Detection of viable but non-culturable *Escherichia coli* O157:H7 by PCR in combination with propidium monoazide

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Abstract

The aim of this study was to evaluate the applicability of the conventional PCR detection method combined with propidium monoazide (PMA) treatment for the detection of viable but non-culturable (VBNC) state *Escherichia coli* O157:H7 in ground beef meatballs. Under low temperature, *E. coli* O157:H7 cells were induced into the VBNC state in ground beef meatballs at -20°C after 152 days. The optimal PMA concentration of $5\text{ }\mu\text{g/mL}$ was obtained in beef meatball samples, which could completely inhibit the DNA amplification on dead cells (10^6 cells/mL) but with no inhibition on viable cells. The established PMA-PCR assay revealed that the VBNC counts exceeded 10^7 CFU/mL in artificial contamination beef samples, which could be used for semi-quantitative detection of VBNC cells in beef meatball samples. This study indicated that the PMA-PCR assay might be a potential method for detection of VBNC state *E. coli* O157:H7 cells in food products.

Keywords Viable but non-culturable · *Escherichia coli* O157:H7 · Propidium monoazide · Detection · Low temperature induction

Introduction

Escherichia coli O157:H7, one of the most notorious food-borne pathogens known to cause gastroenteric infections with a low infectious dose, which is often isolated from fruits, raw vegetables, fresh cheese, raw milk, meat products and undercooked beef (Zhao et al. 2013c; Liao et al. 2016). *E. coli* O157:H7 hidden in food ingredients is usually treated with a series of food processing and storage, such as drying process, ultraviolet sterilization, pulsed light, low-temperature storage, adding preservative and so on. These environmental stresses are able to induce *E. coli* O157:H7 into viable but non-culturable (VBNC) state in which pathogens can remain for a long time and maintain the potential for virulence (Zhang et al. 2015; Li et al. 2016b; Zhao et al. 2017). Makino et al. (2000) showed that VBNC *E.*

coli O157:H7 cells may be involved in an outbreak caused by salted salmon roe in Japan, demonstrated that the possible presence of VBNC pathogens could bring serious risks to food safety and public health. Hence, it is necessary to develop a specific, low-cost and highly sensitive method to detect VBNC *E. coli* O157:H7 cells in food products.

Conventional culture-based techniques, involving enrichment, isolation and confirmation steps, are low-cost and sensitive but also strenuous and time-consuming (Murakami 2012). The polymerase chain reaction (PCR) is a typical technique used for nucleic acid-based amplification, which can identify the enriched target strains within 3 h. However, it cannot distinguish viable cells from dead cells because of that DNA from dead cells can remain intact and lead to false-positive PCR results (Zhao et al. 2013b; Liu and Mustapha 2014). In order to overcome this problem, nucleic acid intercalating dyes have been used as sample pretreatment to selectively remove the DNA from dead bacterial cells, such as propidium monoazide (PMA) (Nocker et al. 2006) or ethidium monoazide (EMA) (Rudi et al. 2005). PMA (or EMA) treatment prior to DNA extraction is a crucial step for the differentiation of viable and dead. PMA (or EMA) can enter the cells with damaged membranes and covalently bind to cellular DNA through visible-light photocatalysis. As a result, the covalent link would inhibit PCR

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amplification of DNA from dead or membrane-injured cells (Nocker et al. 2006; Rudi et al. 2005). However, it has been demonstrated that EMA has highly toxic effect on viable cells, resulting in the death of cells and the inhibition of nucleic acid amplification in viable cells (Pan and Breidt Jr 2008). By contrast, PMA is less toxic than EMA to viable cells and less likely to penetrate VBNC cells with intact membranes (Nocker et al. 2006; Zhu et al. 2012). Hence, PMA is used for the detection of VBNC cells as a sample pretreatment reagent in this study.

Direct viable count (DVC), an identification method of live bacteria based on the substrate absorption ability, was first proposed by Kogure et al. (1979). This assay employed nalidixic acid as a DNA replication inhibitor, and the living cells did not perform separatist activities after absorption of nalidixic acid, causing the cells to become larger and longer, while the dead cells remained the original size. Therefore, the elongated cells can be judged as living cells.

