Methods and Applications

Development of A Novel EvaGreen-Dye Based Recombinase Aided Amplification Assay Using Self-Avoiding Molecular Recognition System Primers

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ABSTRACT

Introduction: Fluorescent probe-based recombinase aided amplification (RAA) offers the advantages of rapidity and simplicity but is limited by the requirement for complex and lengthy probe design, restricting its widespread application.

Methods: A novel EvaGreen dye-based RAA (EvaGreen-RAA) assay utilizing self-avoiding molecular recognition system (SAMRS) primers was developed for the detection of *Pseudomonas fluorescens* (PF) and *Bacillus cereus* (BC) in milk. Conventional RAA was used as a reference method. Sensitivity was evaluated using nucleic acids from recombinant plasmids and simulated milk specimens. Additionally, a dual EvaGreen-RAA assay was investigated for simultaneous detection of mixed BC and PF in simulated milk specimens.

Results: The EvaGreen-RAA demonstrated superior sensitivity compared to conventional RAA, with detection limits of 1 copy/μL versus 10 copies/μL for both BC and PF plasmids, respectively. In simulated milk specimens, EvaGreen-RAA detected BC and PF at concentrations of 100 CFU/mL and 200 CFU/mL, respectively, compared to 400 CFU/mL and 600 CFU/mL for conventional RAA. The dual EvaGreen-RAA assay successfully detected mixed BC and PF in simulated milk specimens at concentrations of 200 CFU/mL for each pathogen.

Conclusion: The EvaGreen-RAA assay demonstrated significant advantages in terms of simplicity and enhanced sensitivity compared to fluorescent probe-based RAA, offering a novel approach for developing multiplex pathogen detection systems using melting curve analysis.

Pseudomonas fluorescens (PF) is one of the primary bacterial agents responsible for spoilage and deterioration of milk and eggs during low-temperature storage, and can lead to shock following human infection in severe cases (*1*). *Bacillus cereus* (BC) represents the most prevalent foodborne pathogen in raw milk and dairy farm environments (*2*). Both bacteria commonly contaminate dairy products as psychrophiles; when humans consume dairy products contaminated with these psychrophiles, they can experience vomiting, diarrhea, and various other illnesses (*3*). With the increasing recognition of protein's nutritional importance, milk has become an indispensable food source for organisms that require external protein intake (*4*).

Currently, most quality inspection departments in dairy enterprises employ traditional culture methods for pathogen detection, a process requiring culture, isolation, purification, and biochemical and serological identification. This approach typically requires 5–7 days before results are available, with exceedingly complex and time-consuming detection steps. PCR-based molecular methods have also been implemented for pathogen diagnosis in dairy products, reducing detection time to 1–2 hours, but these methods require relatively complex thermal cycling instruments and operational procedures. In recent years, isothermal amplification technologies have gained widespread acceptance due to their time efficiency, eliminating the need for complex temperature cycling processes (*5*). Recombinase aided amplification (RAA) offers high sensitivity and specificity, completing nucleic acid amplification at 37–42 °C within 20–30 min; however, a significant limitation of RAA is the requirement for designing complex and lengthy probes (45 nucleotides in length) (*6*–*7*). Although dye-based isothermal amplification methods without fluorescent probes allow result visualization, these approaches exhibit reduced specificity due to non-specific binding of dyes to double-stranded DNA.

The self-avoiding molecular recognition system (SAMRS) employs nucleotide analogs A*, G*, C*, and

T* to substitute for A, G, C, and T at specific positions within primers, where A* pairs with T, T* pairs with A, G* pairs with C, and C* pairs with G. Additionally, SAMRS primers can bind to normal single-stranded DNA. Since SAMRS primers do not bind to each other, the formation of primer dimers can be minimized or even eliminated (*8*).

In this study, we replaced several bases at the 3' terminus of standard RAA primers with unnatural bases to create SAMRS primers and developed an EvaGreen-RAA assay for BC and PF detection using these SAMRS primers. This approach enables real-time observation of reaction results within 30 minutes while eliminating the need to design complex RAA probes, thereby maintaining the sensitivity and specificity of the RAA assay.

