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A propidium monoazide-polymerase spiral reaction (PMA-PSR) designed for direct detection of *Escherichia coli* O157:H7 viable cell

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ABSTRACT

Escherichia coli O157:H7 is a major foodborne pathogen causing food poisoning. Standard detection method for foodborne *E. coli* is plate counting, which is time consuming and omits VBNC cells. PSR is an isothermal amplification technique identifying trace amount DNA. However, its effectiveness is influenced by residual DNA from dead cells. This study aimed at designing a PMA-PSR system for accurate determination of viable cells of *E. coli* O157:H7. Firstly, PSR was optimized in fluorescence indicator, betaine concentration and reaction time. The optimized system could successfully identify *rfbE, stx1 and stx2* genes within 45 min at 65 °C. Secondly, sensitivity and specificity were tested in pure culture and different food samples. With 100% specificity, PSR could determine DNA amount as low as 1.12 pg/μL. Thirdly, a PMA-PSR system was designed for accurate determination of viable cells. Cells were treated with 5 μg/mL PMA to remove residual DNA from dead cells. Pretreated cells were adapted to PSR, thus viable cells were specifically detected. Fourthly, the system was applied in food samples with VBNC cells. Viable cells could be accurately determined in accordance with regular assay. In conclusion, the PMA-PSR system was an efficient tool for accurate determination of *E. coli* O157:H7 viable cells.

1. Introduction

Outbreaks caused by foodborne pathogens have raised attention due to its frequent appearance and big impact (White, et al., 2022). Such foodborne diseases are normally caused by the toxins produced by pathogens. *Escherichia coli* is a common foodborne pathogen widely detected in food products including dairy products, eggs, rice and flour products, and vegetables (Ackers et al., 1998; Gao et al., 2023; Jacob et al., 2013; Marder et al., 2014). Among different serotypes of *E. coli*, O157:H7 is the major enterohemorrhagic serotype leading to varieties of illnesses, hospitalizations and deaths each year (Jiang et al., 2016a; Chart et al., 1991). Differing from other species or serotypes, *Escherichia coli* O157:H7 is required to be strictly undetectable in majority of food types, which enables the applicability of direct detection on viable cells (from either culturable or VBNC cells). Thus, accurate determination of *E. coli* O157:H7 is in need. Standard microbiological detection method in food industry is culturing-based plate counting which is time consuming and overlooks the existence of viable but nonculturable (VBNC) cells (Ferone et al., 2020).

VBNC status is recognized as a microbial survival scheme under

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unfavorable environmental conditions, including high/low temperature, starvation, high salt, high/low pH, light, osmolarity, oxidative stress and UV, which are frequently present in food related environment (Cunningham et al., 2009; Foster, 1999; Guo et al., 2019; Liu et al., 2023; Maertens et al., 2021; Ramaiah et al., 2002; Xu et al., 1982; Yadav et al., 2022). According to recent review, over 100 microbial species including 67 foodborne microorganisms are capable of entering into the VBNC status (Dong et al., 2020). *E. coli* O157 had been proofed to enter into the VBNC status under low temperature and VBNC cells remained virulence genes expression (Liu et al., 2017; Rahman et al., 1996). Furthermore, microbes in the VBNC status are able to recover and regain growth with proper conditions (Pinto et al., 2015). Due to the non-culturable feature of VBNC, traditional culturing-based method fails to determine VBNC cells. With advantages on rapidity, sensitivity, specificity and convenience, nucleic acid amplification techniques (most widely used PCR) have been replacing traditional methods. Propidium monoazide (PMA) in combination with nucleic acid techniques ensure the determination of viable cells, such as PMA-PCR (Li et al., 2020; Liu et al., 2018; Zhong & Zhao, 2017). However, due to its specific equipment requirement, PCR is inapplicable when it comes to on-site test.

Comparing with PCR, isothermal amplification-based methodologiescan be conducted at a constant temperature with advantages on convenience and sensitivity. Currently available isothermal amplification methods include loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), rolling circle amplification (RCA) (Haible et al., 2006), cross priming amplification (CPA) (Zhang et al., 2019), polymerase spiral reaction (PSR) (Liu et al., 2015), strand displacement amplification (SDA) (Walker et al., 1992), and recombinase polymerase amplification (RPA) (Piepenburg et al., 2006). Among these techniques, LAMP is most frequently applied in the determination of various microbes (Zhao et al., 2010). However, LAMP requires multiple primers (4 or 6 primers) which involves critical design and optimization, and thus influences reproducibility (Wong et al., 2018). In the present study, we had applied a novel isothermal amplification method, PSR, which depends on a Bst DNA polymerase to conduct auto cycling strand displacement DNA synthesis with none of denaturation and fewer primers required (2 or 4) (Liu et al., 2015). As an overall advantage of isothermal amplification techniques, no specific and complicated equipment is required. Meanwhile, it yields result with high accuracy, sensitivity and specificity. In recent years, PSR technique has been used to determine different species, including but not limited to *Pseudomonas aeruginosa* (Dong et al., 2015), *Klebsiella pneumoniae* (Wang et al., 2019), *Candida albicans* (Jiang et al., 2016b), *Brucella* spp. (Das et al., 2018) and *Bovine Herpesvirus* (Malla et al., 2018). Thus, PSR could be a potentially applicable method for rapid determination of foodborne pathogen *E. coli* O157:H7.

