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Original Article

# Detection of viable *Salmonella* in ice cream by TaqMan real-time polymerase chain reaction assay combining propidium monoazide

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ABSTRACT

Real-time polymerase chain reaction (PCR) allows rapid detection of *Salmonella* in frozen dairy products, but it might cause a false positive detection result because it might amplify DNA from dead target cells as well. In this study, *Salmonella*-free frozen ice cream was initially inoculated with heat-killed *Salmonella* Typhimurium cells and stored at −18°C. Bacterial DNA extracted from the sample was amplified using TaqMan probe-based real-time PCR targeting the *invA* gene. Our results indicated that DNA from the dead cells remained stable in frozen ice cream for at least 20 days, and could produce fluorescence signal for real-time PCR as well. To overcome this limitation, propidium monoazide (PMA) was combined with real-time PCR. PMA treatment can effectively prevent PCR amplification from heat-killed *Salmonella* cells in frozen ice cream. The PMA real-time PCR assay can selectively detect viable *Salmonella* at as low as $10^3$ CFU/mL. Combining 18 hours of pre-enrichment with the assay allows for the detection of viable *Salmonella* at $10^0$ CFU/mL and avoiding the false-positive result of dead cells. The PMA real-time PCR assay provides an alternative specifically for detection of viable *Salmonella* in ice cream. However, when the PMA real-time PCR assay was evaluated in ice cream subjected to frozen storage, it obviously underestimated the contamination situation of viable *Salmonella*, which might lead to a false negative result. According to this result, the use of enrichment prior to PMA real-time PCR analysis remains as the more appropriate approach.



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## 1. Introduction

Frozen dairy products are popular with consumers. However, *Salmonella* spp. are known for their tolerance of freezing and are widely distributed in frozen dairy products such as ice cream, which could cause serious food safety issues [1,2]. To better control *Salmonella* contamination and consequently to reduce foodborne illnesses, rapid and accurate detection methods are required in frozen dairy products. Although traditionally culture-based methods are widely applied currently, 4–5 days are required to show results after selective plating combined with immunological or biochemical identification [3].

Real-time polymerase chain reaction (PCR) is a promising molecular tool for detecting microorganisms and excluding the contaminated food [4,5]. To date, several real-time PCR-based assays have been developed for the detection of foodborne pathogens in food samples including dairy products [6–10]. However, a problem has grown more prominent along with the development and application of real-time PCR technology in frozen dairy products, in that, intact DNA from dead cells may yield false-positive results because of the sensitivity and indiscrimination of amplification on intact DNA [11]. The dead cells might be caused by the frozen environmental stress [12] or other food safety intervene procedures, and the bacterial DNA may keep intact in the environment for a long time although no viable cell is culturable [11]. The false positive detection results of real-time PCR may cause unnecessary product recalls and economic losses.

Recently, propidium monoazide (PMA) or ethidium monoazide (EMA) treatment before conducting the PCR assay has been used in many reports to discriminate between viable and dead bacterial cells [11,13–15]. These dyes can permeate the membrane-compromised cells and covalently bind to genomic DNA. Following irradiation with visible light, the genomic DNA from dead cells could theoretically be excluded from the PCR system. Previous reports have suggested that PMA penetrates dead bacteria more selectively and effectively than EMA in some bacterial species including *Salmonella* [16], whereas EMA was suggested as more useful for the detection of *Campylobacter* [17]. In the dairy industry, frozen products after heat-based pasteurization are one of the most popularly consumed foods around the world. Although the PMA combined real-time PCR assay has been developed to enhance the detection of live *Salmonella* in food samples [18,19], until now, only a few studies have reported on the application of PMA real-time PCR on the specific detection of viable *Salmonella* cells in dairy products subjected to freezing.

The aim of this study was to investigate the stability of genomic DNA of dead *Salmonella* cells in frozen ice cream, and to use a TaqMan probe based real-time PCR combined with PMA treatment assay for the effective detection of viable *Salmonella* in frozen ice cream.

## 2. Materials and methods

### 2.1. *Salmonella* strain and growth conditions

*S.* Typhimurium ATCC 14028 was used as a representative of *Salmonella* serotypes in this study. The strain was aerobically grown at 37°C, 150 rpm in Brain Heart Infusion broth (Becton Dickinson Co., Sparks, MD, USA), to the later exponential stage (approximately $10^8$ CFU/mL).