METHODS

Strain Collection and Nucleic Acid Extraction

Standard strains of *Bacillus cereus* (ATCC13525) and *Listeria monocytogenes* (ATCC11778) were purchased from the BeNa Culture Collection. Nucleic acid was extracted using the FastPure® Microbiome DNA Isolation Kit (Vazyme Biotech, Nanjing, China).

The Preparation Process of SAMRS Nucleotides

All intermediates and final products were characterized by ^{1}H NMR (Nuclear Magnetic Resonance) or ^{31}P NMR, with spectral data consistent with previously reported findings (*9*). All oligonucleotides containing modified nucleobases were synthesized by Sangon Biotech.

Primer and Probe Design

The B subunit of the DNA gyrase gene (gyrB) and alkaline metalloprotease gene (aprX) were selected as specific targets for the identification of BC and PF, respectively. Highly conserved regions of gyrB and aprX genes were downloaded from NCBI GenBank and subjected to sequence alignment using BioEdit software. The designed primers and probes were evaluated using Oligo7 and Amplifx software. The specific sequences of primers and probes are presented in Table 1.

Construction of Recombinant Plasmids

The target fragments of gyrB and aprX genes were cloned into pUC57 vector for the construction of recombinant plasmids, respectively, which were synthesized by TsingKe Biotech Crop (Beijing, China). Plasmids were quantified using the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States), and the copy number per microliter was calculated according to the equation: copies/reaction=[(6.02 × 10^{23}) × concentration (ng/μL) × 10^{-9}] / [number of bases (bp) × 660]. The lyophilized plasmids were reconstituted by adding 40 μL of TE buffer, quantified, and stored at –80 ℃ until use.

Establishment of RAA and EvaGreen-RAA

The RAA reaction was performed in a total volume of 50 μL, containing a dry powder pellet, 2 μL of RAA standard nucleotide (STD) primer, 4 μL of betaine, 0.6

TABLE 1. Primer and probe sequences used in this study.

Primer/probe	Sequence (5'–3')	Length	Amplicon size (bp)
BC-RAA-F	GTAAAATCCATTACCAAAAATACGAAAGAGG	31	167
BC-SAMRS-F	GTAAAATCCATTACCAAAAATACGAAA*G*A*G*G	31	
BC-RAA-R	TTCACGCATACGAGTTGCTAGCGTATCAAA	30	
BC-SAMRS-R	TTCACGCATACGAGTTGCTAGCGTA*TC*A*A*A	30	
BC-RAA-P	TCATTGGTGACACCGATCAAACAGGAACRA/i6FAMdT/A/idSp/C/iBHQ1dT/CGATTTAAACCAGAT-C3	49	
PF-RAA-F	CGGCAACCCGACCTATAACGACGCAACCTA	30	177
PF-SAMRS-F	CGGCAACCCGACCTATAACGACGCA*A*C*C*TA	30	
PF-RAA-R	TTGGCACCGTAGAGCTTCTGGATCGCCGCAAT	32	
PF-SAMRS-R	TTGGCACCGTAGAGCTTCTGGATCGCCG*C*A*A*T	32	
PF-P	CAGGACACGCGTGGCTATAGCCTCATGAGT/i6FAMdT//idSp/C/iBHQ1dT/GGAGCGAGAGCAACAC-C3	49	

Abbreviation: SAMRS=self-avoiding molecular recognition system; RAA=recombinase aided amplification; FAM=6-carboxyfuorescein; THF=tetrahydrofuran; BHQ=black hole quencher; C3-spacer=3'phosphate blocker; A*, G*, and C* =SAMRS nucleotides.

μL of probe, 5 μL of template, and 29.4 μL of reaction buffer (Amp-Future, Changzhou, China). Additionally, 2.5 μL of magnesium acetate (280 mmol/L) was added to the reaction lid as an initiator for the RAA reaction, followed by brief centrifugation to ensure rapid reaction initiation at 39 ℃ for 30 min.

