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Establishment and evaluation of a rapid detection method on viable cells of *Salmonella enterica*: A potential POCT applicable in various food systems

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ABSTRACT

As a common foodborne pathogen, *Salmonella enterica* can enter into the viable but nonculturable (VBNC) state, thus cause false negative detection by culture-based methods. This study aimed to establish a propidium monoazide (PMA)-polymerase spiral reaction (PSR) for rapid detection on viable cells of *S. enterica*. Firstly, to ensure the stability and effectiveness of PMA-PSR assay, optimization of PSR assay which is critical for stability was performed. The optimized PSR assay was established with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4. Calcein was used as an indicator to ensure naked eye result determination and avoid false positive detection. Secondly, the specificity and sensitivity of PSR assay were examined to ensure effective detection of *S. enterica*. The optimized PSR assay had 100 % specificity and the detection limit was 10 copies of plasmid pINVA and 123 pg/μL of genomic DNA. Thirdly, considering the complexity of food matrix, 12 *S. enterica* frequently contaminated food systems covering liquid and solid foods were included to construct artificially contaminated food models. The detection limit of the optimized PSR assay was 10^3 CFU/mL in liquid food samples and 10^4 CFU/mL in solid food samples. Fourthly, PMA treatment, which is critical for specific identification of viable cells, was combined with PSR assay to establish PMA-PSR assay. Lastly, the PMA-PSR assay was applied in 12 food types to ensure its applicability to detect viable *S. enterica* cells in various food systems. PMA-PSR assay was successfully established to detect VBNC cells of *S. enterica* and applied in various food systems. Considering its rapidity, sensitivity and simplicity, especially naked eye results determination, the PMA-PSR assay is a potential POCT for viable cells detection in various food systems.

1. Introduction

With the increasing globalization of food supply, food safety and traceability are of high priority. Studies (Havelaar et al., 2015; S. S. Li, Peng, Zhou, & Zhang, 2022) have shown that food poisoning caused by foodborne pathogens is a growing public health problem worldwide and a major food safety issue in China (Alcorn & Ouyang, 2012; Sun et al., 2022; Wang et al., 2021). Foodborne illness is an infectious or toxic disease caused by the ingestion of various pathogenic agents (Mei et al., 2022). It exerts a substantial socioeconomic burden on the population

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and healthcare system (Y.-n. Wu et al., 2018). Foodborne pathogens include *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*, etc (Miao, Liang, et al., 2017). *Salmonella* is a group of Gram-negative enteric rods which is widely distributed in nature (Ferrari et al., 2019) and is one of the most common foodborne pathogens worldwide. It is mainly transmitted through contaminated food, water, and feces and is able to cause many disease syndromes, including acute gastroenteritis, septicemia, enteric fever, typhoid, and paratyphoid fever (Darwin & Miller, 1999; Foley et al., 2007; Heredia & Garcia, 2018). Globally, approximately 93.8 million cases of foodborne illness and 155,000 deaths caused by *Salmonella* were reported per year (Eng et al., 2015). According to the report from China foodborne illness surveillance system, 899 food safety incidents related to *Salmonella* were reported nationwide from 2003 to 2017, ultimately resulting in 21,881 illnesses, and 11,351 hospitalizations (W. Li et al., 2020). The food types frequently contaminated by *S. enterica* include vegetables, juice, milk, meat, rice-flour product (Ali & Alsayeqh, 2022; Kowalska, 2023; Manu et al., 2017; Parker et al., 2016; Yuk & Schneider, 2006). Contamination with *S. enterica* is an important factor leading to substandard of food products and causing food safety issues. Therefore, it is significant and essential to establish an effective and easy detection method for the identification of *Salmonella*.

In 1982, Xu et al. identified a special state of bacteria, designated the "Viable but Non-culturable" (VBNC) state (H. S. Xu et al., 1982; Zhou et al., 2020). Bacteria can enter into the VBNC state under environmental stresses such as nutrient deficiency, changes in temperature, and osmotic pressure (Guo et al., 2019; J. Liu, Deng, Li, et al., 2018; J. Liu, Deng, Soteyome, et al., 2018; J. J. Liu, Yang, Kjellerup, & Xu, 2023). Bacteria in this state remain active, but are unable to grow in conventional culture media, resulting in false negative detection (Jiang et al., 2021). Although the bacteria lose the ability to grow on conventional culture media, the VBNC cells maintain intact cell membrane structure, ensuring the integrity of genetic information. Meanwhile, cells entered the VBNC state still have a certain level of metabolic activity and are able to exchange substances inside and outside the cell (Lahtinen et al., 2008; Rahman et al., 1994). It is worth noting that bacteria entered the VBNC state can be restored to a culturable state under appropriate conditions, such as elevated temperature, addition of catalase or special nutrient factors, posing a risk to human health and food safety (Hu et al., 2019; Lin et al., 2017; Miao, Chen, et al., 2017). With the development of molecular biology, nucleic acid amplification technology has gradually developed into an alternative to traditional detection methods with high specificity and sensitivity. Therefore, molecular biology methods have a wide range of prospects for the detection of bacteria in the VBNC state (L. Liu et al., 2019; Ou et al., 2021; G. Xu et al., 2012). Propidium monoazide (PMA)-polymerase chain reaction (PCR) is a method developed over the past few years based on traditional PCR technology for detecting viable bacterial cells (Guan et al., 2021; Zhao, Li, & Xu, 2018). The principle of action is that PMA and EMA can cross-link to the DNA double helix structure in the cell through the damaged cell membrane (dead cells), but not through the intact cell membrane (viable cells), so that the primers cannot recognize the target region, resulting in the inhibition of PCR amplification of DNA molecules, thus enabling the identification of VBNC state and dead organisms (S. S. Chen, Wang, Beaulieu, Stein, & Ge, 2011; Zhao et al., 2013).

Pathogenic microbial detection techniques include culture-based method, immunological technique, and nucleic acid detection (Haesendonck et al., 2014; Ramirez et al., 2006; Xue et al., 2017). Among these, nucleic acid detection technique is widely used in the medical, life science, and food safety fields because of their high sensitivity, few limitations, and ease of operation (Q. Wu et al., 2019). Due to the invention of PCR technology in the mid-20th century (Oste, 1988), real-time fluorescent quantitative PCR (RT-qPCR) (Faraji et al., 2018), loop-mediated isothermal amplification (LAMP) (Roedel et al., 2020), recombinase polymerase amplification (Njamkepo et al.) (Archer et al., 2020), and recombinase-aided isothermal amplification (RAA) (Fan et al., 2020) based on the specific amplification of microbial nucleic acids have developed rapidly with the continuous innovation of molecular biology techniques. Since PCR and its derivatives could not get rid of the restriction of thermal cycling for heating and cooling (R. Gupta et al., 2009; W. Liu et al., 2015), isothermal amplification techniques were gaining increasing popularity among scientists.

Table 1
Strains used in this study.

