradiation damage may have been confined mostly to the DNA, as single strand DNA breaks, with little membrane damage, therefore, the EMA failed to penetrate a portion of the dead cells, resulting in the EMA real-time PCR erroneously indicating viable cells. Another possibility might be that the γ-irradiation dose is insufficient to destroy the target DNA sequences and inhibit the PCR amplification. Therefore, a combination of γ-irradiation and sensitization factors was studied to improve discrimination.

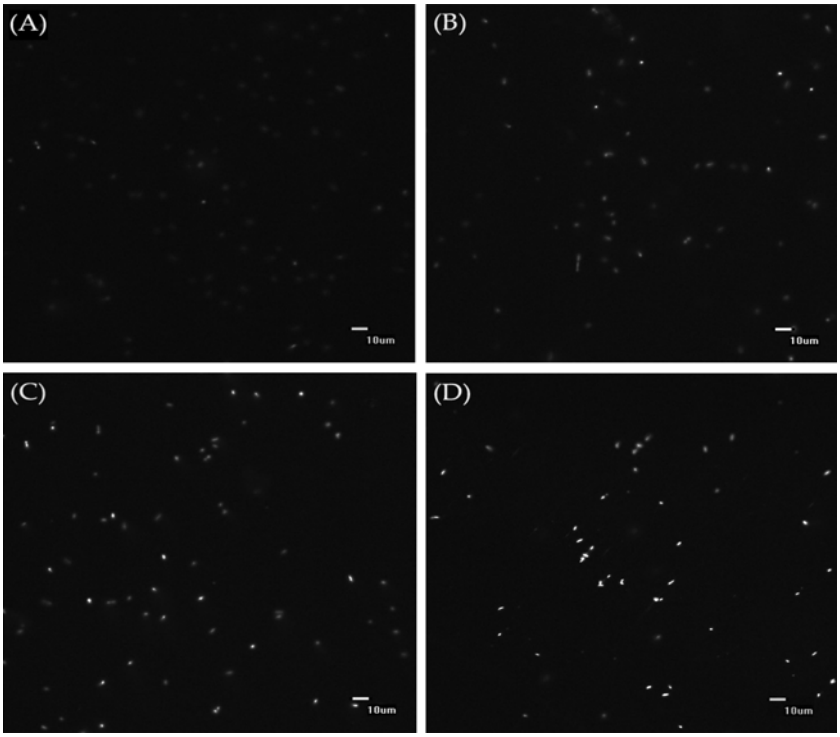

Fig. 1. Confocal microscopic images of *V. vulnificus* stained with EMA. White spots indicate membrane permeability of EMA after various stresses. (A) Non-stressed control cells (ca. 1×10^8 CFU/mL), (B) γ-irradiation, (C) combination of γ-irradiation and cold/heat shock, (D) heat killed cells.

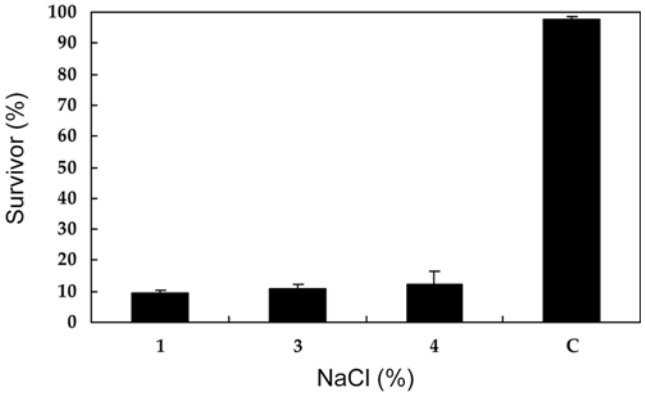

Fig. 2. EMA real-time PCR to determine % discrimination in varying concentrations of NaCl (1, 3, and 4%), and C (control, no irradiation) from *V. vulnificus* irradiated at a dose of 1.08 KGy. Plotted values are the means and SD derived from 3 independent assays.