The EvaGreen RAA reaction differed from the standard protocol by the addition of 2.5 μL of EvaGreen dye, 2 μL of betaine, and 1.2 μL each of SAMRS forward and reverse primers (10 μmol/L). All other reagents remained identical to those described above.

Assessing the Ability of SAMRS Primers to Reduce Primer Dimerization

Serial dilutions of recombinant plasmids (10^1–10^6 copies/μL) were used as templates for probe-free RAA reactions with both STD and SAMRS primers for BC and PF at 39 °C for 30 min. Following amplification, products underwent a melting curve analysis from 60 to 95 °C with a temperature gradient of 0.2 °C/s. Fluorescence signals were detected in the SYBR green channel, and high-resolution melting curves (HRM) were generated by plotting the negative inverse of fluorescence values against temperature. In addition to HRM analysis, primer dimer formation was evaluated using agarose gel electrophoresis (AGE).

Sensitivity and Specificity of RAA and EvaGreen-RAA assays

To validate the sensitivity of both RAA and EvaGreen RAA assays, serial dilutions of BC or PF recombinant plasmids (10^0–10^5 copies/reaction) were used as templates, with DEPC water serving as a negative control. Each reaction was performed in eight replicates.

The specificity of both assays was evaluated using 18 common foodborne pathogens, including BC (ATCC11778), PF (ATCC13525), *Staphylococcus aureus* (ATCC29213), *Listeria monocytogenes* (ATCC19115), *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae* (ATCC11296), *Escherichia coli* (isolated strains), *Staphylococcus epidermidis* (isolated strains), *Enterococcus faecium* (isolated strains), *Enterococcus faecalis* (isolated strains), *Enterobacter cloacae* (isolated strains), *Pseudomonas maltophilia* (isolated strains), *Proteus mirabilis* (isolated strains), *Candida albicans* (ATCC753), *Candida tropicalis* (ATCC750), *Candida parapsilosis* (ATCC22019), *Candida glabrata* (ATCC2001), and *Candida krusei* (ATCC6258). DEPC water was used as a negative control, with each reaction performed in eight replicates.

Preparation of Simulated Milk Specimens and Detection

Standard BC and PF strains were inoculated into Nutrient Broth (NB) and Luria-Bertani (LB) liquid media, respectively, and cultured overnight on a shaker. Bacterial suspensions were quantified spectrophotometrically, with OD values between 0.45–0.5 corresponding to approximately 10^8 CFU/mL. The accuracy of spectrophotometric quantification was verified by direct microscopic enumeration using a bovine abalone blood counting chamber. Following sequential ten-fold dilutions, appropriate bacterial concentrations were added to sterilized milk to prepare simulated specimens. Nucleic acids were extracted from these specimens, and both fluorescent RAA and EvaGreen RAA assays were performed in parallel to determine their respective detection sensitivities.

Dual EvaGreen-RAA Assay for Mixed BC and PF in Simulated Milk Specimens Using Melting Curves

The dual EvaGreen-RAA reaction system comprised 29.4 μL of buffer, 1 μL each of SAMRS forward and reverse primers for PF and BC, 2.5 μL of EvaGreen dye, 2 μL of betaine (5 mol/L), 2.5 μL of magnesium acetate (280 μmol/L), and 2.5 μL of templates (corresponding to 200 CFU/mL) for each BC and PF. The RAA reaction was initially performed at 39 ℃ for 30 min, followed by high-resolution melting curve analysis on a PCR instrument.

RESULTS

SAMRS Primers Reduced or Eliminated Primer Dimerization

When STD primers were used in the RAA system, both high product peaks and non-specific primer dimer peaks at approximately 30–35 °C were observed (Figure 1A and 1E). In contrast, SAMRS primers produced only distinct high product peaks at 84 °C and 91 °C, with complete elimination of non-specific product peaks (Figure 1B and 1F). To validate these findings, amplification products from both methods