Species name	Strain number
Salmonella enterica	ATCC14028, ATCC29629
Escherichia coli O157:H7	ATCC43895, E019, E020, E043, E044
Vibrio parahaemolyticus	ATCC27969, ATCC17802, R40
Vibrio cholerae	R44
Listeria monocytogenes	ATCC19114, ATCC19116
Pseudomonas aeruginosa	ATCC27853, C9, C40
Pseudomonas putida	Guangzhou-Ppu420
Staphylococcus aureus	ATCC23235, Guangzhou-SAU085, Guangzhou-SAU071
Staphylococcus epidermidis	9140-M10
Staphylococcus intermedius	Sint230001
Staphylococcus haemolyticus	Shae230001
Staphylococcus saprophyticus	ATCC27840
Lacticaseibacillus casei	Lcas200001
Levilactobacillus brevis	Lbre200001
Lactiplantibacillus plantarum	ATCC8014
Pediococcus acidilactici	Paci180001
Pediococcus damnosus	Pdam230001
Klebsiella pneumoniae	Kp1, Kp2, Kp3, Kp4, Kp5, Kp6
Bacillus cereus	ATCC14579, ATCC13061, ATCC11778, ATCC12480, ATCC25621
Yersinia enterocolitica	ATCC9610
Cronobacter sakazakii	BAA894
Acinetobacter baumannii	12052313

The polymerase spiral reaction (PSR) is a new nucleic acid isothermal amplification method that takes advantage of the activity of Bst DNA polymerase and the semi-dissociation and semi-equilibrium state of nucleic acid molecules at approximately 63 °C to rapidly amplify DNA molecules at a constant temperature (D. Dong et al., 2015; W. Liu et al., 2015). The method is inspired by LAMP primer design and incorporates the advantages of simple PCR primer design. The PSR primers are based on PCR primers and a sequence is taken from the target sequence and added to the 5′ ends of PCR primer to form a forward and reverse composite primer. This fulfils the requirements of simplicity, rapidity, sensitivity, and specificity for the assay (M. Chen et al., 2022; J. Y. Liu, Huang, et al., 2023). Finally, the experimental results were presented by agarose gel electrophoresis and verified by fluorescent dye indication (V. Gupta et al., 2017; Malla et al., 2018). Therefore, PSR is a potentially valuable tool for the rapid detection of *Salmonella*, and the combination of PMA may achieve the accurate detection of viable cells in VBNC state.

Therefore, the objective of this study was to establish and evaluate a PSR-based assay targeting the *invA* gene for the detection of *S. enterica* viable cells in various food products, providing reliable technical support for real-time detection and field diagnostic procedures for foodborne pathogens.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

A total of 46 strains were used in this experiment, all of which were stored as glycerol stocks at −80 °C. *S. enterica* ATCC14028 and *S. enterica* ATCC29629 were used as the target strains and 44 non-*S. enterica* strains were used as non-target strains (Table 1). All strains were streaked from glycerol stocks on tryptic soy agar (Huankai Biotech Co. Ltd, Guangzhou) plate to yield single colonies. The DNA was extracted from

Table 2
Primer sequences used in this study.

Target gene	Primer name	Primer sequence
invA	*invA*-Ft	CACAAAGATGATAATGATGCCAATACTGGAAAGGGAAAGCC
	invA-Bt	CCGTAGTAATAGTAGAAACACGACAGAGCGGAGGATAAA-
	invA-IF	TCATCGCACCGTCAAA
	invA-IB	TGGCGGTATTTCGGTGGG

overnight cultures by inoculating a single colony into 3 mL of tryptic soy broth (TSB) (Huankai Biotech Co. Ltd, Guangzhou) and incubated in a constant temperature shaker at 37 °C and 200 rpm for 24 h. A Rapid Extraction Kit for Bacterial Genomic DNA (Dongsheng Biotech Co. Ltd, Guangzhou, China) was used to rapidly extract DNA from the bacterial genome according to the manufactures' instructions. The extracted DNA was stored at −20 °C. The concentration of the DNA was determined by ultra-micro UV spectrophotometer and the OD_{260}/OD_{280} value of the aqueous template DNA solution was controlled to be 1.8–2.0.

2.2. Positive control plasmid construction

The housekeeping gene *invA* of *S. enterica* was used as target in this study. The complete sequence of *invA* gene was amplified by high fidelity PCR mix (Enzyvally Biotech, Guangzhou, China) and subsequently purified using a PCR purification kit (Itail Biotech Co. Ltd., Guangzhou, China). The purified PCR product was sequenced by Sanger sequencing (IGE Biotech Co. Ltd., Guangzhou, China) and the sequence was compared with the original *invA* gene sequence to ensure 100 % identity. The verified PCR product was then cloned into a T-vector (Sangon Biotech Co. Ltd., Shanghai, China) to serve as positive control pINVA in the optimization and evaluation of the PSR assay. Sanger sequencing was performed again on the plasmid to ensure the *invA* gene has no mutation.

2.3. Primer design

The specific fragment was obtained by BLAST comparison for the *S. enterica* housekeeping gene *invA*. The primers for the PSR amplification reactions were designed using Primer Premier 5 (Table 2), with Ft/Bt as the detection primer pair and IF and IB as the acceleration primer pair. All primers were synthesized by IGE Biotech Co. Ltd., Guangzhou, China, with primers Ft and Bt purified by HPLC and primers IF and IB purified by PAGE. The primers were solubilized in ddH_2O and stored at −20 °C. The initial concentration of the primers was 100 nM.

2.4. Establishment of the PSR assay with chromogenic reaction

The PSR system was developed and established using the *S. enterica*-specific gene *invA* as target gene with the genomic DNA of strain *S. enterica* ATCC14028 and plasmid pINVA as templates. The PSR reaction system consisted of reaction solution, primers, Bst DNA polymerase, template DNA, and chromogenic reagent. Reaction solution included KCl, $(NH_4)_2SO_4$, $MgSO_4$, Tween 20, Tris-HCl, dNTP mix, betaine solution. Chromogenic reagent included 1 mM $MnCl_2{\cdot}4H_2O$ and 50 μM calcein. The 26 μL PSR reaction system consisted of 12.5 μL reaction solution, 2 μL primers, 2 μL template DNA, 1 μL Bst DNA polymerase, 1 μL chromogenic reagent, ddH_2O (make up to 26 μL). The above PSR system was incubated in a metal bath at 65 °C for 60 min. The result was determined by chromogenic reaction (positive: green, negative: orange) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band). The results determined by agarose gel electrophoresis was to ensure the accuracy and scientific soundness. Concerning the complication of agarose gel electrophoresis and the requirement to open lid and exposure the PSR system into the environment, naked eye observation by chromogenic reaction was included to provide PSR assay as a potential onsite POCT detection application and lower false positive determination possibility by avoiding open lid after reaction.

2.5. Optimization of reaction time, temperature, betaine and chromogenic reagent

Four key factors impacting the effectiveness of PSR assay were included in the optimization, including reaction time (10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min), temperature (59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C), betaine (concentration at 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M) and chromogenic reagent (calcein and Mn^{2+} ratio at 1:1, 1:2, 1:4, 1:6, 1:8, 1:12, 1:16, 1:20). The optimization of reaction time, temperature and betaine was to ensure the stability of the reaction, while the optimization of chromogenic reagent was to ensure chromogenic reaction to be observed clearly by naked eyes. For experimental design, the conditions (reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.8 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:8) which had been widely used were set as control conditions in each optimization step (Chaudhary et al., 2025; Haridas et al., 2010; Sharma et al., 2022; X. X. Wu, Chen, Yang, Ning, & Liu, 2022; Zhang et al., 2022). Upon acquiring all the optimized conditions, optimized conditions were set as control conditions to repeat all optimization steps. For the first round of optimization, in reaction time optimization, reaction temperature, betaine, and chromogenic reagent were set as 65 °C, 0.8 M, and calcein and Mn^{2+} ratio at 1:8, while reaction was terminated at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, respectively. In reaction temperature optimization, reaction time, betaine, and chromogenic reagent were set as 60 min, 0.8 M, and calcein and Mn^{2+} ratio at 1:8, reaction temperature was set at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, respectively. In betaine concentration optimization, reaction temperature, reaction time, and chromogenic reagent were set as 65 °C, 60 min, and calcein and Mn^{2+} ratio at 1:8, while calcein concentration was set at 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, respectively. In chromogenic reagent optimization, reaction temperature, reaction time, and betaine were set as 65 °C, 60 min, and 0.8 M, while chromogenic reagent was set as calcein and Mn^{2+} ratio at 1:1, 1:2, 1:4, 1:6, 1:8, 1:12, 1:16, 1:20, respectively. The conditions with both bright ladder band in 1.5 % agarose gel electrophoresis and clear orange to green in chromogenic reaction was selected as optimized conditions. The optimized conditions were used as control conditions in the second round of optimization. If optimized conditions acquired in the second round were conflict with the first round, a third round of optimization using the optimized conditions from the second round as control conditions were required. The final optimized conditions were determined until the results were consistent in two continuous rounds of optimization. All experiments were performed in triplicate to ensure reproducibility.