### 2.2. Inoculation of dead *Salmonella* cells into frozen ice cream

Pasteurized ice cream was used as a representative dairy product in this study. The ice cream was obtained from a local factory and stored at −18°C prior to use. After thawing, 9 mL of ice cream was transferred into sterile plastic tubes and stored at −18°C in a refrigerator. To obtain dead *Salmonella* cells, the cell suspensions were heated at 70°C for 30 minutes in a water bath. The cell death was confirmed by incubating on Brain Heart Infusion agar at 37°C for 48 hours. One milliliter dead *S.* Typhimurium cell suspension of $10^8$ CFU/mL prior to heat inactivation was inoculated individually into 9 mL of thawed ice cream, which were then stored at −18°C for 20 days. On Day 0, Day 5, Day 10, Day 15, and Day 20, three tubes were taken for analysis.

### 2.3. PMA treatment and genomic DNA extraction

A 20-mM PMA stock solution (Biotium Inc., Hayward, CA, USA) in the amount of 1.25 μL was added into 1-mL *Salmonella* cell suspension to reach a final concentration of 25 μM. The mixture was incubated in the dark at room temperature for 10 minutes to allow PMA to penetrate the dead cells and bind to the DNA [16]. Next, the sample was incubated in ice for 1 minute and then exposed to 650 W halogen light for 5 minutes. The sample was placed about 20 cm from the light source and laid horizontally on ice to avoid excessive heating [20]. After photoinduced cross-linking, cells were collected by centrifugation. The genomic DNA was extracted from *Salmonella* cells after PMA treatment, as well as cells without PMA treatment, using the DNeasy Blood and Tissue kit (Qiagen, Valencia, USA) according to the manufacturer's recommendations. The DNA quantity (A260) and quality (ratio of A260/A280) were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

### 2.4. Real-time PCR

The StepOnePlus system (Applied Biosystems, Foster City, USA) was used in this study. The 20-μL reaction mixture contained 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 200 nM concentration of each primers and probes, $1.2 \times 10^4$ copies of internal amplification control (IAC), and 2 μL sample DNA. The primer and TaqMan probe

sets targeting *invA* gene and IAC followed our previously established multiplex real-time PCR system [21]. The IAC was a 79-bp DNA fragment amplified from a long oligonucleotide (TGGAAGCAATGCCAAATGTGTATGTGGT GGCATTGTCTTCTCCCGTTGTAACTATCCACTGAGATGTGTT AGGCGCGCC). *invA* is a virulence gene encoding an invasion protein and exclusively exists in almost all *Salmonella* spp. It has been proven to be *Salmonella* specific in our previous study [21]. The amplicon length of targeting *invA* gene was only 75 bp for the purpose of ensuring amplification efficiency. To evaluate the real-time PCR amplification efficiency and detection sensitivity, later exponential S. Typhimurium cells were inoculated into 10 mL thawed ice cream to reach final concentrations of $10^0$–$10^8$ CFU/mL. These samples were individually mixed with 90 mL peptone water. Then, two sets of 1 mL homogenate were collected from each sample. One set was treated with PMA prior to DNA extraction; the other was directly subjected to DNA extraction. Each sample was analyzed in triplicate. Next, a linear standard curve was drawn by plotting $C_t$ values generated from real-time PCR against S. Typhimurium cell concentrations in ice cream (log CFU/mL).

### 2.5. *Evaluation of PMA combined real-time PCR in the detection of viable Salmonella cells after enrichment when dead cells existed*

Viable S. Typhimurium cell suspensions were inoculated into 10 mL thawed *Salmonella*-free ice cream to reach final concentrations ranging from $10^0$ CFU/mL to $10^2$ CFU/mL. These samples were individually mixed with 90 mL peptone water containing $10^6$ CFU/mL of dead S. Typhimurium cells prior to heat inactivation. The mixtures were incubated at 37°C for 18 hours. At 6 hours and 18 hours, three sets of 1 mL cell suspensions were collected from each sample. Two sets were applied to the real-time PCR assay, in that one set was treated with PMA prior to DNA extraction, and the other set was directly subjected to DNA extraction. The third set was used for the selective *Salmonella* cell enumeration by plating onto xyloselysine–deoxycholate agar (Becton Dickinson Co.). The xyloselysine–deoxycholate agar was incubated at 37°C for 2 days prior to enumeration.