Up to date, application of PSR technique on the determination of *E. coli* O157:H7 has not been studied. This study aimed at developing an accurate determination method for *E. coli* O157:H7 viable cells and virulence factors based on PMA-PSR targeting *rfbE* (housekeeping genes), *stx1* and *stx2* (virulence genes) genes. The optimization on PSR system, specificity and sensitivity determination, as well as the application of PMA-PSR on the monitoring of *E. coli* O157:H7 viable cells in frozen food samples (rice cake and steamed bun) were performed.

2. Materials and methods

2.1. Bacterial strains

In this study, a total of 23 commonly used foodborne bacterial strains were included. Except for *Lactobacillus casei* cultured in MRS broth (OXOID, UK), the strains were cultured in TSB broth (Huankai Microbial, China) upon DNA extraction and artificial food contamination. Amongst, 5 *E. coli* O157:H7 strains were used, including E019 (isolated from egg), E020 (isolated from milk), E043 and E044 (isolated from beef). Other strains were either standard strains or food originated strains (*L. casei* isolated from beer and *S. aureus* from meat samples).

2.2. PSR primer design

Three targets (*rfbE*, *stx1*, *stx2*) were selected and primers were designed using software Primer Premier 5. A set of primers (Ft and Bt), in combination with another set of acceleration primers were designed for each target gene (Table 1). Upon applying in experiments, primer sequences were aligned by BLAST against the GenBank database to secure their specificity.

2.3. DNA extraction from pure culture (PC)

Genomic DNA samples extracted from the PC of all the strains were used as templates for PSR process. PCs were prepared by incubating isolated colony in TSB or MRS broth for 16 h. DNA samples were extracted from PCs using a bacterial DNA isolation Kit (Dongsheng Biotech, China) following the instructions provided by manufacturer. The DNA concentration and optical densities at 260 nm and 280 nm were determined by adding 1 μL each DNA sample on a Nano Drop 2000 (Thermo Scientific, USA). Only qualified DNA was applied in further experiments.

2.4. Artificial contamination model and DNA extraction

According to the national standard (China, GB 19295-2021), 25 g of frozen food samples (rice cake and steamed bun from a local food company) were added to 225 mL of 0.9% NaCl solution (Aladdin Co., Ltd, China) and mixed with *E. coli* O157:H7 cultures as artificially contaminated frozen food samples (ACFFS). Final concentrations of 10–10^8 CFU/mL (10-fold serial dilution) were used. 1 mL of each ACFFS mixture (10–10^8 CFU/mL) was adapted to centrifugation at 1,000 rpm for 10 min with supernatant further transferred and adapted to centrifugation at 12,000 rpm for 10 min. Cell pellet was treated by saline solution and ethyl acetate (Sigma-Aldrich, USA). The culture was further adapted to centrifugation at 12,000 rpm for 10 min. The cells were washed twice followed by DNA isolation by the bacterial DNA isolation kit (Dongsheng Biotech, China) as described above.

2.5. Acquisition of VBNC cells in food sample

To acquire VBNC cells, induction the entry of *E. coli* O157:H7 cells into the VBNC status was performed simulating frozen food storage condition. *E. coli* O157:H7 ATCC 43895 was subcultured in TSB for 16 h to enrich enough cells (~10^9 CFU/mL). The culture was diluted with either saline or food homogenate to 10^8 CFU/mL. The subcultures were

Table 1
List of oligonucleotide primer sequence.

Target gene	Primer	Sequence (5′-3′)	Size	Position
rfbE	Ft	ttggcatcgtgtggacagggt-AGGACCGCAGAGGAAAGA	39	791-770, 697-715
	Bt	tgggacaggtgtgctacggtt-TCCACGCCAACCAAGATC	39	770-791, 846-829
	IF	GTTTCGATGAGTTTATCTGC	20	755–735
	IB	TCAAAAGCACCCTATAGCTG	20	806–826
sxt1	Ft	cagtgttgtacgaaatcccc-GAGCGATGTTACGGTTTG	38	190-171, 111-128
	Bt	cccctaaagcatgttgtgac-AGGCAGGACACTACTCAA	38	171-190, 277-260
	IF	CTGTATTTGCCGAAAAC	17	169–153
	IB	ACATTGAACTGGGGAAG	17	242–258
stx2	Ft	ctcttcagccagtcgtcgtg-CAACAGCGACATCATCCG	38	616-597, 555-572
	Bt	gtgctgctgaccgacttctc-ATTCCTTCCCGTAACAACT	39	597-616, 697-679

separated into multiple tubes and stored at −20 °C for VBNC status induction. The culturability and viability of the cells were monitored following the same principle using different tubes.