2.6. Evaluation on specificity and sensitivity

Specificity and sensitivity are critical for a detection method. In this study, the specificity of PSR assay with *invA* target and corresponding primers was evaluated by including 44 non-*S. enterica* strains as negative samples. Two *S. enterica* strains ATCC14028 and ATCC29629, as well as plasmid pINVA were used as positive samples. Concerning sensitivity, plasmid pINVA at 10,000 copies, 1000 copies, 100 copies, 10 copies, and 1 copy, and the concentration of genomic DNA isolated from *S. enterica* strain ATCC14028 pure culture at 12.3 ng/μL, 1.23 ng/μL, 123 pg/μL, 12.3 pg/μL, and 1.23 pg/μL were included to determine limit of detection of the PSR assay. All experiments were performed in triplicate to ensure reproducibility.

2.7. Evaluation of applicability and sensitivity in various food products

In this study, to test the applicability of PSR assay in detecting

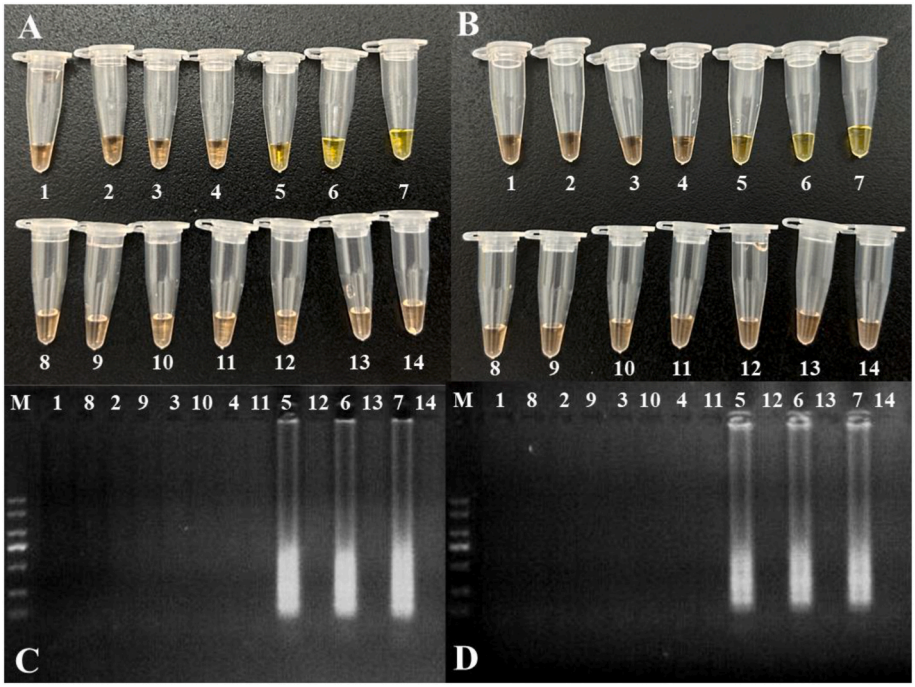

Fig. 1. Reaction time optimization of PSR assay using plasmid pINVA (A, C) and *S. enterica* ATCC14028 (B, D) by chromogenic reaction (positive: green, negative: orange) (A, B) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (C, D). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–7, positive reactions with reaction time at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, respectively; lane/tube 8–14, negative controls with reaction time at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, respectively. The PSR system was set with reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

S. enterica cells in various food products, 12 types of food products covering liquid (milk, egg, carrot juice, tomato juice, apple juice) and solid food samples (rice and flour product, pepper, tomato, beef, chicken wing, meatball) were included to construct artificial contamination food model. The rationale for selection the 12 types of food products was to cover *S. enterica* frequently contaminated food types and ensure diversity. Vegetables, juice, milk, meat, rice-flour product are frequently contaminated by *S. enterica*. 2–3 types of vegetables, juice, and meat were included considering they are more diverse. The artificial contamination food model was constructed as follows. Food samples (25 g/25 mL) were weighed and ground thoroughly, and added to 225 mL sterile saline solution to obtain food homogenate. To avoid interference by the natural accumulation of bacteria, autoclaving was performed. For evaluation of sensitivity, artificially contaminated food samples were prepared by adding *S. enterica* cells at different concentrations (10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, and 10 CFU/mL) to the sterilized food homogenate. Food samples without *S. enterica* contamination were used as the negative control. Afterward, genomic DNA was isolated from the artificially contaminated food samples and adapted to PSR assay. All experiments were performed in triplicate to ensure reproducibility.

2.8. Establishment of PMA-PSR assay for VBNC cells

The VBNC state of *S. enterica* was induced by oligotrophic medium (sterile saline) at low temperature (−20 °C). *S. enterica* cells were washed three times with sterile saline and then resuspended in fresh sterile saline. The suspensions were added separately to 1.5 mL centrifuge tubes and subsequently induced at −20 °C in freezer. The culturable cell number of *S. enterica* was determined using a plate count method and the cells were considered nonculturable when no colony was presented after 3 days of continuous incubation. Thereafter, viable cells were determined using the LIVE/DEAD® BacLight™ kit in combination with fluorescent microscope to determine the presence of VBNC state.

The samples confirmed to be in the VBNC state were added to 1.5 mL centrifuge tubes, followed by adding PMA working solution (500 μg/mL) and incubated for 10 min at room temperature without light. Then the mixture was incubated for 5 min at a distance of 15 cm from a 650 W halogen lamp to allow sufficient cross-linking of PMA reagent with DNA. The treated samples were centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. Rapid extraction of bacterial genomic DNA for PMA-PSR amplification was then performed.

2.9. Evaluation of PMA-PSR in various food products

The obtained *S. enterica* cells in the VBNC state, as well as regular culturable cells and dead cells (heat killed) were included in the artificial contamination food model. Heat killed dead cells were prepared by autoclaving *S. enterica* culture (121 °C, 15 min) and verified by plate counting and live/dead viability test. The artificial contamination food model was constructed as mentioned above. To avoid impurities in the food products that could affect the cross-linking ability of PMA with DNA molecules, the artificially contaminated food samples were washed three times with saline to reduce the turbidity of the sample. PMA was added to 1 mL of the artificial contaminated food samples to achieve a final concentration of 5 μg/mL, and the samples were then subjected to PMA treatment, followed by DNA extraction and PSR assay.