It has been demonstrated that the PCR assay combined with PMA is able to effectively discriminate viable pathogens from dead pathogens in food samples, such as milk (Wang et al. 2014), potable and source water (Singh et al. 2013), vegetables (Elizaquível et al. 2012; Dinu and Bach 2013), beef (Liu and Mustapha 2014), raw seafood (Zhu et al. 2012), infant food products (Li et al. 2016a) and so on. The aim of this study was to evaluate the applicability of PMA-PCR for the detection of VBNC cells which were incubated at low temperature in artificially contaminated beef meatball samples, and to achieve the semi-quantitative detection of VBNC cells. Finally, DVC assay would be used to verify the PMA-PCR results and to evaluate the efficiency of PMA-PCR.

Materials and methods

Bacterial strains and culture conditions

E. coli O157:H7 ATCC 43895 obtained from the American Type Culture Collection (ATCC, USA) were grown on Difco™ Tryptic Soy Agar (TSA; Becton Dickinson and Co., Sparks, MD, USA) at 37 °C for 24 h, and incubated overnight in Bacto™ Tryptic Soy Broth (TSB; Becton Dickinson and Co., Sparks, MD, USA) at 37 °C in shaking bed (110 rpm).

Low temperature induction of VBNC *E. coli* O157:H7 in beef meatballs

The frozen beef meatballs (Yayouwang Food Co., Ltd., Shantou, China) were thawed and cut into small pieces, then shredded under domestic meat grinder (ANMIR, China). The shredded beef meatballs were sterilized at 121 °C

for 20 min. The 1.5 mL micro-centrifuge tubes containing 1 mL of cell suspension from mid-logarithmic growth phase (6.4×10^8 CFU/mL) were centrifuged at $10,000 \times g$ for 5 min. After removing the supernatant, cells were washed twice with 1 mL of 0.85% (w/v) sterile saline solution, and then centrifuged at $10,000 \times g$ for 5 min. The 0.5 g of high temperature sterilized beef balls were sub-packed to micro-centrifuge tubes mixed with 0.85% (w/v) saline solution. After shaking fully, the tubes were, respectively, incubated at 4 and -20 °C until the cells were induced into the non-culturable state. The culture number of cells in beef balls was counted by spread plate method every 8 days, 100 µL of cell suspension was added to 900 µL of 0.85% (w/v) saline solution and was serially tenfold diluted. Finally, 100 µL of proper content gradient diluent suspension was added to the plate and keep the number of colonies on the plate between 30 and 300.

Preparation of dead cell suspension

100 µL of cell suspension from mid-logarithmic growth phase (6.4×10^8 CFU/mL) was added to 900 µL of 0.85% (w/v) saline solution and serially diluted to 10^6 CFU/mL. The dilution cell suspension was heated at 100 °C for 3 min in a water bath to kill bacteria, and then cooled down to room temperature. The dead cell suspension was incubated on TSA at 37 °C for 24 h, setting parallel experiments to ensure all the cells died.

PMA treatment of different cell samples

1 mg of PMA (Biotium, USA) was dissolved in 200 µL of 20% (v/v) dimethyl sulfoxide (DMSO) to obtain the PMA stock solution (5.0 mg/mL) and stored at 4 °C in the dark. Then, the PMA stock solution was tenfold diluted to obtain PMA working solution (0.5 mg/mL). Various cell samples incubated in diverse culture medium were listed from “Pure culture samples”—“The non-culturable cells of *E. coli* O157:H7 in beef balls” sections. After shaking and dark incubation, the transparent Eppendorf tubes contained 500 µL of mixture were placed horizontally on ice and exposed to a 650 W halogen lamp (220 V; OSRAM Light-bulb Company, Munich, Germany) at a distance of 15 cm for 5 min, shaking gently to ensure uniform exposure of the suspension to light.

Pure culture samples

A series of volumes of PMA working solutions added to 500 µL culture aliquots contained 10^6 CFU/mL of viable (or dead) cell suspension, to obtain the final PMA concentrations of 0, 1, 3, 5, 10, 15, 20 and 25 µg/mL for viable cells (or 0, 1, 3, 5, 7.5, 10, 15, 20 µg/mL for dead cells).