The culturability was determined by traditional culturing based plate counting method. The stored culture was 10-fold serially diluted to be plated on TSA and incubated at 37 °C for 24 h. Number of colonies was recorded as culturable cell number. Nonculturability was designated when the number of colonies was <1 for at least three days (Deng et al., 2015). The commonly used technique in VBNC studies, Live/Dead BacLight bacterial viability kit (Invitrogen, USA) in combination with fluorescence microscopy (Zeiss, Germany) was used to verify the existence of viable cells (Liu et al., 2017).

2.6. Development of PSR system

The PSR system is composed of 8 U of Bst DNA polymerase (NEB, USA), 0.7 M of betaine (Sigma-Aldrich, USA), 20 mM of TRIS hydrochloride (Tris-HCl), 10 mM of Potassium chloride (KCl) (Aladdin Co., Ltd., China), 10 mM of Ammonium Sulfate ($(NH_4)_2SO_4$) (Aladdin Co., Ltd., China), 8 mM of Magnesium sulfate ($MgSO_4$) (Aladdin Co., Ltd., China), 0.1% of Tween-20, 1.4 mM of Deoxy-ribonucleoside triphosphate (dNTP) (Sigma-Aldrich, USA), 1.6 μM of Ft and Bt primers (each), 0.8 μM of IF and IB primers (each) (optional) and chromogenic agent (optional). Chromogenic agent was made by mixing calcein (Sigma-Aldrich, USA) with Mn^{2+} (Aladdin Co., Ltd., China). Chromogenic agent was added before the reaction for visualize result determination and avoid cross contamination due to open lid, otherwise, agarose gel electrophoresis was required. Upon mixture, 2 μL of template DNA was added in the system and the final volume of the system was 25 μL without chromogenic agent and 26 μL with chromogenic agent. The reaction was occurred at 65 °C (in thermostatic equipment or water bath) (Bio-Rad, USA) for 60 min with primers IF and IB and 90 min without acceleration primers. To terminate the reaction, incubation at 80 °C for 2 min was required. Concerning result determination, 2% agarose gel electrophoresis (Bio-Rad, USA) and observation by naked eye under visible light were both acceptable. Laddering or strip pattern band was considered as positive in agarose gel electrophoresis. With chromogenic agent, an orange to green color change was recorded as positive.

2.7. Optimization of the PSR system

Three factors (chromogenic agent mixture, betaine concentration, reaction time) were included for optimization of the PSR system. Concerning chromogenic agent, different ratios (1:20 to 1:2) of calcein (1 mM): Mn^{2+}(2–20 mM) were used to get the most efficient color change. The fluorescence color change principle of calcein is mainly due to that Mn^{2+} and Mg^{2+} compete for the binding site with pyrophosphate ions. Thus, the reaction system will experience color change from orange to green after amplification. If the ratio of calcein to Mn^{2+} concentration is too high, the own fluorescence of calcein would be too high and we cannot accurately interpret positive reaction. If the ratio of calcein to Mn^{2+} concentration is too low, the color change may not occur. Therefore, it is important to explore the appropriate concentration ratio of calcein to Mn^{2+} in the PSR reaction system. Secondly, betaine which contributes to DNA melting, was used at different concentrations. Thirdly, different lengths of reaction time (5–60 min) were considered to acquire the minimum time.

2.8. Determination of reaction specificity and sensitivity in PC

Twenty-three reference strains, including 5 *E. coli* O157:H7 strains (positive) and 18 other strains (negative) were used to evaluate the specificity of PSR system. PCs of these strains were prepared in TSB or MRS broth at 37 °C for 16 h. Template DNA was extracted as mentioned above and, optimized PSR system was used. PSR results were comparatively validated by standard PCR assay.

Two *E. coli* O157:H7 strains were selected base on their virulence gene carriage to test the sensitive of the optimized PSR system. Strain ATCC 43895 was positive for *rfbE* and *stx1* and strain E019 was positive for *rfbE* and *stx2*. Ten-fold dilutions for the DNA samples of the two strains were prepared and added into the PSR system, respectively. Meanwhile, regular PCR was used as a control.