3. Results

3.1. Optimized PSR assay establishment

In the detection of viable cells by PMA-PSR assay, the stability and accuracy of PSR assay is utmost important. To establish a reliable PSR assay, the major factors including reaction time, temperature, betaine concentration and chromogenic reagent were optimized. Firstly, 7 positive reactions each for plasmid pINVA and DNA of *S. enterica* ATCC14028 as template with corresponding negative control reactions

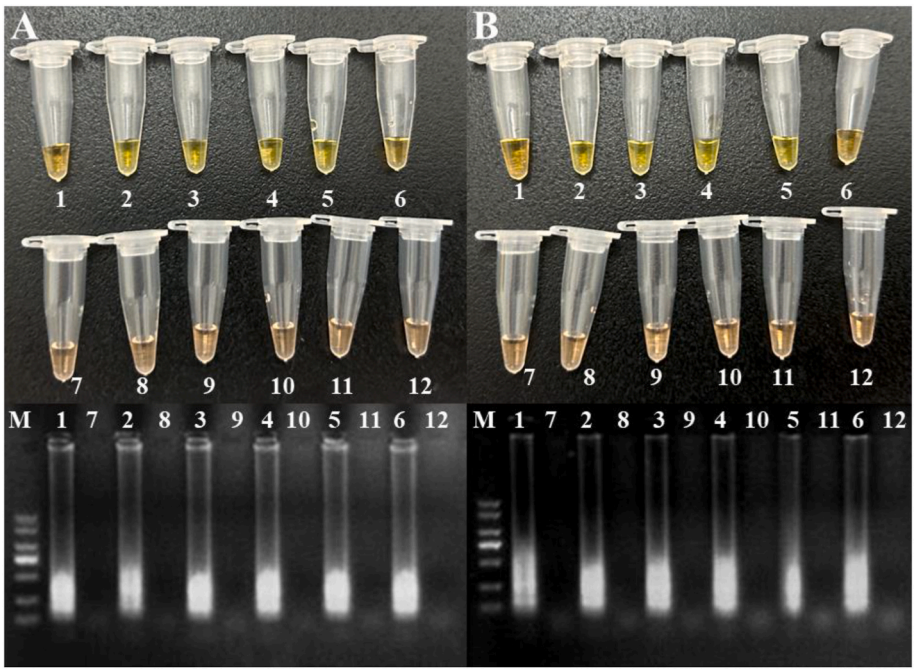

Fig. 2. Reaction temperature optimization of PSR assay using plasmid pINVA (A, C) and *S. enterica* ATCC14028 (B, D) by chromogenic reaction (positive: green, negative: orange) (A, B) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (C, D). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–6, positive reactions with reaction temperature at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, respectively; lane/tube 7–12, negative controls with reaction temperature at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, respectively. The PSR system was set with reaction time at 60 min, betaine concentration at 0.8 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:8.

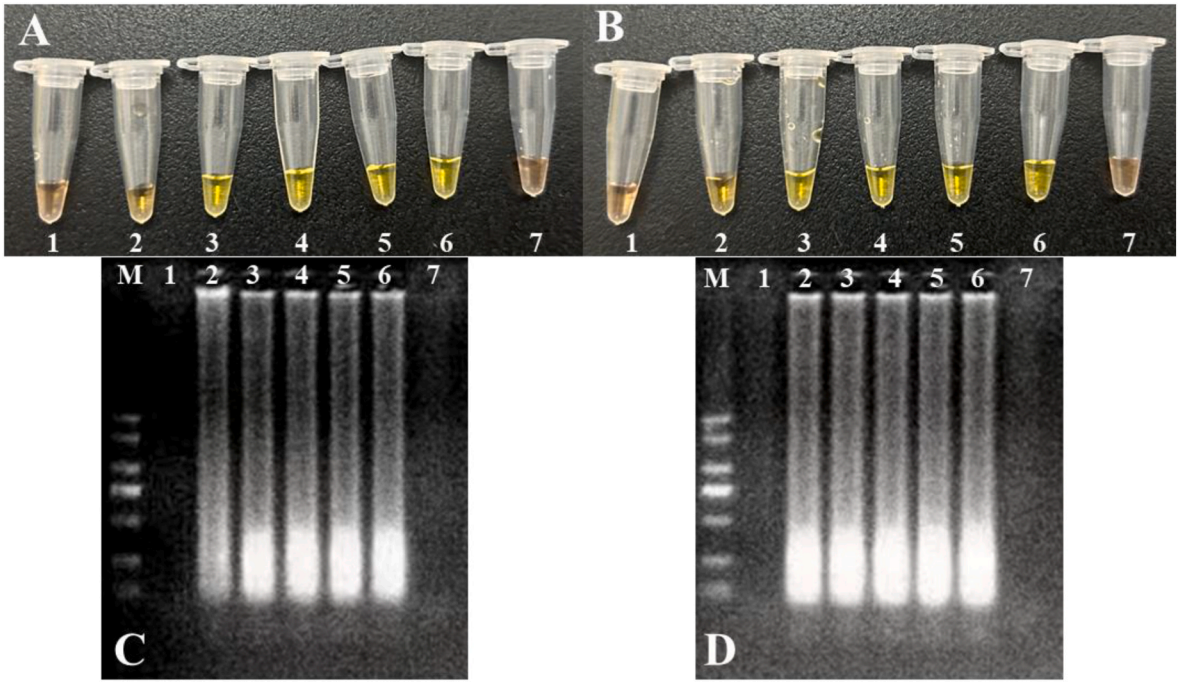

Fig. 3. Betaine concentration optimization of PSR assay using plasmid pINVA (A, C) and *S. enterica* ATCC14028 (B, D) by chromogenic reaction (positive: green, negative: orange) (A, B) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (C, D). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–6, PSR systems with betaine concentration at 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, respectively; lane 7, negative control. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

terminated at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, respectively, were included to examine the optimal reaction time of PSR assay (Fig. 1). All negative controls and positive reactions terminated at 10–40 min showed orange color in chromogenic reaction and no band in agarose gel electrophoresis. Positive reactions terminated at 50–70 min had a color change from orange to green in chromogenic reaction (the

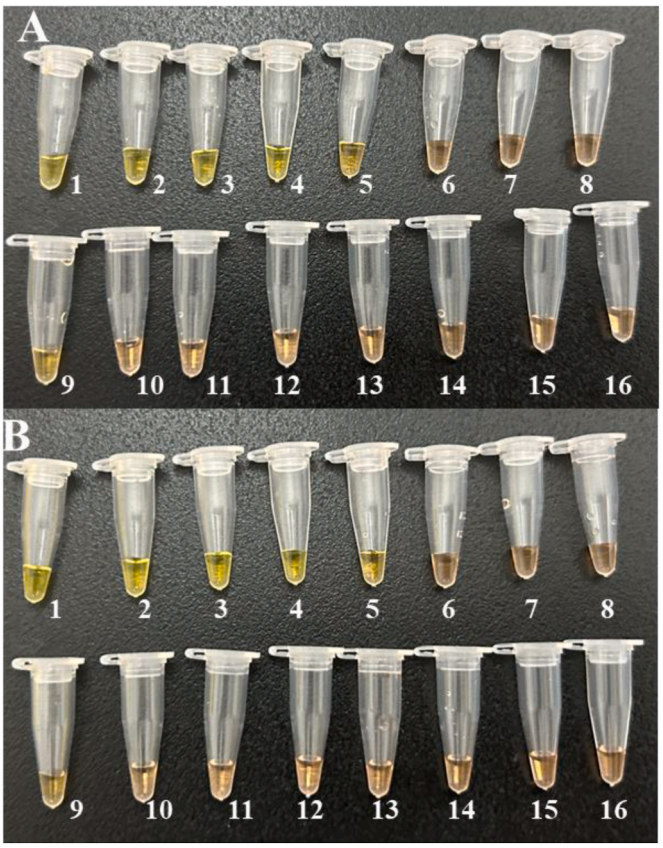

Fig. 4. Chromogenic reagent (positive: green, negative: orange) optimization of PSR assay using plasmid pINVA (A) and *S. enterica* ATCC14028 (B). Tube 1–8, positive reactions with calcein and Mn^{2+} ratio at 1:1, 1:2, 1:4, 1:6, 1:8, 1:12, 1:16, 1:20, respectively; tube 9–16, negative controls with calcein and Mn^{2+} ratio at 1:1, 1:2, 1:4, 1:6, 1:8, 1:12, 1:16, 1:20, respectively. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M.

color change in reaction terminated at 50 min was relatively weak) and ladder pattern bands in agarose gel electrophoresis. The results indicated 60 min was the optimal reaction time for PSR assay targeting *invA* gene in *S. enterica*.