Artificial contaminated beef meatball samples

In order to obtain the effective inhibition concentration of PMA on the amplification of pure culture cells, various volumes of PMA working solution were mixed with viable (or dead) cell suspension (10^6 CFU/mL) from contaminated beef meatballs to obtain the optimal PMA concentration.

Mixture samples of viable and dead cells in beef meatball

To evaluate this PMA-PCR method on mixture samples of viable and dead cells in beef meatball, 500 μ L of dead cell suspension (10^8 cells/mL) was, respectively, added to the 1.5 mL micro-centrifuge tubes which contained 500 μ L of viable cell suspension with different concentration gradients (10^8 , 10^7 , 10^6 , 10^5 CFU/mL). The mixture of viable and dead cells was collected by centrifugation ($10,000\times g$, 5 min). After removing the supernatant, the cells were suspended in 500 μ L of 0.85% (w/v) saline solution and were vibrated for a few seconds. The 0.5 g of high temperature sterilized beef balls were sub-packed into micro-centrifuge tubes. The tubes were vigorously shaken to ensure adequate mixing. The mixture suspension was 100-fold diluted to obtain the cell concentration of 10^6 and 10^6 (meaning that 10^6 CFU/mL of viable cells was mixed with 10^6 cells/mL of dead cells), 10^5 and 10^6 , 10^4 and 10^6 and 10^3 and 10^6 cells/mL.

The non-culturable cells of *E. coli* O157:H7 in beef balls

The non-culturable cell suspension from beef balls was added to 0.85% (w/v) saline solution and serially diluted to 10^6 CFU/mL, and then was manipulated by PMA treatment with the optimized concentration.

PCR

According to the gene sequence of *rfbE* in GenBank (GenBank ID S83460), the primers were designed by Primer Premier, Version 5.0 (Premier Inc., Palo Alto, CA) and were synthesized by Bioengineering (Shanghai) Co. Ltd. The initial concentration of primers was 100 μ mol/L. The sequences of primers used for the PCR assay were listed in Table 1. DNA template was prepared according to the previous publication (Zhao et al. 2013c).

The final concentrations of each component in the PCR reaction mixture (25 μ L) were 0.2 mM of dNTP, 0.44 μ M of

each primer, 1 μ L of template DNA, 0.06 U/ μ L of Taq DNA polymerase (TaKaRa Biotech, Dalian, China), 17 μ L of ddH₂O and 2.5 μ L of $10\times$ Taq DNA polymerase buffer, respectively. After a denaturation at 94 °C for 5 min, the PCR mixtures were subjected to 30 cycles of amplification at 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min, using a thermal cycler (TC-96/G/H(b)A; Bori Technology Co. Ltd., Hangzhou, China).

Quantification of PCR product

After the PCR reaction, 10 μ L of PCR products mixing with 2 μ L of loading buffer were added to 2% agarose gel which was dyed with Golden view nucleic acid. The electrophoretic voltage was 75 V with $0.5\times$ TAE buffer. Finally, the gel picture was captured by BD-3000 gel imaging system.

The Gel-Pro analyzer procedure (Version 4.0; Media Cybernetics Inc., USA) was used to quantify the fluorescence intensity of PCR product. The DNA yield of treated samples was obtained from DNA yield in percent of the value of untreated samples with a removed background value. Error bars in diagrams represent standard deviations from three independent replicas.

Verification of VBNC cells

The DVC assay was used to test whether these non-culturable cells were alive or dead. The non-culturable cell suspension was serially tenfold diluted to a proper concentration gradient. Nalidixic acid (Biotium, USA) and yeast extract (Becton Dickinson and Co., Sparks, MD, USA) were added to the diluent cell suspension and the final concentrations were 0.002% (w/v) and 0.025% (w/v), respectively. After incubation period at 37 °C for 12 h, the mixture was filtered through a 0.2 μ m pore size polycarbonate filter, and then was stained in the dark for 5 min with an orange acridine solution (Sigma, USA) which final concentration was 0.01% (w/v). Finally, a cover-slip was added to the air-dried filter before observation with a fluorescence microscope (Olympus, U-HGLGPS, China). The counting of obvious elongated cells was completed in ten random microscope fields, and the calculation was based on the above method (Besnard et al. 2000). The above procedure was also applied to the dead cells treated with heating, which were visually compared with VBNC cells from beef meatballs.