2.9. Determination of reaction sensitivity in ACFFS

In order to determine the sensitivity of the optimized PSR system in detecting *E. coli* cells from ACFFS (rice cake and steamed bun), final concentrations of *E. coli* strain ATCC43895 and E019 at 10–10^8 CFU/mL (10-fold serial dilution) were used. The genomic DNA of *E. coli* cells were isolated from ACFFS and used as reaction template. Nuclease free dH_2O was used to replace DNA template as negative control. Three target genes (*rfbE, stx1* and *stx2*) were selected to evaluate the sensitivity in ACFFS. Meanwhile, regular PCR was used as a control.

2.10. Verification of optimized PSR system in combination with PMA in ACFFS

To develop PMA-PSR system for determination of viable cells (including VBNC cells) of *E. coli* O157:H7, the VBNC culture was enriched in PCs and ACFFS, respectively. ACFFS with cells in the VBNC status were washed more than three times by 0.9% NaCl to avoid the influence of complicated components in food. The VBNC cultures with dead cells were processed by PMA agent in final concentration at 5 μg/mL. Then, the processed culture was sat without light at 25 °C for 10 min. The cultures were placed on ice with tubes horizontally exposed to the halogen light (650 Watt). The distance of tubes from the lamp was exactly 15 cm. After 15 min, the PMA could combine the DNA from dead cells (Chen et al., 2020). Subsequently, the PMA processed culture was adapted to centrifugation at 10,000 rpm for 5 min to acquire cell pellet. The cells were washed twice with PBS and adapted to DNA extraction as mentioned above. The DNA from ACFFS was applied in the optimized PSR system.

3. Results

3.1. PSR system with optimized fluorescence indicator and betaine concentration

As fluorescence indicator plays a vital role in judgement of result, the best ratio of calcein and Mn^{2+} have been optimized. The DNA from *E. coli* O157:H7 strain ATCC43895 at a concentration of 112 ng/μL was used as template and *rfbE* gene was used as a target in the PSR system. With a ratio of calcein and Mn^{2+} at 1:2, the reaction tube has presented green without color change. While with the ratio of calcein and Mn^{2+} at 1:4, a green to orange color change was present, indicating a positive result (Fig. 1). Thus, the optimal calcein and Mn^{2+} was 1:4. As for the dosage of betaine, obvious color change occurred with betaine concentration at 0.6 M and more obvious at 0.7 M (Fig. 1). Thus, 0.7 M was used as optimal betaine concentration. Concerning the reaction time, with primer IF and IB, the amplification products could be visually detected with fluorescence indicator until 40 min (Fig. 2A), and ladder-like or stripe band was shown using gel electrophoresis until 30 min (Fig. 2B). The results determination by color change based on fluorescence indicator were relatively more reliable when the reaction ran as long as 45 min. Without primers IF and IB, positive reaction could be judged in 90 min according to results of gel electrophoresis (data not shown), demonstrating that the designed primers IF and IB successfully accelerated the PSR reaction. Importantly, the PSR system can only be performed in the existence of both primer Ft and Bt.

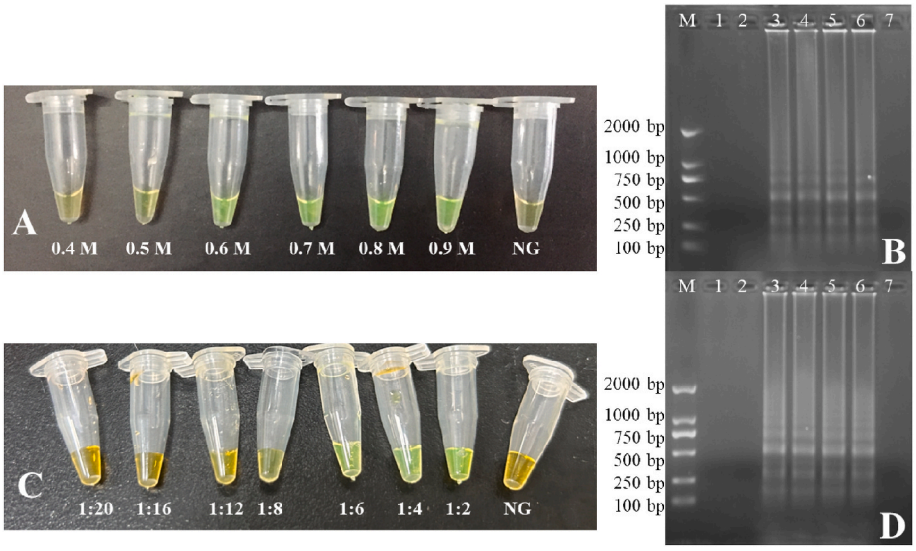

Fig. 1. The results of PSR system with different betaine concentration (A, B) and calcein and Mn^{2+} ratio (C, D). Both visual detection with fluorescence indicator (A, C) and agarose gel electrophoresis (B, D) were applied to determine the results. For the label in figures, M refers to 2000 bp DNA marker, NG refers to negative control. 1–7 in figure B refer to betaine concentration at 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, negative control, respectively. 1–7 in figure D refer to calcein and Mn^{2+} ratio at 1:20, 1:16, 1:12, 1:8, 1:6, 1:4, 1:2, negative control, respectively.