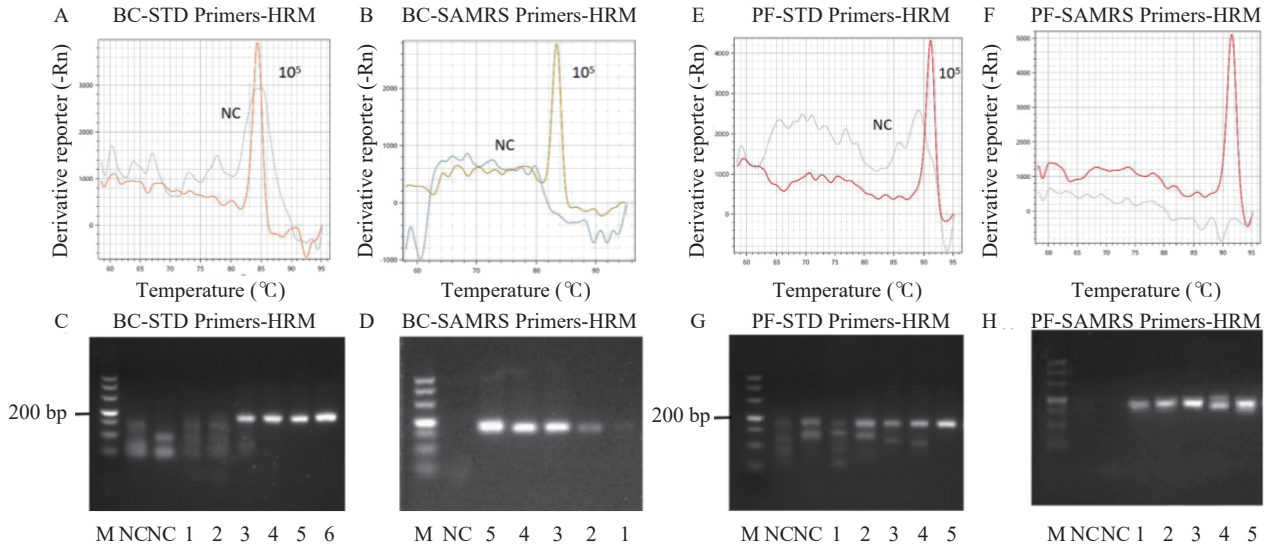

FIGURE 1. Effect of SAMRS primers on primer dimerization. (A) Merged HRM results of the STD primer for BC; (B) Merged HRM results of SAMRS primers for BC; (C) Merged HRM results of the STD primer for PF; (D) Merged HRM results of SAMRS primers for PF; (E) AGE results of STD primers for BC; (F) AGE results of SAMRS primers for BC; (G) AGE results of STD primers for PF; (H) AGE results of SAMRS primers for PF.
Note: Lane M: Marker; Lanes 1–6: 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies/μL BC or PF recombinant plasmids.
Abbreviation: SAMRS=self-avoiding molecular recognition system; AGE=agarose gel electrophoresis; STD=standard nucleotide; HRM=high-resolution melting curves; NC=negative control; BC=*Bacillus cereus*; PF=*Pseudomonas fluorescens*.

were analyzed by agarose gel electrophoresis (3%). STD primers generated prominent dimer bands (Figure 1C and 1G), whereas SAMRS primers yielded no detectable dimer formation (Figure 1D and 1H). These results conclusively demonstrate that incorporating SAMRS primers into the RAA reaction system effectively inhibited or completely eliminated primer dimer formation.

Sensitivity of RAA and EvaGreen-RAA Assays Using Recombinant Plasmids and Simulated Milk Specimens

The sensitivity of the RAA reaction was 10 copies/μL for both BC and PF (Figure 2A and 2B), while the EvaGreen-RAA reaction demonstrated enhanced sensitivity at 1 copy/μL for both pathogens using recombinant plasmids (Figure 2C and 2D). In simulated milk specimens, the detection sensitivity of the RAA assay for BC and PF was 400 CFU/mL and 600 CFU/mL (Figure 2E and 2F), respectively, whereas the EvaGreen-RAA assay exhibited superior sensitivity at 100 CFU/mL and 200 CFU/mL (Figure 2G and 2H), respectively. These results demonstrate that the utilization of SAMRS primers consistently achieves enhanced detection sensitivity compared to STD primers in equivalent assay conditions.