Concerning reaction temperature, 65 °C was the most widely used but other temperatures ranging from 60 °C to 65 °C had also been used in different studies (Das et al., 2018; Milton et al., 2021; Sharma et al., 2022). In this study, more varieties in reaction temperature were examined. A total of 6 positive reactions each for plasmid pINVA and DNA of *S. enterica* ATCC14028 as template with corresponding negative control reactions with temperature at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, respectively, were included (Fig. 2). All negative controls showed orange color in chromogenic reaction and no band in agarose gel electrophoresis. Positive reactions with temperature at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C all had orange to green color change in chromogenic reaction (the color change in reactions at 59 °C and 69 °C were relatively weak) and ladder pattern bands in agarose gel electrophoresis. The results indicated temperatures ranging from 61 °C to 67 °C were suitable for PSR assay targeting *invA* gene in *S. enterica*.

Thirdly, PSR systems with betaine concentration at 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, respectively were performed with plasmid pINVA and DNA of *S. enterica* ATCC14028 as template (Fig. 3). Orange to green color change in chromogenic reaction (the color change in reaction with betaine concentration at 0.5 M was relatively weak) and ladder pattern bands in agarose gel electrophoresis (The band in reaction with betaine concentration at 0.5 M and plasmid pINVA as template was relatively weak) were shown in PSR systems with betaine concentrations ranging from 0.5 M to 0.9 M. The results indicated betaine concentrations ranging from 0.6 M to 0.9 M were more appropriate for PSR assay targeting *invA* gene in *S. enterica*.

In addition, the calcein and Mn^{2+} ratio in chromogenic reagent is critical for chromogenic reaction to enable naked eye result observation. In this study, positive reactions with plasmid pINVA and DNA of *S. enterica* ATCC14028 as template and negative controls were performed using chromogenic reagent with calcein and Mn^{2+} ratio ranging from 1:1 to 1:20 (Fig. 4). Orange color was observed in all negative controls and positive reactions using chromogenic reagent with calcein and Mn^{2+} ratio at 1:12, 1:16, and 1:20. Weak orange to green color change was shown in positive reaction using chromogenic reagent with calcein and Mn^{2+} ratio at 1:8. A clear visible change from orange to green color was observed in positive reaction using chromogenic reagent with calcein and Mn^{2+} ratio at 1:1, 1:2, 1:4, and 1:6. However, the negative control using chromogenic reagent with calcein and Mn^{2+} ratio at 1:1 had slight weak green which could influence result determination. Thus, chromogenic reagent with calcein and Mn^{2+} ratio at 1:2, 1:4, and 1:6 was suitable for naked eye result observation in PSR assay targeting *invA* gene in *S. enterica*.

In summary, the optimized PSR assay was established with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

3.2. PSR assay with high specificity and sensitivity

To evaluate the specificity of the optimized PSR assay targeting *invA* gene in *S. enterica*, plasmid pINVA and genomic DNA extracted from 2 strains of *S. enterica* and 44 strains of non-*S. enterica* bacteria were used as DNA template, and sterile water was used to replace DNA template as a negative control (Fig. 5). The results were interpreted by chromogenic reaction (Fig. 5A) and agarose gel electrophoresis (Fig. 5B). Orange to green color change in chromogenic reaction and ladder pattern bands in agarose gel electrophoresis were shown in PSR systems with plasmid pINVA and genomic DNA extracted from 2 strains of *S. enterica*. While negative control and PSR systems with genomic DNA extracted from the 44 strains of non-*S. enterica* bacteria showed orange color in chromogenic reaction and no band in agarose gel electrophoresis. The results demonstrated high specificity of the optimized PSR assay and designed primers targeting *invA* gene for the detection of *S. enterica*.

To determine the sensitivity of the PSR assay, different concentrations of plasmid pINVA (10,000 copies, 1000 copies, 100 copies, 10 copies, 1 copy) and genomic DNA of *S. enterica* ATCC14028 (12.3 ng/μL, 1.23 ng/μL, 123 pg/μL, 12.3 pg/μL, 1.23 pg/μL) were applied as template (Fig. 6). Orange to green color change in chromogenic reaction and ladder pattern bands in agarose gel electrophoresis were shown in PSR systems with plasmid pINVA at 10 to 10,000 copies and genomic DNA at 123 pg/μL to 12.3 ng/μL. While negative control and PSR systems with plasmid pINVA at 1 copy and genomic DNA at 1.23 pg/μL to 12.3 pg/μL showed orange color in chromogenic reaction and no band in agarose gel electrophoresis. The results showed the detection limit of the optimized PSR assay was plasmid pINVA at 10 copies and genomic DNA at 123 pg/μL. It indicated that the optimized PSR assay for the detection of *S. enterica* has a high sensitivity.

3.3. The application of PSR assay in various food systems

In view of the complex composition of the food matrix, which has influence on the detection of *S. enterica*, the optimized PSR assay was applied for the detection of artificially contaminated food models including liquid (milk, egg, carrot juice, tomato juice, apple juice) and solid food samples (rice and flour product, pepper, tomato, beef, chicken wing, meatball). *S. enterica* ATCC14028 artificial food contamination models were built by subculturing cells at different concentrations (10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, 10 CFU/mL) into various food systems. Subsequent DNA isolation and PSR assay were