### 2.6. *Evaluation of PMA combined real-time PCR in ice cream during frozen storage*

The later exponential bacterial culture was added to thawed pasteurized ice cream to form $10^8$ CFU/ml of initial cell concentration. After thoroughly mixed by vortexing, 5 mL ice cream was distributed into Eppendorf plastic tubes. Next, the tubes were refrigerated at −18°C. At predesigned time points, three tubes were taken out to determine the viable cell number by plating onto TSAYE (tryptic soy agar with yeast extract) agar (Land Bridge Technology Co., Ltd, Beijing, China), followed by thawing at 4°C. The TSAYE agar was incubated at 37°C for 24 hours prior to enumeration. The tubes after 30 days and 55 days of storage were also evaluated using real-time PCR assay and viable cell counting.

## 3. Results and discussion

Heat inactivation following frozen storage is a common condition in the dairy industry for inactivating harmful microorganisms and ensuring safety of many frozen dairy products. Because there was little information on the stability of genomic DNA in frozen dairy products, the study started with assessing the persistence of genomic DNA of heat-inactivated *Salmonella* cells in ice cream stored at −18°C. Pasteurized ice cream was inoculated with heat-killed S. Typhimurium cells. During freezing, bacterial DNA was extracted and subjected to the TaqMan real-time PCR assay targeting the *invA* gene [21]. As shown in Fig. 1, comparing to the viable *Salmonella* cells, the $C_t$ values generated from the heat-inactivated cells increased during frozen storage. However, a $C_t$ value of 27.9 could still be generated from the residue genomic DNA on Day 20. The result agrees with previous reports that real-time PCR assay cannot differentiate between DNA from dead and living cells [22,23]. Whereas the cells are heat inactivated, the residue S. Typhimurium DNA can remain stable in frozen ice cream for at least 20 days and may cause false-positive results for PCR-based assays.

In order to overcome the limitation of real-time PCR, a PMA treatment step was added prior to DNA extraction to eliminate the influence of dead cells. The concentration of PMA was selected as 25 μM, which was the optimized concentration (in another study) that has been proven to be sufficient to remove the dead *Salmonella* cells [23]. The PMA real-time PCR assay was applied to ice cream inoculated with viable S. Typhimurium cells ranging from $10^0$ CFU/mL to $10^8$ CFU/mL. The real-time PCR results from PMA-untreated and PMA-treated samples are shown in Fig. 2. Two standard curves exhibited a very similar linear relationship between the $C_t$ value and the concentration of S. Typhimurium in ice cream, both with high coefficients of determination ($R^2$, 0.9994 compared with 0.9974). The real-time PCR assay had a linear quantitative detection and a detection limit of $10^3$ CFU/mL, regardless of whether the sample was treated with PMA. The EMA real-time PCR assay established by Wang and Mustapha [24] could

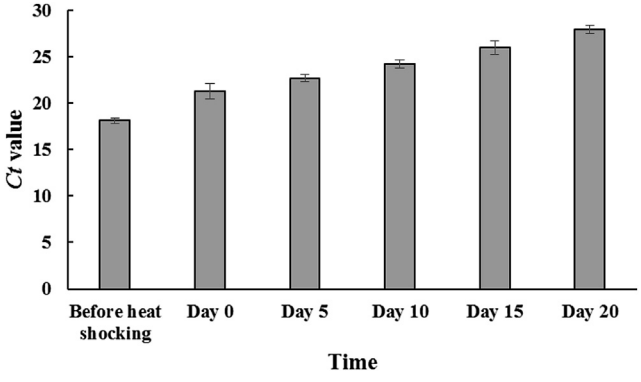


**Fig. 1 – Real-time polymerase chain reaction $C_t$ value change of DNA from dead *Salmonella* Typhimurium in dairy during storage at −18°C. Data are means of three separate determinations, and error bars represent ± standard deviation.**

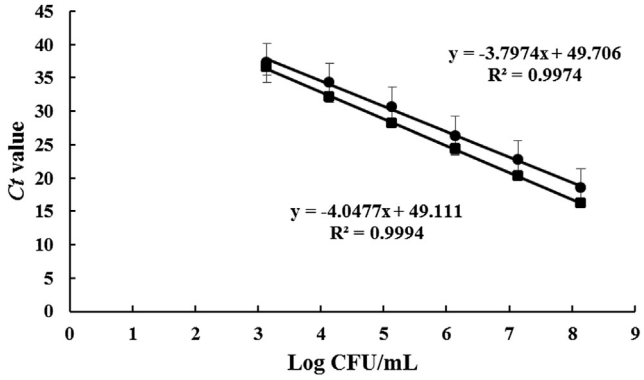


**Fig. 2 – Standard curves for detection of viable *Salmonella* Typhimurium in artificially contaminated ice cream by real-time PCR (squares) and propidium monoazide real-time PCR (circles). Data are means of three separate determinations, and error bars represent ± standard deviation. PCR = polymerase chain reaction.**