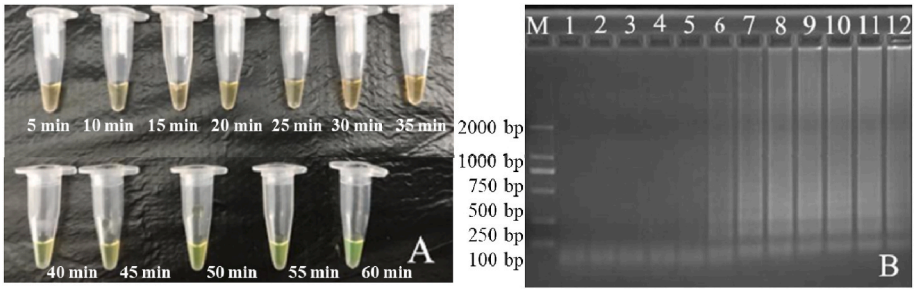

Fig. 2. Optimization of PSR system in reaction time. (A) Visual detection with fluorescence indicator, negative results (orange color) shown in 5–35 min and positive results (green color) shown in 40–60 min. (B) Agarose gel electrophoresis, Lane M: 2000 bp DNA marker, 1–12 refer to reaction time at 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, and 60 min, respectively.

3.2. Sensitivity and specificity of PSR system in PCs and ACFFS

The detection limits of PSR system were identified as 11.2 pg/μL for *rfbE* (Fig. 3), 1.12 pg/μL for *stx1,* 680 pg/μL for *stx2*. It indicated the sensitivity of the designed PSR system was 10–100 times higher than regular PCR system (detection limit for *rfbE, stx1* and *stx2* genes were 1.12 ng/μL, 112 pg/μL and 6.8 ng/μL, respectively). To determine specificity, the optimized PSR system were applied on 5 *E. coli* O157:H7 strains and 18 other strains. Consistently, positive results were only appeared in the 5 *E. coli* strains (Fig. 4).

Furthermore, sensitivity of the optimized PSR system was determined in ACFFS (rice cake and steamed bun). The limit of detection of PSR system targeting *rfbE* gene for *E. coli O157: H7* cells in ACFFS was 10^3 CFU/mL, approximately 100 times of regular PCR (10^5 CFU/mL)

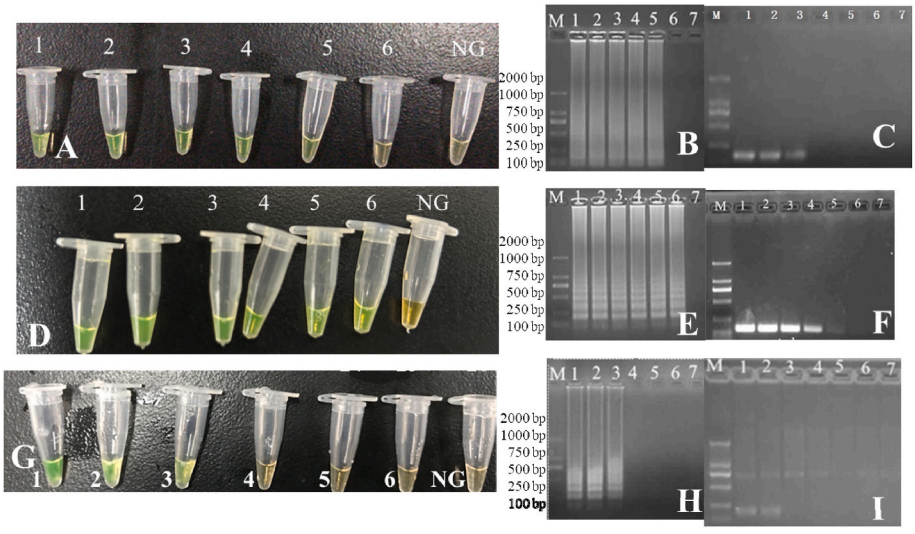

Fig. 3. Sensitivity determination of the optimized PSR system in detection of *E. coli* O157:H7 targeting *rfbE* gene (A, B) and its virulence factors *stx1* (D, E) and *stx2* (G, H) in pure cultures. Both visual detection with fluorescence indicator (A, D, G) and agarose gel electrophoresis (B, E, H) were applied to determine the results. PCR was served as a control (C, F, I). For the label in figures, in (A) and (B), tube/lane 1–6 refer to DNA concentration at 112 ng/μL, 11.2 ng/μL, 1.12 ng/μL, 112 pg/μL, 11.2 pg/μL, 1.12 pg/μL, respectively. In (C), tube/lane 1–6 refer to DNA concentration at 68 ng/μL, 6.8 ng/μL, 680 pg/μL, 68 pg/μL, 6.8 pg/μL, 680 fg/μL, respectively. 7/NG refers to negative control. M refers to 2000 bp DNA marker.