Table 1 The specific primer sequences of *E. coli* O157:H7

Primer	Sequence (5'–3')	Size (bp)	Position ^a	Product size (bp)
EF	AACAGTCTTGTAACAAGTCCA	20	905–924	193
EB	GGTGCTTTTGATATTTTCCG	21	1077–1097	

^aThe positions are determined by the coding sequences of the *rfbE* gene (GenBank ID S83460)

Fig. 1 The culturable graph of *E. coli* O157:H7 low temperature induced in beef balls. Downward-pointing arrows indicate culturability results below the level of detection. Error bars represent standard deviations from two independent replicates

Results

Low temperature induction of the non-culturable *E. coli* O157:H7 cells in beef meatballs

The number of culturable cells in beef meatballs at 4 °C maintained at 10^8 CFU/mL (Fig. 1), indicated that *E. coli* O157:H7 retained highly culturable ability at 4 °C and failed to enter the non-culturable state. At − 20 °C, the cells in beef pellets remained at 10^8 CFU/mL on days 0–8, and then decreased to 10^7 CFU/mL from day 16 and retained the order of magnitude up to day 72. When the induction time reached day 80, the culturable growth curve of *E. coli* O157:H7 showed a marked decline trend. On days 152–156, the cells could not be cultured on TSA, indicating that the cells of *E. coli* O157:H7 in beef meatballs were fully induced into the non-culturable state. However, the non-culturable cells have to be verified if alive or dead by PMA-PCR and DVC assay.

PCR detection limit

The results showed that the detection limit was 10^3 CFU/mL (5.8×10^3 CFU/mL) in TSB and 10^4 CFU/mL (3.2×10^4 CFU/mL) in beef meatballs, respectively (Fig. 2). Thus, although the detection limit in beef meatballs was an order of magnitude lower than that in TSB, the DNA in cells from artificial contaminated beef meatball samples was normally amplified at the bacterial concentration of 10^8 – 10^4 CFU/mL. Although the results indicated that there were minor differences between these two samples, the PMA-PCR detection method based on the pure culture samples could be applied to the beef meatball samples.

Fig. 2 PCR detection limit for bacteria in TSB (a) and beef meatballs (b). Lane M, DL2000 DNA Marker; Lanes 1–8, various concentrations of bacterial suspension (10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10^1 CFU/mL)

Effect of PMA concentration on DNA amplification for viable and dead cells

In order to evaluate the inhibitory effect of PMA on *E. coli* O157:H7 cells (10^6 CFU/mL), a series of PMA concentrations were adopted to pre-treat the viable and dead cells from TSB. PMA had an obvious inhibitory effect on viable cells when the concentration reached 10 µg/mL, and the amplification reaction was completely inhibited by the PMA with a concentration of 20 µg/mL (Fig. 3a), and thus the PMA concentration range for cells should be less than 10 µg/mL. The amplification of dead cells was obviously impeded by 1 µg/mL PMA and completely inhibited by 5 µg/mL PMA (Fig. 3b). Since 5 µg/mL PMA was unable to effect the amplification of viable cells, the optimal concentration of PMA for dead cells from TSB was 5 µg/mL. The optimization of PMA concentration within 0–5 µg/mL would be the focal point in the application of PMA-PCR detection in beef meatball samples.