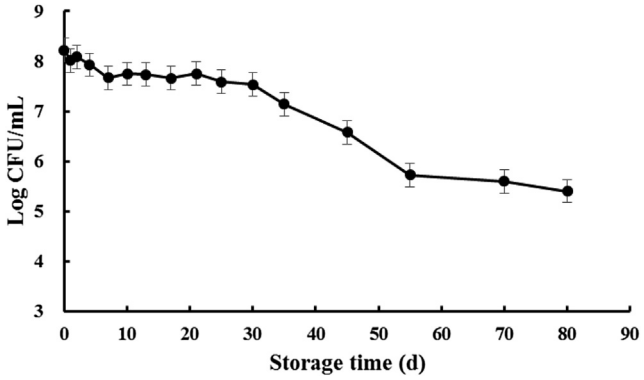


**Fig. 3 – Viable numbers of *Salmonella* Typhimurium in ice cream during frozen storage. Data are means of three separate determinations, and error bars represent ± standard deviation.**

detect *Salmonella* at as low as $10^5$ CFU/mL in chicken rinse and egg broth. The lower detection limit in the present study might be attributable in part to the high specificity of PMA compared to EMA, whereas the capability of EMA to penetrate viable bacterial cells could cause DNA loss [16]. It should also be noted that the penetration effectiveness of dyes through cell membrane depends on the bacterial species. However, PMA could not fully reduce the signal from dead *Campylobacter* cells [25] as well as dead *Listeria monocytogenes* cells [22].

However, the chemical composition of food matrices may affect the detection sensitivity of real-time PCR. Compared with lettuce, ice cream contains more protein and fat, which might interfere with the amplification of DNA by PCR [26]. Still, the present detection limit was comparable to that of $10^3$ CFU/g obtained from lettuce [23], which should be attributed to the high efficiency of silica column-based genomic DNA extraction method from dairy products [27].

Because the contamination dosage of foodborne pathogen in frozen dairy products is normally very low as $10^0$–$10^2$ CFU/mL [28], and the quantification of live cells by PMA–PCR in the presence of high levels of dead cells proved difficult when the concentration of live cells was low [29], it should be better if an enrichment step is added prior to the real-time PCR assay. In the present study, *Salmonella* cells ranging from $10^0$ CFU/mL to $10^2$ CFU/mL were innoculated into ice cream, which was proven to be free of viable *Salmonella* cells but was pre-innoculated with $10^6$ CFU/mL of dead cells prior to heat inactivation. As shown in Table 1, when compared to the detection limit without pre-enrichment step, the PMA–real-time PCR could detect as low as $10^1$ CFU/mL and $10^0$ CFU/ml of initial *Salmonella* cells after 6 hours and 18 hours of enrichment, respectively. The existing dead *Salmonella* cells did not show a significant influence on the efficiency of PMA–PCR for the detection of viable *Salmonella*, which was coincident with previous reports [11,23]. However, if there was no PMA treatment step prior to DNA extraction, the real-time PCR assay could also obtain positive fluorescence signals within 40 cycles from the nonviable *Salmonella*-contaminated ice cream samples, which was inconsistent with the results tested by culture-based assay. The results could be explained by the fact

**Table 1 – Detection of low concentrations of viable *Salmonella* Typhimurium in frozen ice cream by real-time PCR and PMA real-time PCR after enrichment when $10^6$ CFU/mL of dead cells existed.**

| Enrichment time (h) | Spiked cell concentration (CFU/g) | Real-time PCR result [a] | PMA real-time PCR result [a] | Traditional culture-based viable cell count (CFU/mL) [b] |
|---|---|---|---|---|
| 6 | $10^2$ | 31.5 ± 0.2 | 36.8 ± 0.3 | $5.34 \times 10^3$ |
| | $10^1$ | 31.1 ± 0.4 | 37.1 ± 0.8 | $9.66 \times 10^2$ |
| | $10^0$ | 32.6 ± 0.4 | >40 | 0 |
| | 0 | 32.2 ± 0.5 | >40 | 0 |
| 18 | $10^2$ | 16.6 ± 0.4 | 16.5 ± 0.4 | $6.17 \times 10^8$ |
| | $10^1$ | 16.3 ± 0.3 | 16.2 ± 0.1 | $2.68 \times 10^8$ |
| | $10^0$ | 17.5 ± 0.8 | 18.4 ± 0.7 | $3.25 \times 10^7$ |
| | 0 | 33.1 ± 0.2 | >40 | 0 |

CFU = colony forming units; PCR = polymerase chain reaction; PMA = propidium monoazide; XLD = xyloselysine–deoxycholate.
[a] Results were obtained from three repeated experiments, and were expressed as average $C_t$ value ± standard deviation. Values higher than 40 indicate that no visible signal was observed within 40 cycles of amplification.
[b] Viable cells were counted on selective XLD agar plates after the enrichment.