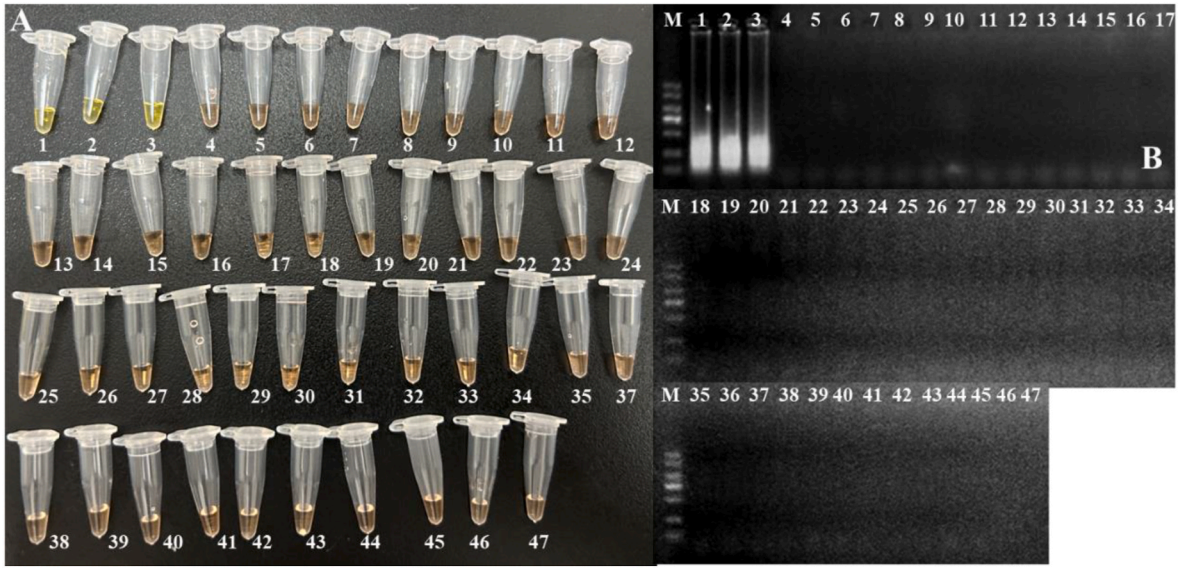

Fig. 5. Specificity test of PSR assay for detection *S. enterica* strains with i*nvA* gene by chromogenic reaction (positive: green, negative: orange) (A) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (B). M: DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–3, plasmid pINVA, *S. enterica* ATCC14028, *S. enterica* ATCC29629, respectively; lane/tube 4–46, 5 *Escherichia coli* O157:H7 (ATCC43895, E019, E020, E043, E044), 3 *Vibrio parahaemolyticus* (ATCC27969, ATCC17802, R40), 1 *Vibrio cholerae* (R44), 2 *Listeria monocytogenes* (ATCC19114 and ATCC19116), 3 *Pseudomonas aeruginosa* (ATCC27853, C9, C40), 1 *Pseudomonas putida* (Guangzhou-Ppu420), 3 *Staphylococcus aureus* (ATCC23235, Guangzhou-SAU085, Guangzhou-SAU071), 1 *Staphylococcus epidermidis* (9140-M10), 1 *Staphylococcus intermedius* (Sint230001), 1 *Staphylococcus haemolyticus* (Shae230001), 1 *Staphylococcus saprophyticus* (ATCC27840), 1 *Lacticaseibacillus casei* (Lcas200001), 1 *Levilactobacillus brevis* (Lbre200001), 1 *Lactiplantibacillus plantarum* (ATCC8014), 1 *Pediococcus acidilactici* (Paci180001), 1 *Pediococcus damnosus* (Pdam230001), 6 *Klebsiella pneumoniae* (Kp1, Kp2, Kp3, Kp4, Kp5, Kp6), 6 *Bacillus cereus* (ATCC14579, ATCC13061, ATCC11778, ATCC12480, ATCC25621), 1 *Yersinia enterocolitica* (ATCC9610), 1 *Cronobacter sakazakii* (BAA894), and 1 *Acinetobacter baumannii* (12052313), respectively; lane/tube 47, negative control. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

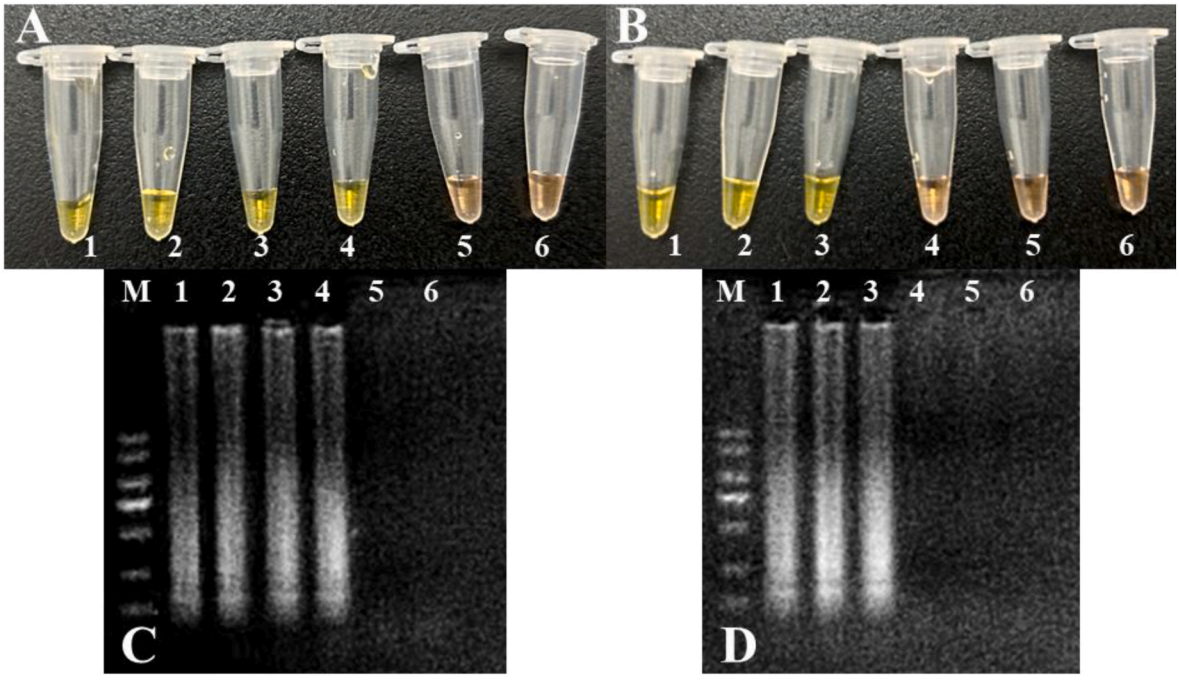

Fig. 6. Sensitivity test of the PSR assay using plasmid pINVA (A, C) and *S. enterica* ATCC14028 (B, D) by chromogenic reaction (positive: green, negative: orange) (A, B) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (C, D). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–5 in A, plasmid at 10,000 copies, 1000 copies, 100 copies, 10 copies, 1 copy, respectively; lane/tube 1–5 in B, genomic DNA concentration at 12.3 ng/μL, 1.23 ng/μL, 123 pg/μL, 12.3 pg/μL, 1.23 pg/μL; lane/tube 6, negative control. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