Optimization of PMA concentration in beef meatball samples

Based on the concentration range of TSB, the optimized concentration of PMA could be obtained by the application of PMA-PCR in beef meatball samples, which were shown in Fig. 3c. It was seen that 1–4 µg/mL of PMA significantly inhibited DNA amplification of dead cells. However, the amplification was completely inhibited by the PMA with the

Fig. 3 Efficiency of different PMA concentrations on the amplification of DNA from 10^6 cells. Error bars represent standard deviations from three independent replicates **(a)** Viable cells from TSB. **b** Dead cells from TSB. **c** Optimization of PMA concentration in beef meat-

ball samples. Lane M, DL2000 DNA Marker; Lanes 1–6, varying PMA concentrations on dead cells; Lanes 7–12, varying PMA concentrations on viable cells

concentration of 5 $\mu\text{g/mL}$ (Lane 6, Fig. 3c). The amplified products from viable cells were significantly decreased by 10 $\mu\text{g/mL}$ of PMA (Lane 12, Fig. 3c), and the others were amplified normally. The inhibitory effect of PMA on the cells from beef meatballs was consistent with that from TSB. Therefore, the concentration of 5 $\mu\text{g/mL}$ PMA was determined to be suitable for discrimination of VBNC cells from beef meatball by this PMA-PCR method.

Application of PMA-PCR detection in the non-culturable cells in beef meatball samples

It was possible for the non-culturable cells to have a mixture state of viable and dead cells in frozen beef meatballs (see the discussion section). Therefore, PMA-PCR was used for the detection of mixed cells at different bacteria concentration gradient through the simulation of mixture state of non-culturable cells existed in contaminated beef meatballs. At the same time, the non-culturable cells were confirmed and identified by PMA-PCR method whether in

the viable state. If the target sequence of the non-culturable cells was amplified, the approximate concentration range of VBNC cells could be obtained by the contrast with mixed cells. Finally, it was shown that the samples of 10^6 and 10^5 CFU/mL of viable cells mixed with 10^6 cells/mL of dead cells could be both amplified the target band under the PMA concentration of 5 $\mu\text{g/mL}$ (lane 2 and 3, Fig. 4), the samples contained 10^4 and 10^3 CFU/mL of viable cells failed to be amplified (lane 4 and 5, Fig. 4), indicating that the detection limits of PMA-PCR (5 $\mu\text{g/mL}$ of PMA) for the viable cells mixed with 10^6 cells/mL of dead cells in frozen beef meatballs was 10^5 cells/mL. Moreover, the target sequences of non-culturable *E. coli* O157:H7 cells from three independent beef meatball samples were amplified in lanes 6–8 (Fig. 4), which meant that there were at least 10^5 cells/mL viable cells and 10^6 cells/mL dead cells in the diluted beef meatballs suspension. The results mentioned above revealed that the concentration of VBNC cells in the beef meatball samples was more than 10^7 cells/mL.

Fig. 4 Evaluation of PMA-PCR in the detection for VBNC cells incubated in frozen beef meatballs. Error bars represent standard deviations from three independent replicates. Lane M, DL2000 DNA Marker; Lane 1, the negative control (DNA extract buffer as a template of PCR); Lanes 2–5, varying concentration of viable cells mixed with 10^6 cells/mL of dead cells (10^6 , 10^5 , 10^4 and 10^3 CFU/mL); Lanes 6–8, three independent samples of non-culturable cells

Verification of VBNC cells by DVC assay

In this study, heat treated dead cells and non-culturable cells were verified by DVC assay and observed with fluorescence microscope, and the representative pictures were showed in Fig. 5a, b, respectively. The detection results showed that the percentage of elongated cells was 12.1%, namely, the number of VBNC cells was about 6.3×10^7 cells/mL. The number of VBNC cells detected by PMA-PCR was consistent with that detected by DVC assay, revealing that PMA-PCR could achieve the quantitative detection of VBNC cells in approximate range.

Discussion

In this study, *E. coli* O157:H7 cells were induced to the VBNC state in ground beef meatballs at -20°C after 152 days, however, the culturable cells incubated at 4°C were maintained at 10^8 CFU/mL. Compared with previous literatures, Masmoudi et al. (2010) showed that 10^8 CFU/mL *Staphylococcus aureus* spp. collected in exponential growth phase were induced into VBNC state by incubating in natural filtered-sterilized seawater at 4°C after 210 days. Patrone et al. (2013) showed that the 10^8 cells/mL of *Campylobacter jejuni* 241 and *Campylobacter jejuni* ATCC33291 incubated in fresh water at 4°C were induced to VBNC state after 48 and 46 days, respectively. These results suggested that the cells incubated at 4°C under nutrition conditions of beef meatballs may spend more time entering VBNC state or fail to enter this state until death.