**Table 2 – Evaluation of PMA real-time PCR in the quantitative detection of viable *Salmonella* Typhimurium in artificially contaminated ice cream during frozen storage.**

| Storage time (d) | Plate count (CFU/mL) | Real-time PCR[a] | PMA real-time PCR[a] |
|---|---|---|---|
| 0 | $(1.85 \pm 0.12) \times 10^8$ | $(4.57 \pm 0.28) \times 10^8$ | $(1.09 \pm 0.22) \times 10^8$ |
| 30 | $(3.39 \pm 0.36) \times 10^7$ | $(3.04 \pm 0.34) \times 10^8$ | $(1.65 \pm 0.20) \times 10^6$ |
| 55 | $(5.37 \pm 0.27) \times 10^5$ | $(1.92 \pm 0.13) \times 10^8$ | $(8.98 \pm 0.24) \times 10^3$ |

CFU = colony forming units; PCR = polymerase chain reaction; PMA = propidium monoazide.
[a] The genome copies were calculated from the standard curves in Fig. 2.

that the positive signals were most probably derived from the heat-killed dead cells because 6 hours of pre-enrichment was not sufficient to obtain a detectable viable cell concentration, which was also confirmed by the traditional culture-based viable cell count.

As shown in Fig. 3, when artificially contaminated ice cream was stored at −18°C, the viable *Salmonella* cell number counted by plating assay showed a slight change within the first 30 days of storage. However, there was a significantly faster decrease in viable cell number between 30 days and 55 days of storage ($p < 0.05$), which subsequently remained at a steady level. The total decline in viable cell number within 80 days was 2.82 $\log_{10}$ CFU/mL. It was previously known that inactivation of microorganisms by freezing was achieved through the physical and chemical damaging effects on cell membranes and possibly through the formation of ice crystals [30].

The frozen ice cream was sampled at 30 days and 55 days to evaluate the efficiency of PMA combined with real-time PCR assay. The results in Table 2 show that although the viable cell number decreased during frozen storage, the quantitative real-time PCR assay showed a constant value at 30 days and 55days, compared to that at initial storage. The results determined by PMA real-time PCR assay also showed a decreasing trend along with the decline of viable *Salmonella* during storage; however, it should be noted that the values determined by PMA real-time PCR assay were obviously lower than those obtained with plating, which was confirmed by a difference of almost 1.8 $\log_{10}$ CFU/mL between the two assays. Our research underlines the fact that PMA may reduce the signal of viable bacteria at low concentrations and confirms the results of another recent study [13]. Moreover, it is known that PMA is able to penetrate only dead bacteria with compromised cell walls/membranes, and, following TaqMan real-time PCR assay, all viable bacteria are exposed to give a fluorescence signal, and the quantitative result should coincide with that given by plating count. The current results strikingly illustrate that PMA might eliminate bacterial cells not only based on the cell wall/membrane integrity [31]. Therefore, the PMA real-time PCR process may cause false negative testing results for ice cream, especially when it is contaminated by a low dose of *Salmonella*. These false negative results are not acceptable in a foodborne pathogen detection system, where zero tolerance is the rule, as is the case for *Salmonella*. Based on the results, for a TaqMan real-time PCR foodborne pathogen detection system, it is not recommended to omit the enrichment step prior to PMA treatment, in its current form.

We have developed a method for the detection of viable *Salmonella* in frozen ice cream using TaqMan probe based real-time PCR followed by PMA treatment. PMA treatment can effectively prevent PCR amplification from heat-killed *Salmonella* cells. The PMA real-time PCR assay can selectively detect viable *Salmonella* at as low as $10^3$ CFU/mL. Combining an 18-hour enrichment step with the assay allows for the detection of viable *Salmonella* at $10^0$ CFU/mL, and avoiding the false-positive result of dead cells. However, the PMA real-time PCR assay obviously underestimated the contamination situation of viable *Salmonella* in ice cream during frozen storage, which might lead to a false negative detection result. At this stage, the use of enrichment prior to PMA real-time PCR analysis remains as the more appropriate approach.

## Conflicts of interest

All authors declare no conflicts of interest.

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