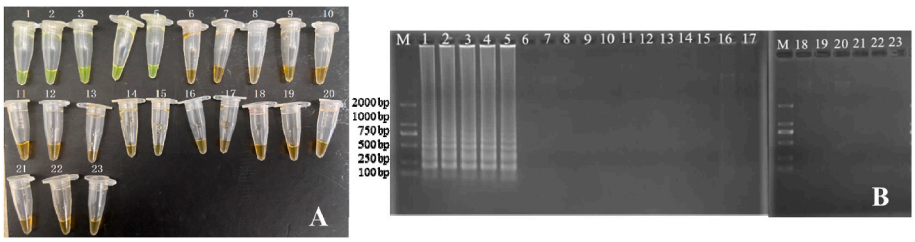

Fig. 4. Specificity determination of the optimized PSR system in detection of *E. coli* O157:H7 targeting *rfbE* gene. Both visual detection with fluorescence indicator (A) and agarose gel electrophoresis (B) were applied to determine the results. For the label in figures, tube/lane 1–5 refer to *E. coli* O157:H7 strains E019, E020, E034, E044, ATCC43895, respectively, and 6–23 refer to *Staphylococcus aureus* ATCC23235, ATCC6358, 10,071, 120,335, 121,271, *Salmonella* ATCC29629, ATCC19585, ATCC14028, ATCC13076, *Listeria monocytogenes* ATCC15313, ATCC19113, ATCC19114, ATCC19115, ATCC19116, ATCC19118, *Vibrio parahaemolyticus* ATCC27969, ATCC17802, *Lactobacillus casei* L. c, respectively. M refers to 2000 bp DNA marker.

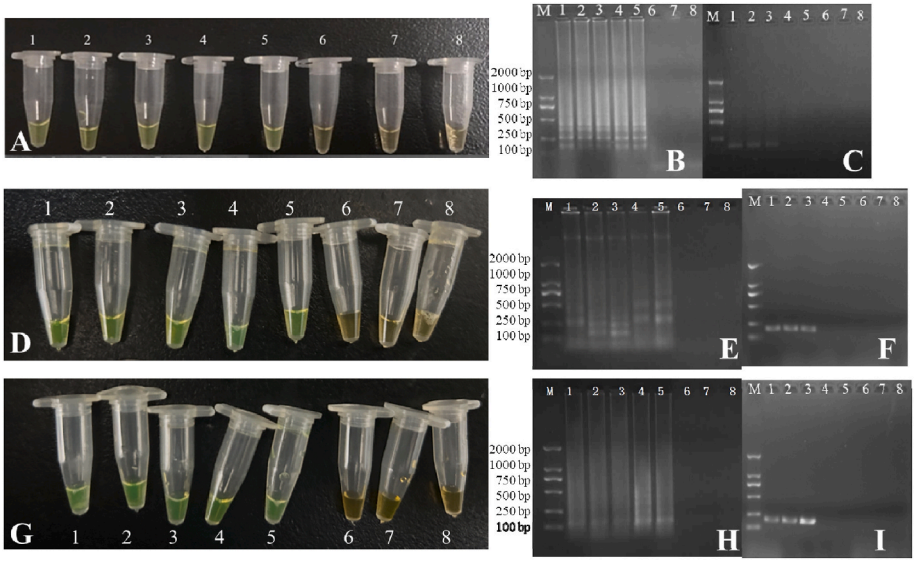

Fig. 5. Sensitivity determination of the optimized PSR system in detection of *E. coli* O157:H7 targeting *rfbE* gene (A, B) and its virulence factors *stx1* (D, E) and *stx2* (G, H) in artificially contaminated food samples. Both visual detection with fluorescence indicator (A, D, G) and agarose gel electrophoresis (B, E, H) were applied to determine the results. PCR was served as a control (C, F, I). For the label in figures, tube/lane 1–8 refer to *E. coli* O157:H7 cell concentration at 10^7 CFU/mL, 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, 10^1 CFU/mL, negative control, respectively. M refers to 2000 bp DNA marker.