Fig. 5 Representative pictures of dead cells (a) and VBNC cells (b) with DVC assay visualized by fluorescence microscopy. The white arrows point to some of elongated living cells of *E. coli* O157:H7

The PCR reaction is so sensitive that if there are other macromolecules (such as proteins or fat) in the reaction system which may obstruct the amplification reaction of target sequences (Rossen et al. 1992). Moreover, the DNA polymerase derived from *Thermotoga maritima* (Ultma) was verified to be the most susceptible to PCR inhibitors that present in cheese and meat samples (Al-Soud and Rådström 1998). Therefore, there may be significant differences in the detection results of *E. coli* O157:H7 cells between TSB and beef meatballs. However, it was found that the detection limit of cells in beef meatballs was only one order of magnitude less than that in TSB. An extraction procedure with hot base solutions treatment might be the major reason to reduce the inhibition effect significantly in food samples (Rossen et al. 1992).

The cell concentration in the procedure of PMA treatment is a crucial parameter of PMA-PCR detection. In this study, PMA working solution was added to 500 μL culture aliquots (10^7 CFU/mL viable or dead cells) from TSB to final concentrations of 0, 1, 3, 5, 10, 20, 30, 40 and 50 $\mu\text{g/mL}$. The results showed that the amplification of DNA from 10^7 cells/mL of dead cells was completely inhibited by 40 $\mu\text{g/mL}$ of PMA (lane 9, Fig. 6), indicating the higher concentration of cells lead to the more consumption of PMA. The data of PMA concentration in different experimental projects are also shown in Table 2, which illustrates that the PMA concentration is determined by the bacterial concentration.

Fig. 6 Effect of various PMA concentrations on 10^7 cells/mL of viable (a) and dead (b) cells from TSB. Error bars represent standard deviations from three independent replicates. Lane M DL2000 DNA Marker, NC negative control

Hence, the cell concentration of 10^6 cells/mL was used in the process of PMA treatment in this study. The same cell concentration performed with PMA treatment was also reported in the previous papers (Vesper et al. 2008; Singh et al. 2013; Liu and Mustapha 2014).

It was necessary to consider that the non-culturable cells might be a mixture state of viable and dead cells. When PMA-PCR was used for the viability identification for non-culturable cells, and thus, the experiment should be performed to investigate the efficiency of PMA on different

concentrations of viable cells discriminated from dead cells. Because of the initial concentration of 10^8 CFU/mL in the low temperature induction, it was generally assumed that the existences of viable and dead cells in this condition are 0.5×10^8 and 0.5×10^8 cells/mL (means that 0.5×10^8 CFU/mL of viable cells and 0.5×10^8 cells/mL dead cells), 1×10^7 and 9×10^7 cells/mL, 1×10^6 and 9.99×10^7 cells/mL, 1×10^5 and 9.999×10^7 cells/mL, 1×10^4 and 9.9999×10^7 cells/mL, 1×10^3 and 9.99999×10^7 cells/mL, 1×10^2 and 9.999999×10^7 cells/mL, 1×10^1 and 9.9999999×10^7 cells/mL or 0 and 1×10^8 cells/mL, respectively. The above hypothesis is based on the fact that the viable counts in VBNC state were less than the dead counts since the dead cells accounted for the majority in the mixture state when some cells into the VBNC state which is shown in the study. For instance, Zhao et al. (2013a) showed that viable cell counts was still 10^6 cells/mL while the level of total cell counts of 10^8 cells/mL decreased to undetectable levels on plate. Moreover, Liu et al. (2008) showed that 3×10^6 *E. coli* O157:H7 cells incubated in deionized water were not detected on the culture plates at room temperature after 13 weeks, and the viable cell counts were determined at 3×10^4 cells by the staining method. According to the assumption, it could be generally deduced that the number of dead cells in the mixture was close to 10^8 cells/mL. Therefore, the effect of PMA-PCR on the mixture cells from contaminated beef balls was also investigated. In addition, the samples of non-culturable cells need to be tenfold serially diluted to 10^6 cells/mL in sterile saline solution since the optimized concentration of 10^6 cells/mL.

