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Discrimination of live and dead cells of *Escherichia coli* using propidium monoazide after sodium dodecyl sulfate treatment

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ABSTRACT

In this study, we evaluated and improved the effectiveness of PMA-qPCR using the microbial model for simulating the process of heat sterilization in the food industry. Heat-induced decay of *Escherichia coli* was measured by traditional techniques including a culture-based method and PMA-qPCR. After heat treatment at a set temperature of 90 °C for 35 s, quantification discrepancies were observed between plate counts and PMA-qPCR. The PMA-qPCR method was then improved by treating twice with PMA to inhibit the DNA from dead cells in *E. coli* suspension. Moreover, cell suspensions were treated with sodium dodecyl sulfate (SDS) before PMA treatment, which was useful for further developing the effectiveness of PMA-qPCR to detect live *E. coli*. Thus, the novel combination of double PMA treatment and SDS treatment was established in this study as a strategy to improve the effectiveness of PMA-qPCR. In conclusion, we evaluated and improved the PMA-qPCR method for discriminating live and dead cells of foodborne pathogens using the microbial models established in our study.

1. Introduction

Food safety problems have been highlighted following outbreaks of foodborne illnesses, which result in adverse health consequences or death. Food safety problems should be encountered with establishment of preventive controls with science-based standards and detection technologies. Currently, since conventional culture methods are labor-intensive, time-consuming, and viable but nonculturable cells cannot be detected, alternative methods for detecting and monitoring microorganisms are expected to be applied to the food chain in order to prevent biological hazards. Real-time quantitative PCR (real-time qPCR), one of the rapid and sensitive methods for quantifying microorganisms, plays an important role in microbial testing and would be expected to replace the culture assay in the food industry (Elizaquível, Sánchez, & Aznar, 2012; McKillip & Drake, 2004). However, using qPCR to distinguish between viable and dead bacterial cells is enormously difficult due to the persistence of DNA, even after loss of cell viability. Therefore, qPCR may generate false-positive results and overestimate the biological hazards, especially when the population of dead cells is larger than that of live cells (Lee & Levin, 2006; Wang & Levin, 2006).

Utilizing qPCR in conjunction with the DNA-intercalating dye, propidium monoazide (PMA), has been considered a promising and easy-method for discriminating between live and dead cells of foodborne pathogens (Nocker, Sossa-Fernandez, Burr, & Camper, 2007). PMA penetrates the membrane-damaged cells, forms covalent bonds with DNA under subsequent illumination, and finally inhibits amplification of the bound DNA in qPCR. Thus, only the DNA from viable cells with an intact membrane can be amplified and detected in the subsequent qPCR process. In addition, an alternative DNA-intercalating dye, ethidium bromide monoazide (EMA), was also reported as a similar reagent (Delgado-Viscogliosi, Solignac, & Delattre, 2009; Nogva, Dromtorp, Nissen, & Rudi, 2003; Rudi, Moen, Drømtorp, & Holck, 2005). However, qPCR combined with the treatment with EMA (EMA-qPCR) was found to underestimate the number of live cells, possibly due to injury to viable cells (Cawthorn & Witthuhn, 2008; Fittipaldia, Nockerb, & Codonya, 2012; Flekna et al., 2007; Lee & Levin, 2009; Nocker, Cheung, & Camper, 2006; Pan & Breidt, 2007; Rueckert, Ronimus, & Morgan, 2005). Compared to EMA, the use of PMA allows accurate measurement of cell number. However, when bacterial cells are exposed to mild heat stress, the cell membrane injuries are sometimes not enough for PMA penetration even after loss of

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viability. In such a case, PMA-qPCR could overestimate the number of live cells (Lee & Levin, 2009; Løvdal, Hovda, Björkblom, & Møller, 2011; Yang, Badoni, & Gill, 2011; Wang, Gill, & Yang, 2014).

Despite this fact, most previous studies concerning the development of PMA-qPCR or EMA-qPCR have used a mixture of untreated live cells and completely heat-killed cells (Chen & Chang, 2010; Hellein et al., 2012; Martin, Raurich, Garriga, & Aymerich, 2013; Nocker et al., 2006, 2007). The dead cells were prepared by heating for a relatively long time, at ≥70 °C for ≥10 min, and the membranes were then considered to be completely disrupted. However, thermal death of microorganisms is a very complex process that may cause an uncertain degree of membrane injury, which directly influences the effectiveness of PMA-qPCR. All these factors need to be explored in detail and addressed completely.

This study involved experiments using *Escherichia coli* to develop and evaluate the qPCR method in combination with PMA treatment (PMA-qPCR). For the accurate discrimination of live cells from dead cells, the samples were treated with modest membrane-destabilizing agents like sodium dodecyl sulfate (SDS), which has no precedent for being used in cell membrane destruction to improve the permeability of heat-killed cells to PMA.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A laboratory strain of *E. coli* was used in this study. *E. coli* was grown in Bacto-trypticase soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA) at 30 °C for 18 h until the logarithmic-growth phase was achieved. To harvest the cells, 10 ml of the suspension was transferred to a 50-ml centrifuge tube, centrifuged at 15,000 × *g* for 3 min at 4 °C and resuspended in physiological saline at a volume equal that of the displaced TSB. After performing a 10-fold dilution, the suspensions were spread on appropriate plates of Bacto-trypticase soy agar (TSA; Becton Dickinson). Bacterial counts were determined by counting colonies after the plates were incubated at 37 °C for 24 h.