Specificity of RAA and EvaGreen-RAA Assays Using Common Foodborne Pathogens

Both the RAA and EvaGreen-RAA assays demonstrated high specificity for BC and PF detection, with no cross-reactivity observed against 16 other common foodborne pathogens (Table 2).

Dual EvaGreen-RAA Assay for Mixed BC and PF in Simulated Milk Specimens Using Melting Curves

Amplification products with distinct nucleotide compositions exhibit characteristic melting temperature (Tm) values. High-resolution melting curve analysis of the dual EvaGreen-RAA amplification products revealed two clearly distinguishable product peaks at 84 °C and 91 °C (Figure 3). These distinct peaks corresponded precisely to the individual melting curves of BC and PF, demonstrating that the EvaGreen-RAA assay with HRM analysis can effectively identify and differentiate mixed BC and PF contamination in simulated milk specimens at concentrations as low as 200 CFU/mL for each pathogen.

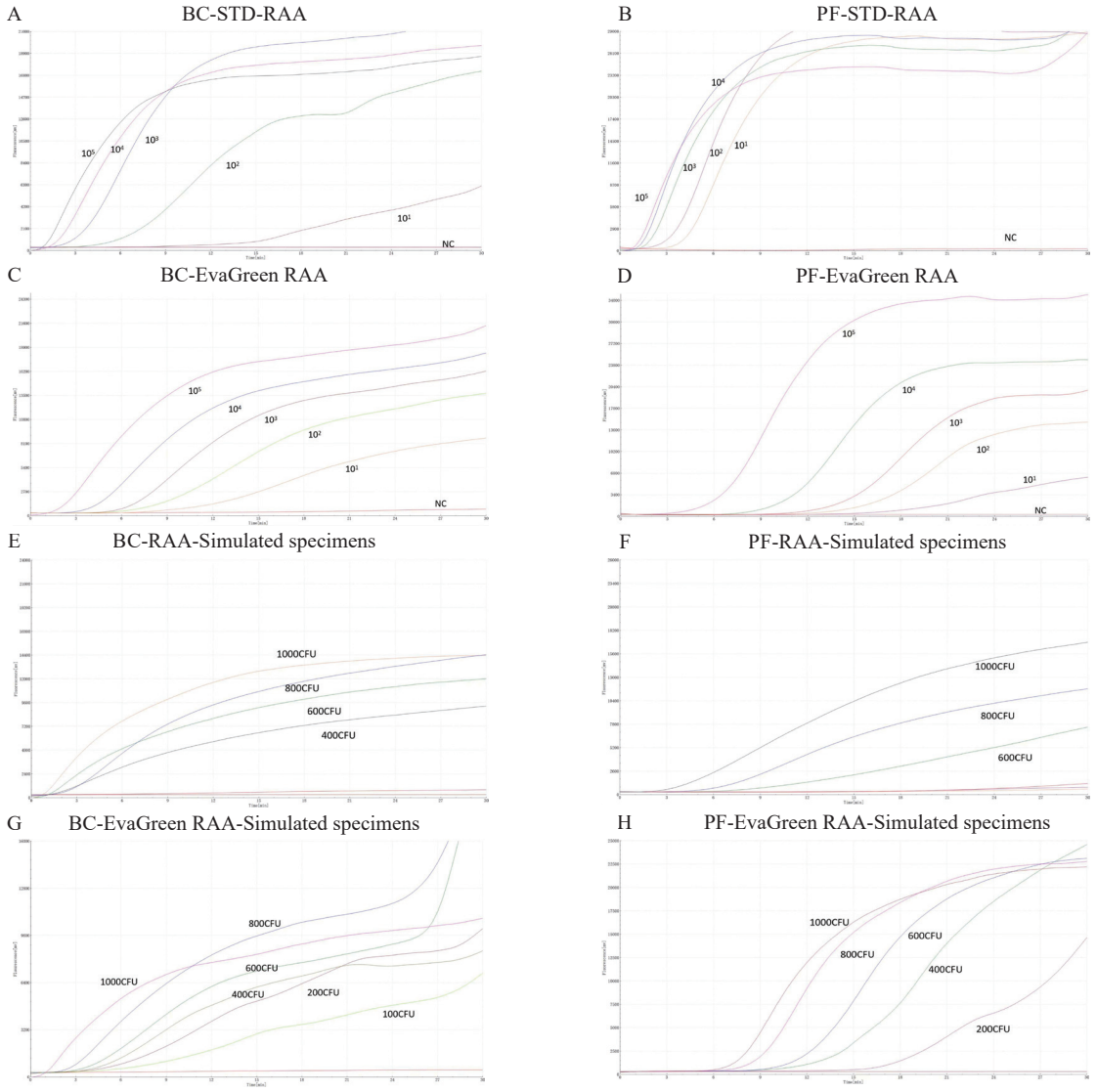

FIGURE 2. Sensitivity assessment of RAA and EvaGreen-RAA on recombinant plasmids. (A) The sensitivity of RAA for BC was 10 copies/μL; (B) The detection sensitivities of RAA for PF was 10 copies/μL; (C) The sensitivity of EvaGreen-RAA for BC was 1 copy/μL; (D) The detection sensitivities of EvaGreen-RAA for PF was 1 copy/μL. Detection of RAA and EvaGreen-RAA on simulated milk specimens; (E) The detection sensitivity of RAA for BC in simulated specimens was 400 CFU/mL; (F) The detection sensitivity of RAA for PF in simulated specimens was 600 CFU/mL; (G) The detection sensitivity of EvaGreen-RAA for BC in simulated specimens was 100 CFU/mL; (H) The detection sensitivity of EvaGreen-RAA for PF in simulated specimens was 200 CFU/mL.
Abbreviation: RAA=recombinase aided amplification; BC=*Bacillus cereus*; PF=*Pseudomonas fluorescens*.

DISCUSSION