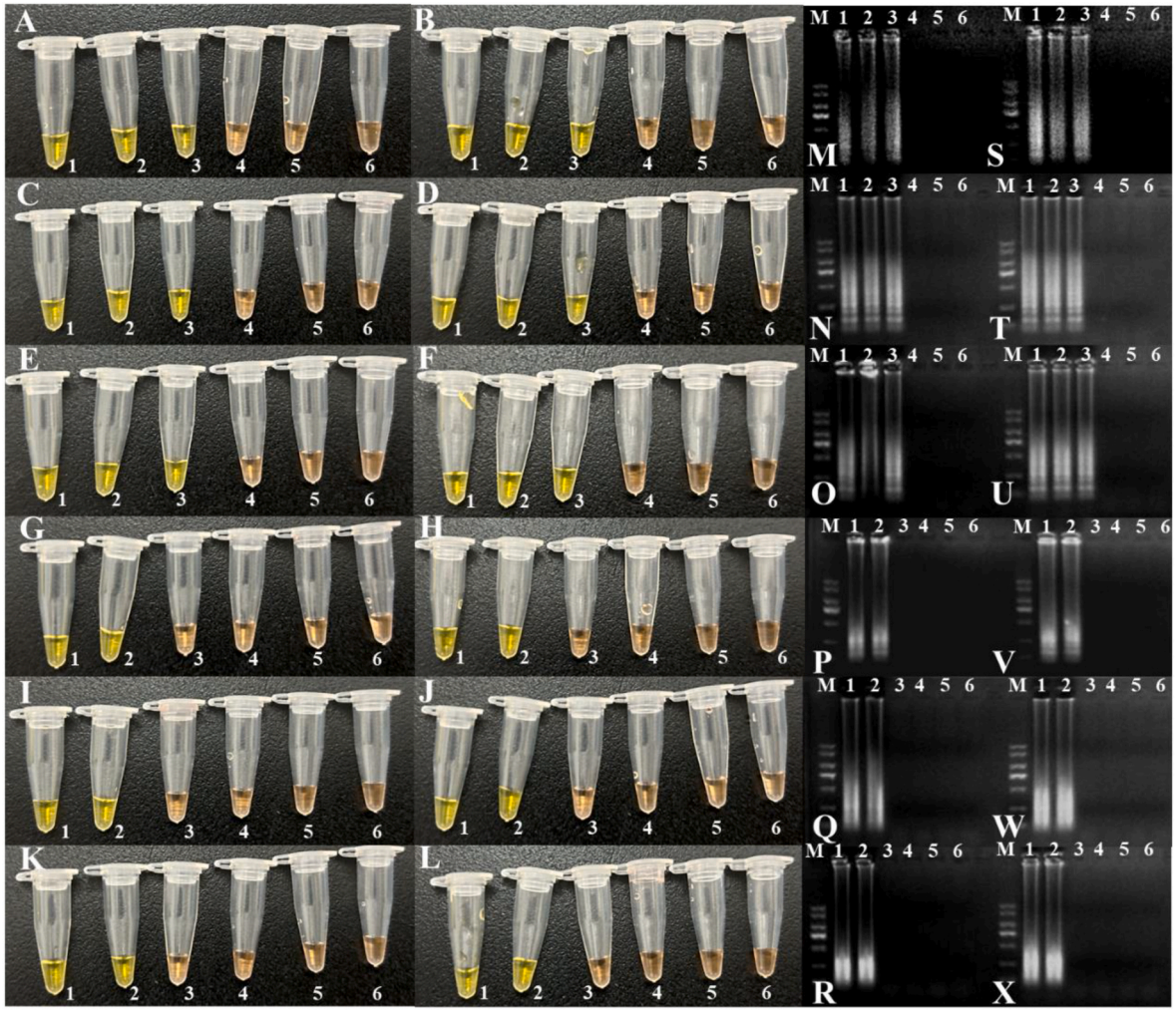

Fig. 7. Sensitivity test of the PSR assay using *S. enterica* ATCC14028 artificial food contamination models (A, M: milk, B, N: egg, C, O: carrot juice, D, P: orange juice, E, Q: tomato juice, F, R: apple juice, G, S: rice and flour product, H, T: pepper, I, U: tomato, J, V: beef, K, W: chicken wing, L, X: meatball) by chromogenic reaction (positive: green, negative: orange) (A–L) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (M–X). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–5, 10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, 10 CFU/mL; lane/tube 6, negative control. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

performed on the artificially contaminated food models. The results showed that the optimized PSR assay was applicable in the detection of *S. enterica* from the 12 types of food system (Fig. 7). Interestingly, the detection limit of the optimized PSR assay was 10^3 CFU/mL in all liquid food samples and 10^4 CFU/mL in all solid food samples, possibly due to the more complicated matrix of solid food samples. This demonstrated that the optimized PSR assay is effective for the detection of *S. enterica* in various food systems and has a strong practical value especially in liquid food samples.

3.4. PMA-PSR for VBNC cells detection in various food systems

In order to investigate whether PMA combined with the optimized PSR assay can be used to detect *S. enterica* cells in the VBNC state, culturable cells, VBNC cells, and dead cells were treated with PMA, followed by DNA extraction and optimized PSR assay. In addition, to examine the applicability of the PMA-PSR assay in various food systems, the detection of culturable cells, VBNC cells, and dead cells in artificially contaminated food models including liquid (milk, egg, carrot juice, tomato juice, apple juice) and solid food samples (rice and flour product, pepper, tomato, beef, chicken wing, meatball) were conducted (Fig. 8). Orange to green color change in chromogenic reaction and ladder pattern bands in agarose gel electrophoresis were shown in PMA-PSR systems with DNA from culturable cells and VBNC cells as template. While orange color in chromogenic reaction and no band in agarose gel electrophoresis were shown in negative control and PMA-PSR systems DNA from dead cells as template. The above results indicated that the PMA-PSR assay was successfully established to detect VBNC cells of *S. enterica* and applied in various food systems.

4. Discussion

S. enterica, as one of the most important pathogens causing foodborne diseases in humans and animals, causes economic loss to China's livestock farming industry every year. At present, the traditional culture-based method for *S. enterica* detection requires a long detection period and causes false negative detection due to the presence of VBNC cells (Foddai & Grant, 2020). Immunological detection methods or molecular biological detection techniques require special laboratory equipment and professional operators, thus greatly limiting their application in on-site detection. Therefore, the development and application of rapid, low-cost, specific, and highly sensitive detection techniques remain a hot topic. In addition, the entry of foodborne pathogenic bacteria into the VBNC state poses a threat to the food

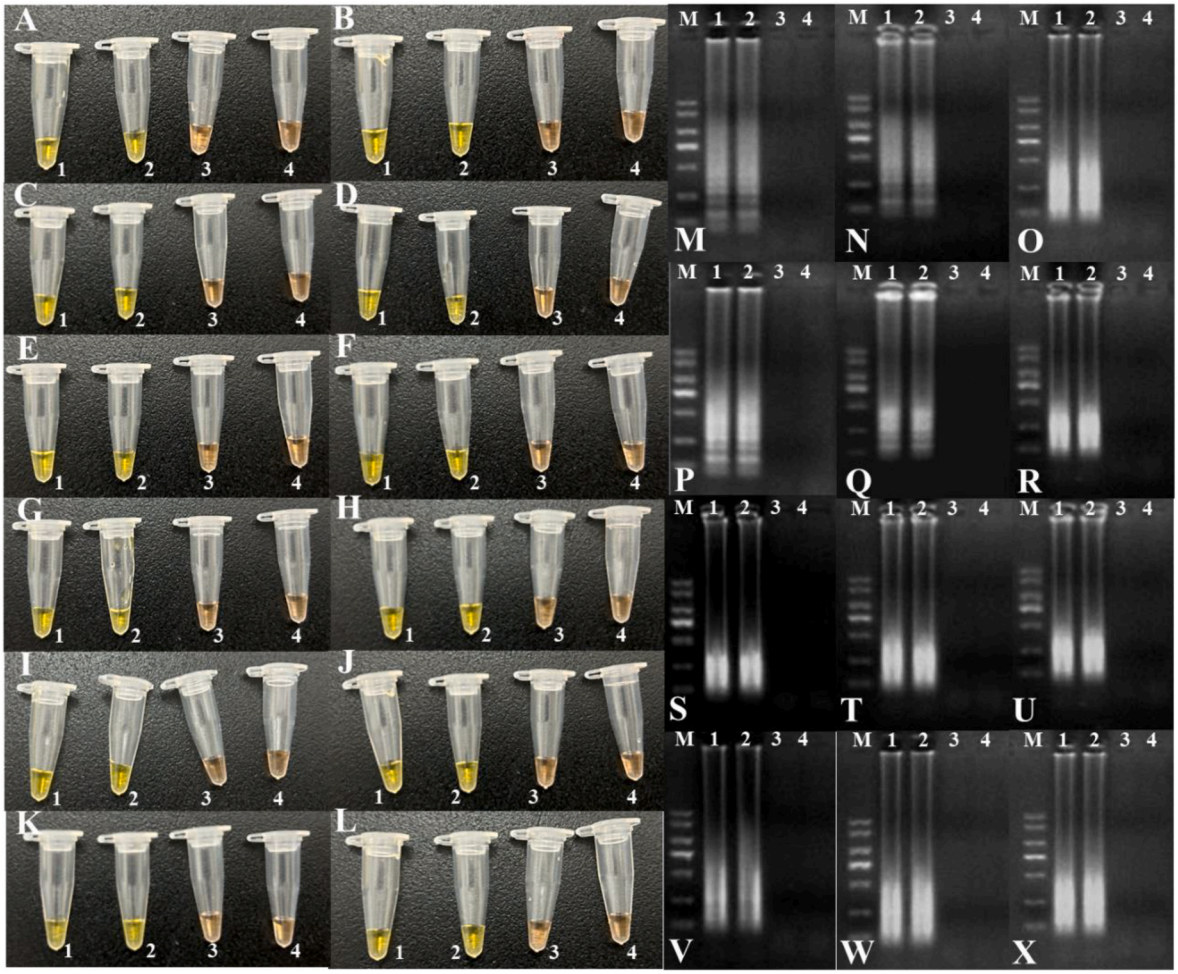

Fig. 8. Application test of PMA-PSR assay on detecting VBNC cells using artificial food contamination models (A, M: milk, B, N: egg, C, O: carrot juice, D, P: orange juice, E, Q: tomato juice, F, R: apple juice, G, S: rice and flour product, H, T: pepper, I, U: tomato, J, V: beef, K, W: chicken wing, L, X: meatball) by chromogenic reaction (positive: green, negative: orange) (A–L) and 1.5 % agarose gel electrophoresis (positive: ladder pattern band, negative: no band) (M–X). M, DNA marker (lanes from top to bottom refer to 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); lane/tube 1–3, culturable cells, VBNC cells, dead cells, respectively; lane/tube 4, negative control. The PSR system was set with reaction time at 60 min, reaction temperature at 65 °C, betaine concentration at 0.6 M and chromogenic reagent with calcein and Mn^{2+} ratio at 1:4.