Osmotic effectiveness of NaCl for discriminating γ-irradiated *V. vulnificus* by the EMA real-time PCR

Chemicals like salt, inorganic ions, organic acids, etc, can be used to reduce the dose required to kill radiation resistant organisms (4). Pre-sensitization with salt (NaCl) treatment followed by irradiation may delay the Ct values since osmosis may affect weak cell membranes during γ-irradiation, allowing EMA to more easily penetrate the cell membranes and inhibit DNA amplification. Treatment with 1, 3, and 4% NaCl plus a 1.08 KGy dose resulted in a reduction of about -1 log CFU by the EMA real-time PCR (Fig. 2). These results indicate a quantitative difference in Na^+ did not have a significant effect on discrimination of irradiated *V. vulnificus*. *Vibrio* species is a well-known pathogen that requires around 2% NaCl for an optimal environment (16,17). A Na^+-rich or poor environment is a disadvantage for marine bacteria resulting in osmotic stress (17). Our study concluded that irradiation produced effective discrimination for *V. vulnificus* at 1, 3, and 4% NaCl but no significant difference ($p>0.01$); therefore, the study of the synergistic effect of osmosis and irradiation was discontinued.

Effectiveness of discrimination of γ-irradiated *V. vulnificus* by different cold and heat shock treatments

γ-Irradiated *V. vulnificus* was held on ice (0°C) for 15 min, at 40°C for 10 min, and cold/heat combination (0°C for 15 min, 37°C for 15 min, and 40°C for 10 min) for irradiated dead cells to undergo sufficient membrane damage by cold shock and heat shock (Table 1). Without temperature shock, irradiated *V. vulnificus* demonstrated 93.2% discrimination and with cold shock 95% discrimination (-1.3 log reduction). Irradiated *V. vulnificus* with heat shock had slightly higher discrimination (96.6%, -1.47 log reduction) than cold shock (Table 1). Except for heat-killed cells (100%, -3 log reduction), the greatest discrimination (98.2%, -1.74 log) resulted from the synergistic effect of the combination cold and heat shock treatment (Table 1). From these results, we hypothesized several possibilities for cell membrane damage. Membrane damage is typical in cells exposed to cold or heat treatment, therefore, combining the cold and heat shock resulted in synergistic effects similar to that in freezing and thawing causing considerable change to the membrane (18,19). Loss of lipopolysaccharide (LPS) and conformational change in the outer membrane has been seen in cold or heat injured Gram-negative *Escherichia coli* and *Pseudomanas* (18,20). A similar phenomenon might have occured in *V. vulnificus*. Such gross changes in the wall, which resulted in increased permeability of EMA, suggest the creation of small pores in the outer membrane. In this study, the EMA real-time PCR was used to discriminate between γ-irradiated and non-irradiated *V. vulnificus*. γ-Irradiation and sensitization factors such as cold and heat shock treatment could extend not only the self-life, but also improve the screening method by the EMA real-time PCR. These studies are currently being extended to detect γ-irradiated *V. vulnificus* in clam tissue by real-time PCR.

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Table 1. EMA real-time PCR discrimination of γ-irradiated and non-irradiated cells with different treatment on *V. vulnificus*[1)]

Treatment[2)]	%[3)]	-Log[4)]	Ct value	CFU/PCR[5)]	Log CFU[6)]
Control	0	0	29.8±0.2	10^3	3.0
Control+EMA	5.0	-0.02±0.01	32.4±0.5	950±30	3.0
Ir+EMA	93.2	-1.15±0.13	38.4±0.6	70±17	0
Ir+C+EMA	95	-1.30±0.06	39.1±0.3	50±8	0
Ir+H+EMA	96.6	-1.47±0.15	39.9±0.7	34±14	0
Ir+C+H+EMA	98.2	-1.74±0.10	41.2±0.5	18±5	0
Heat killed	100	-3.0	No Ct value	0	0

[1)]Values are the means derived from 3 independent assays
[2)]Ir, C, and H represents γ-irradiation, cold shock, and heat shock, respectively.
[3)]Ability to discriminate dead (%) by EMA real-time PCR.
[4)]Reduction of log genomic target from 3 log (10^3 genomic target/ real-time PCR).
[5)]Genomic target CFU/EMA real-time PCR.
[6)]Log CFU from plate counts.

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