(Fig. 5). Targeting *stx1* and *stx2* genes, PSR system could also reach accurate determination of *E. coli O157: H7* cells at concentration of 10^3 CFU/mL in ACFFS. While the sensitivity of regular PCR was much lower (10^5 CFU/mL). No false positive and false negative detection were observed, revealing high specificity of the optimized PSR system to determine *E. coli* O157:H7 cells in ACFFS.

3.3. Application of PMA-PSR system for determination of viable cells

Upon induction of the VBNC status in saline and frozen food sample at −20 °C, we successfully acquired VBNC cells. The determination of VBNC status was conducted by plate counting and viability kit combined with fluorescence microscopy. After *E. coli* cells were determined to be nonculturable by traditional plate counting, the viability kit was used to determine the existence of viable cells under fluorescence microscope. The VBNC cell culture were processed by PMA reagent and DNA extraction kits as mention above and adapted in PSR system targeting *rfbE, stx1* and *stx2* genes. An obvious color change was present in VBNC tubes with fluorescence indicator (Fig. 6). Thus, the PMA-PSR system was successfully developed for determination of *E. coli* viable cells (including VBNC cells) from the mixture of viable and dead cells targeting at *rfbE, stx1* and *stx2* genes.

4. Discussion

Foodborne pathogen *E. coli* O157:H7 is widely spreading worldwide and has aroused attention (Pinto et al., 2015). Thus, it is urgent to develop a high-performance method to detect and monitor the foodborne bacteria to reduce food safety issues. Specificity, sensitivity, simplicity, expense and detection time are important aspects to be evaluated. The standard to identify microorganisms in food industry is culturing based plate counting method which requires long incubation time with low sensitivity. Moreover, it is not able to determine the VBNC cells which are viable but non-culturable. Nucleic acid amplification techniques (mostly widely used PCR) have been encouraged to be an alternative to detect foodborne microbes *in situ*. However, it failed to be adopted due to its lack of convenience with specific equipment. Furthermore, it has been reported that components in food samples could serve as inhibitors for PCR (Hu et al., 2018).

PSR, a novel isothermal amplification-based detection method (Liu et al., 2015), has been recognized as an alternative for PCR considering its convenience with no specific equipment required. In this study, we applied PSR technique targeting three genes including *rfbE* (O157 antigen gene), *stx1* and *stx2* (Shiga toxin encoding genes) to determine *E. coli* O157:H7 cells and virulence factors from food samples. Results from a total of 5 *E. coli* O157:H7 strains and 18 other strains indicated that the PSR system had high levels of sensitivity and specificity. It is worth noting that Shiga toxin 1 (*Stx1*) encoded by *stx1* can cause diseases in gastrointestinal tract including commonly diagnosed diarrhea, and may develop into hemolytic uremic syndrome which is serious sequelae of the infection (Saeedi et al., 2017). The detection of *stx1/2* gene, which is recognized as health risk factor in the field of food contamination, is extremely important and urgently necessary. Thus, we optimized the PSR system for accurate and efficient determination of *E. coli* O157:H7, also specifically for its virulence factors in this study. We applied the optimized PSR system to determine *E. coli* O157:H7 cells isolated from ACFFS (rice cake and steamed bun) with specificity at 100%. The PSR result could be visually obtained within 45 min by