Table 3
Comparison of PMA-PSR with other detection methods.

Method	Specificity	Sensitivity	Time	Advantage	Disadvantage	Reference
Culture-based methods	-*	Low	1–7 days	Low cost	Time consuming, determine culturable cell number only	Foddai and Grant (2020)
Direct viable Count	–	Low	5–6 h	Fast	Lack of accuracy, limit to specific species	(K. Dong et al., 2020)
LIVE/DEAD bacterial viability kit	–	Low	3–4 h	Differentiate live and dead cells	Toxic, mis-identify hollow but membrane-enclosed vessels as viable cells, result determination relies on microscopy or flow cytometry	Kumar and Ghosh (2019)
RT-PCR	+++	High	2–3 h	High specificity	RNA not stable, high cost, require RT-PCR machine	Sheridan et al. (1998)
PMA-PCR	+	High	30 min + 2 h**	Low cost	Require PCR machine, result determination reply on gel electrophoresis, non-quantitative	Nocker et al. (2006)
PMA-qPCR	++	High	30 min + 2–3 h	Quantitative	Require qPCR machine, high cost	Kibbee and Örmeci (2017)
PMA-LAMP	++	High	30 min + 60 min	Fast, high sensitivity, naked eye result determination, no specific machine required	Non-quantitative, complex primer design	Yan et al. (2017)
PMA-PSR	+++	High	30 min + 60 min	Fast, high sensitivity, simple, naked eye result determination, no specific machine required	Non-quantitative	This study

*The symbol "-" refers to "non-specific", "+" refers to the specificity of PCR, which is high, symbols "++" and "+++" represent the specificity level which is relatively higher than PCR.
**For the time format A + B, A refers to the time for PMA treatment, B refers to the time for the amplication reaction.

industry and food safety. The commonly used methods for the detection of bacteria in VBNC state include Direct Viable Count (DVC), LIVE/-DEAD bacterial viability kit, flow cytometry, immunology, and molecular biology (Cappelier et al., 2005; Khan et al., 2010; Kogure et al., 1979; G. M. G. M. Liu, Han, Li, & Song, 2006). With the development of molecular biology, nucleic acid amplification technology has gradually developed into an alternative to traditional culture-based detection methods with high specificity and sensitivity. Therefore, molecular biology methods have a wide range of prospects in the VBNC state of bacteria.

In recent years, the application of propidium monoazide (PMA) in combination with nucleic acid amplification technology has provided a new idea for the detection of VBNC state microorganisms (Ou et al., 2021; Zhao et al., 2013; Zhou et al., 2020). The main principle is that PMA can cross-link with the DNA double helix structure in the cell through the damaged cell membrane (dead cells), but not through the intact cell membrane (viable cells), so that the primers cannot recognize the target region, resulting in the inhibition of PCR amplification of DNA molecules, thus achieving the identification of VBNC state and dead state bacteria. On the other hand, PSR is a new nucleic acid detection technique for pathogenic microorganisms based on LAMP and PCR (Sharma et al., 2022; Wu et al., 2019). It solves the problem of complex primer design for LAMP on the one hand and is more sensitive than PCR on the other. In particular, compared to PCR and RT-qPCR, PSR retains a high degree of specificity and sensitivity, does not rely on complex instrumentation, and can amplify a large number of target genes in a short period of time (Table 3) (Liu, Huang, et al., 2023). Thus, in this study, we proposed establishing a rapid PMA-PSR detection method on viable cells of *S. enterica* as a potential POCT applicable in various food systems.

In order to ensure the stability and effectiveness of the PMA-PSR assay, firstly the optimization of the PSR assay which is critical for the stability was performed by changing the key factors including reaction time, temperature, betaine concentration and chromogenic reagent. Secondly, the specificity and sensitivity of the PSR assay were examined to ensure *S. enterica* was effectively detected. Thirdly, considering the complexity of food matrix, 12 different types of food covering liquid and solid food samples were included to construct artificially contaminated food models and examine the applicability of PSR assay in various food samples. Fourthly, PMA treatment, which is critical for the specific identification of viable cells, was combined with the optimized PSR assay to establish the PMA-PSR assay. Lastly, the PMA-PSR assay was applied in the detection of VBNC cells in 12 different types of food samples to ensure its applicability in various food systems. In addition, during the PSR reaction, a large amount of DNA and insoluble magnesium pyrophosphate are synthesized, resulting in an increase in turbidity of the solution, which can be observed as a specific "ladder band" by gel electrophoresis when the turbidity reaches a certain concentration. Therefore, the main detection methods for PSR amplification products are turbidimetry and gel electrophoresis. In order to meet the requirements for a rapid and simple assay, we use calcein as an indicator. The positive reaction turns calcein from its original orange color to a green color, so the reaction does not require opening the tube from start to finish, which avoids false positives, the test time is short, the color change is directly observed, and the results can be read by the naked eye. This is an important application for improving the rapid detection and screening of infectious diseases at POCT level. In our study, the method has a high successful test rate when using the optimized conditions. In practice, chromogenic reagent is suggested to add in the reaction system to avoid open lid and false positive detection. Each sample is recommended to test in triplicate to ensure stability and accuracy.

5. Conclusion

Due to its high risk in pathogenicity, *S. enterica* is required to be strictly non-detectable in routine pathogens screening in foods. However, conventional culturing methods are unable to detect VBNC cells. Microbial cells in VBNC state have been previously documented to be able to produce harmful substances as normal cells do. Thus, considering the high pathogenicity of *S. enterica*, their VBNC cells should also be required to be non-detectable. However, a POCT like assay for rapid and on-site identification of *S. enterica* VBNC cells are currently unavailable. In the present study, the PSR assay is simple in primer design, requires only one enzyme, does not require repeated sample addition, can be completed at a constant temperature, and has good specificity, sensitivity, and ease of operation. The amplification results can be read directly by visual observation without the use of a turbidimeter, which is convenient and fast. Then, we have connected the PMA assay with PSR processing to achieve a PMA-PSR assay to directly identify the VBNC cells of *S. enterica*. This study provides a novel method for the rapid detection of *S. enterica* in the field of food hygiene inspection. Considering its rapidity, convenience (in result determination) and simplicity (free of particular equipment), the established PMA-PSR assay is demonstrated to be a potential POCT for VBNC cells detection, which should not be limited within *S. enterica* but also applicable to other non-detectable pathogens. Although acquiring high sensitivity, the PMA-PSR assay is easy to cause false positive detection and fails to be applied as quantitative detection method. These are the limitation of this assay and remain to be explored. Although chromogenic reagent used in this study can avoid open lid and false positive detection, it requires cautious experimental performance. A more sensitive tool (for example biosensor) is to be developed and evaluated to determine the signals or chemicals produced during PSR thus ensure quantitative detection.

CRediT authorship contribution statement

Junyan Liu: Writing – review & editing, Writing – original draft, Visualization, Methodology. **Wei Hong:** Writing – review & editing, Writing – original draft, Validation, Investigation. **Jingxuan Zhao:** Writing – review & editing, Validation, Resources, Data curation. **Haoyue Xue:** Writing – original draft, Software. **Thanapop Soteyome:** Writing – review & editing, Formal analysis. **Lei Yuan:** Writing – review & editing, Visualization. **Gamini Seneviratne:** Writing – review & editing, Supervision. **Zhenbo Xu:** Writing – review & editing, Validation, Supervision, Conceptualization.

Informed consent

Not applicable.

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.

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Data availability

Data will be made available on request.

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