PMA and cell concentration were the most important factors affecting the detection results. The amount of PMA depends on the number of background dead cells. Therefore, if the dosage of PMA is determined, the appropriate concentration of the dead bacteria does not cause false-positive results. However, if the concentration of dead cells exceeds the appropriate range at a certain PMA concentration, false positive results will be occurred since the superfluous nucleic acid cannot be combined with PMA or

Table 2 Optimization of PMA concentrations in different experimental projects

Experiment project	The final concentrations of PMA (μg/mL)													
	0	1.0	2.0	3.0	4.0	5.0	7.5	10.0	15.0	20.0	25.0	30.0	40.0	50.0
DNA yield in % of untreated sample														
10^7 cells/mL of viable cells from TSB (Fig. 5a)	100.0	80.7	—	105.9	—	97.1	—	83.7	—	93.5	—	97.1	92.7	46.7
10^7 cells/mL of dead cells from TSB (Fig. 5b)	100.0	21.4	—	13.5	—	7.0	—	10.3	—	8.6	—	3.9	0	0
10^6 cells/mL of viable cells from TSB (Fig. 3a)	100.0	109.9	—	102.0	—	71.2	—	12.6	5.4	0	0	—	—	—
10^6 cells/mL of dead cells from TSB (Fig. 3b)	100.0	11.4	—	8.4	—	0	0	0	0	0	—	—	—	—
Viable cells from beef meatball samples (Fig. 3c)	100.0	104.1	—	98.7	—	108.0	83.9	37.6	—	—	—	—	—	—
Dead cells from beef meatball samples (Fig. 3c)	100.0	18.1	16.4	16.8	7.1	0	—	—	—	—	—	—	—	—

“—” means that the concentration of PMA was not used in the experiment

cannot be precipitated completely. In this study, the results showed that the target sequence of non-culturable cells from three independent replicates was amplified with the size of 193 bp (Lanes 6–8, Fig. 4), revealing that the VBNC cells beef meatball samples were more than 10^7 cells/mL, which was consistent with the number of viable bacteria (6.3×10^7 cells/mL) detected by DVC assay, therefore, false positive results were ruled out.

PMA can only enter into the cells with damaged membranes and covalently binds to cellular DNA, which was previously described (Nocker et al. 2006; Pan and Breidt 2008; Zhu et al. 2012), indicating that PMA preferentially played an inhibition role on dead cells. However, it was found that the detection limit of this PMA-PCR method for mixture cells was 10^5 CFU/mL in frozen beef meatballs. PMA-PCR cannot detect viable cells from dead cells when the viable counts were less than 10^5 CFU/mL, suggesting that PMA inhibited the amplification of DNA from both dead and viable cells simultaneously when the ratio of dead and viable count was greater than 10:1. Similarly, Liu and Mustapha (2014) showed that the ground beef sample contaminated with low concentrations (10^4 – 10^0 cells/g) of viable and 10^6 dead cells/g generated negative results using PMA real-time PCR.

Conclusion

According to previous reports, the PMA-PCR method can only achieve qualitative identification for pathogens rather than quantitative detection. However, the PMA-PCR assay established in this study was used not only for the viability identification of the non-culturable *E. coli* O157:H7 cells but also for the quantification of VBNC cells. The results revealed that these non-culturable cells were still alive and the VBNC counts were more than 10^7 cells/mL, which was consistent with the number of viable bacteria (6.3×10^7 cells/mL) detected by DVC assay, indicating that the PMA-PCR method was suitable for the identification of viable *E. coli* O157:H7 in frozen beef balls. Although the PMA-PCR method in this study can only detect such a high concentration of VBNC cells, the method still has a potential value because of the achievement of semi-quantitative detection for VBNC cells by non-quantitative analysis instrument.

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Compliance with ethical standards

Conflict of interest Junliang Zhong declares that he has no conflict of interest. Xihong Zhao declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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