While probe-based RAA fluorescence assays demonstrate sensitivity and specificity comparable to real-time PCR, they require probes of considerable length (45–52 bp) with a tetrahydrofuran site positioned centrally within the sequence. These requirements substantially increase both the complexity of probe design and associated costs, particularly when targeting highly variable pathogens. Furthermore, multiplexed RAA reactions present significant challenges due to interference between different probe and primer sets within a single reaction vessel.

Previous investigations have demonstrated the utility of SAMRS in various isothermal amplification techniques, including recombinant polymerase amplification (RPA) and deconjugate-dependent amplification (HDA), where strategic placement of SAMRS nucleotides at the 3' end of primer-binding sites effectively prevents the formation of most primer

TABLE 2. Bacterial strains used in the specificity test.

Strains and origins	RAA (BC)	EvaGreen-RAA (BC)	RAA (PF)	EvaGreen-RAA (PF)
Bacillus cereus	Pos (8/8)	Pos (8/8)	Neg (0/8)	Neg (0/8)
Pseudomonas fluorescens	Neg (0/8)	Neg (0/8)	Pos (8/8)	Pos (8/8)
Staphylococcus aureus	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Listeria monocytogenes	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Pseudomonas aeruginosa	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Klebsiella pneumoniae	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Escherichia coli	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Staphylococcus epidermidis	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Enterococcus faecium	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Enterococcus faecalis	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Enterobacter cloacae	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Pseudomonas maltophilia	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Proteus mirabilis	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Candida albicans	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Candida tropicalis	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Candida parapsilosis	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Candida glabrata	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)
Candida krusei	Neg (0/8)	Neg (0/8)	Neg (0/8)	Neg (0/8)