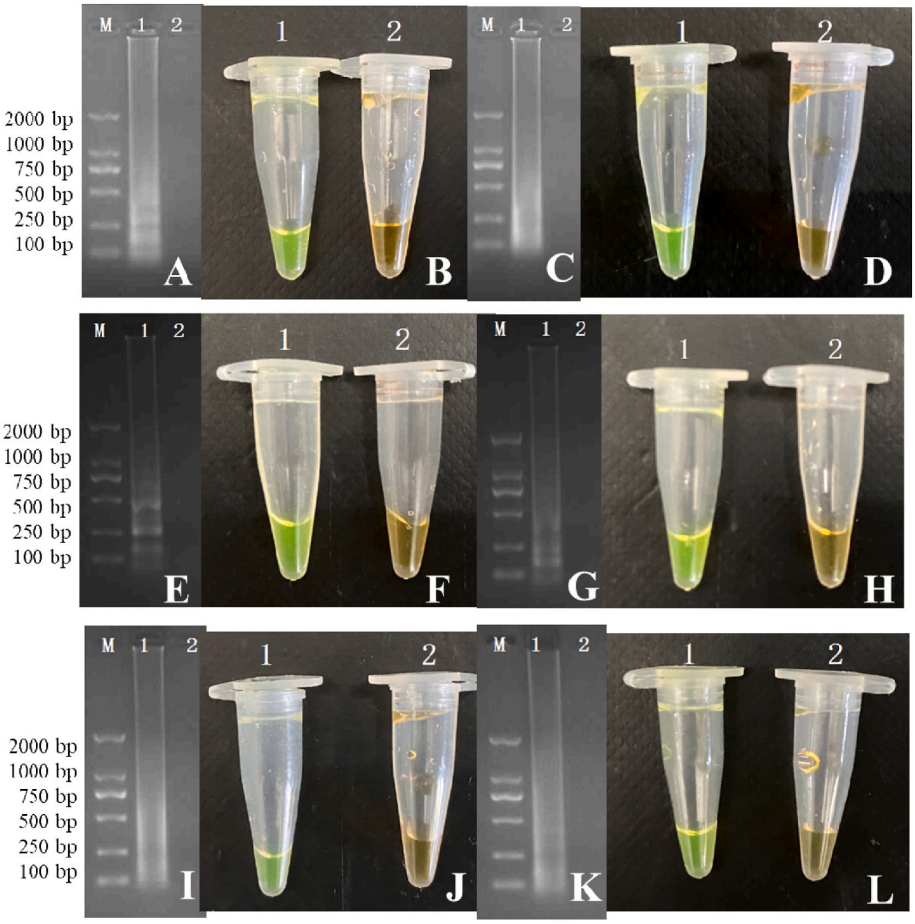

Fig. 6. Determination of viable cells from the mixture of VBNC cells and dead cells of *E. coli* O157:H7 targeting *rfbE* gene (A–D) and its virulence factors *stx1* (E–H) and *stx2* (I–L) in pure cultures (A, B, E, F, I, J) and artificially contaminated food samples (C, D, G, H, K, L) by PMA-PSR system. Both visual detection with fluorescence indicator (B, D, F, H, J, L) and agarose gel electrophoresis (A, C, E, H, I, K) were applied to determine the results. For the label in figures, 1: mixture of VBNC cells and dead cells; 2: dead cells; M: 2000 bp DNA marker.

fluorescence indicator (with primer IF and IB) after optimization. The detection limits showed approximately 100 times of regular PCR, regardless of the cells in PC or ACFFS.

Regular nucleic acid amplification could have false positive detection by amplifying the residual DNA from dead cells. Nucleic acid amplification methods combined with PMA or EMA agent is a new technique to identify viable bacterial cells. EMA and PMA are able to permeate into membrane compromised cells to bind DNA and inhibit its amplification (Nocker et al., 2006). Upon EMA/PMA treatment prior to DNA extraction, viable cells are easily identified at the species level by DNA amplification targeting of species-specific genes. The VBNC status of the foodborne microbes increase the threat to food safety. Many studies have confirmed the fact that VBNC cells still maintain metabolic capacity and produce toxins, but it is difficult to be detected by conventional methods. Thus, this study also designed a PMA-PSR detection system to accurately and efficiently determine *E. coli* O157:H7 viable cells (including VBNC cells). The optimized PMA-PSR system could identify viable cells regardless of the cells in PC or in ACFFS. As far as our knowledge can reach, this is the first report of optimized PSR system to determine *E. coli* O157:H7 and its virulence factor as well as the combination of PMA with PSR to determine viable cells including VBNC cells of *E. coli* O157:H7 from ACFFS.

5. Conclusions

We designed PSR specific primer sets targeting housekeeping gene *rfbE*, virulence factors *stx1* and *stx2* to effectively detect *E. coli* O157:H7. The PSR system was further optimized on calcein: Mn^{2+} ratio (1:4), betaine concentration (7 mM) and reaction time (45 min). And the optimized PSR systems were applied for determination of *E. coli* O157:H7 cells in ACFFS with high specificity and sensitivity. Furthermore, PMA-PSR system was designed to accurately and efficiently determine the viable cells of *E. coli* O157:H7 in PC and ACFFS, avoiding the influence of dead cells and food components on result determination. Thus, acquiring the advantage of rapidity, high sensitivity and specificity, simple and cost effective, PMA-PSR system was proofed as efficient tool for accurate determination of pathogen *E. coli* O157:H7 viable cells and its virulence factors.

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CRediT authorship contribution statement

Junyan Liu: Investigation, Writing – original draft, Funding acquisition. **Tengyi Huang:** Validation, Visualization. **Riyi Xu:** Methodology, Investigation. **Zhufang Xiang:** Methodology, Investigation. **Thanapop Soteyome:** Resources. **Xiangyi Chen:** Methodology. **Qianfei Zhang:** Software. **Qiurong Huang:** Methodology. **Zixu Wu:** Software. **Yunyi Huang:** Formal analysis. **Gongliang Liu:** Supervision. **Chunyun Qu:** Formal analysis. **Gamini Seneviratne:** Data curation. **Birthe V. Kjellerup:** Supervision. **Zhenbo Xu:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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