2.2. Heat treatment

To prepare the heated *E. coli* samples, 1300 μl of 10^{-2}-fold diluted cell suspensions (6–7 log CFU/500 μl) was transferred from the glass tubes to 1.5-ml microcentrifuge tubes and was heat-treated for 35 s or 45 s using a standard laboratory heat block with a set temperature of 90 °C. The tubes were then placed on ice immediately. Since the viable cells were expected to reduce by 2 log or 4 log units during this heating period based on our preliminary experiments, a 100-μl aliquot of each cooled suspension was diluted in 900 μl of physiological saline to 10^{-3}-fold. A 100-μl aliquot of each dilution was then spread on TSA plates and incubated at 37 °C for 24 h for enumeration. At the same time, a 500-μl aliquot of each cooled suspension was transferred to a new 1.5-ml microcentrifuge tube to be treated with PMA. Another 500-μl aliquot obtained from the same microcentrifuge tube was used as the control (without PMA).

2.3. PMA treatment of bacterial suspensions

Propidium monoazide (Biotium Inc., Hayward, CA) was dissolved in sterile distilled water to form a 4 mM stock solution, and was then stored at −20 °C in the dark until use. The tested *E. coli* suspensions were prepared as described above. Each 500 μl aliquot of the prepared bacterial suspension was treated with 6.25 μl PMA at a final concentration of 50 mM in the dark for 10 min, and then exposed to UV light at a 15-cm distance for 15 min. The samples were centrifuged at 15,000 × *g* for 3 min at 4 °C. For samples with a single PMA treatment, the cell pellets obtained were stored at −20 °C and subjected to DNA extraction as described below. For samples of double PMA treatment, the obtained pellets were resuspended in 500 μl of physiological saline, and again treated with PMA in the dark for 10 min followed by exposure to UV light for 15 min. Finally, the samples were centrifuged at 15,000 × *g* for 3 min at 4 °C and stored at −20 °C until DNA extraction.

2.4. DNA extraction

The pellets of PMA-treated and untreated cells were subjected to DNA extraction using the NucleoSpin Tissue kit (Takara Bio, Shiga, Japan), in accordance with the manufacturer's instructions. The pellet of each sample was resuspended in 180 μl Lysis Buffer T1 and 25 μl Proteinase K, and incubated at 56 °C for 1–3 h, followed by incubation with 200 μl Buffer B3 at 70 °C for 10 min. DNA was isolated by the addition of 210 μl ethanol (96–100%) to each sample. The samples were then transferred to the NucleoSpin Tissue Column and centrifuged for 1 min at 11,000×*g*. The flow-throughs were discarded and the columns were placed back into the Collection Tube. The samples were washed several times with 500 μl Buffer BW and 600 μl Buffer B5. Then, 50 μl of prewarmed Buffer BE (70 °C) was added and incubated at room temperature for 1 min. Finally, pure DNA was eluted by centrifugation.

2.5. Real-time PCR

The qPCR for *E. coli* was performed with an ABI PRISM 7900HT sequence detection system (Life Technologies, Carlsbad, CA, USA). The 50-μl reaction volume contained 25 μl of SYBR Premix Ex TaqTM II (Tli RNaseH Plus) (Takara Bio) and 5 μl of DNA purified from each sample. The primers used were, ECN1254F (5′-GCA AGG TGC ACG GGA ATA TT-3′) and ECN1328R (5′-CAG GTG ATC GGA CGC GT-3′) at a final concentration of 10 pmol/μl (Takahashi et al., 2009). Thermal cycling conditions included an initial 1-min denaturation step at 95 °C followed by 40 cycles of repeated denaturation at 95 °C for 15 s and annealing and polymerization at 63 °C for 30 s.

The DNA samples from serially diluted cells were purified to prepare six dilution points ranging from 1 × 10^3 to 1 × 10^8 CFU/500 μl as an external standard. The data were plotted as qPCR threshold cycles (Ct) against the plate counts of cells to determine the calibration curve of estimation for *E. coli* cell numbers with measured fluorescent signals.

2.6. Treatment of cell suspensions with SDS before PMA treatment

The tested *E. coli* suspensions were prepared as described above. A 500-μl aliquot of the suspensions was transferred to 1.5-ml microcentrifuge tubes as viable cell samples. Another 500 μl of the suspensions was transferred to 1.5-ml microcentrifuge tubes and heat-treated at 90 °C for 3 min, to prepare the dead cells. Plate count was conducted to confirm that no cells had survived in the heat-treated suspensions. SDS (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in distilled water to obtain a 5000 ppm stock solution and was then sterilized by autoclaving. The prepared suspensions of live and dead cells were centrifuged at 15,000 × *g* for 3 min at 4 °C and resuspended in serially diluted SDS. The samples were incubated in a water bath at 37 °C for 30 min after addition of reagents. PMA treatment was performed after treatment of the cell suspensions with SDS. According to the results obtained from qPCR, the maximum amount of SDS to be used inhibiting the amplification of dead *E. coli* cells but not viable cells with PMA-qPCR, was optimized.