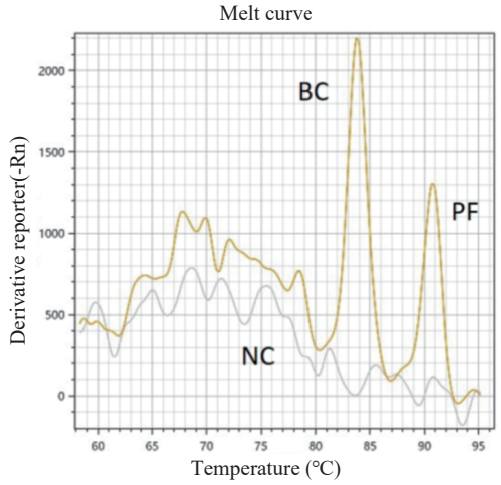

FIGURE 3. Dual EvaGreen-RAA assay using melting curves for mixed BC and PF in simulated milk samples with a concentration of 200 CFU/mL for each pathogen.
Abbreviation: RAA=recombinase aided amplification; NC=negative control; BC=*Bacillus cereus*; PF=*Pseudomonas fluorescens*.

dimers (*10*–*12*). In contrast to previously reported approaches, our study retained the T base at the 3' terminus of primers while selectively replacing only A, G, or C bases, thereby enhancing the stability of the 3'-end of the SAMRS primer.

Eva-green dye exhibits preferential binding to the minor groove region of all dsDNA double helices, offering advantages of enhanced sensitivity and cost-effectiveness. However, its non-specific binding capacity to DNA double strands, including primer dimers, can potentially generate false positive results (*13*–*14*). The integration of SAMRS primers in our study effectively addressed this non-specificity issue. Both AGE and melting curve analyses confirmed that SAMRS primers successfully eliminated primer dimer formation.

The workflow of the EvaGreen-RAA assay, illustrated in Supplementary Figure S1 (available athttps://weekly.chinacdc.cn/), demonstrates enhanced sensitivity and quantitative capability, as the amplicons generated by the reaction consist exclusively of target products, corroborating our most recent findings (*15*). Furthermore, the EvaGreen-RAA assay enables dual-target detection through HRM curve analysis by exploiting the differential Tm values of distinct amplification products. However, this study did not optimize primer concentration or the number of non-natural bases incorporated. An additional limitation concerns the persistent challenges in synthesizing non-natural bases. Despite these constraints, the EvaGreen-RAA assay provides a valuable platform for simple, rapid, and sensitive detection of pathogens of public health concern in primary laboratory settings.

The EvaGreen-RAA assay utilizing SAMRS primers

demonstrates superior sensitivity and specificity for detecting *B. cereus* and *P. fluorescens* in under 30 minutes, eliminating the need for complex RAA probe design. This approach provides a novel strategy for multiplexed pathogen detection in isothermal amplification systems.

Conflicts of interest: No conflicts of interest.

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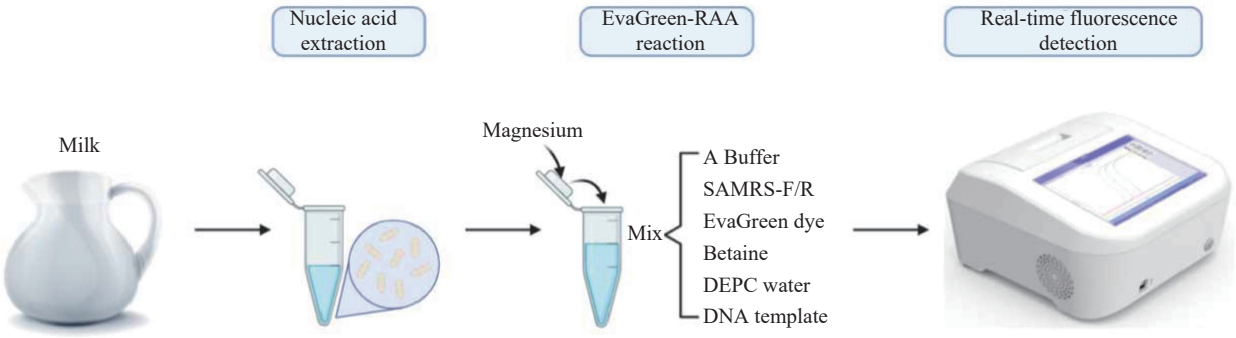
SUPPLEMENTARY MATERIAL



SUPPLEMENTARY FIGURE S1. Workflow diagram of an EvaGreen-RAA assay.
Note: An EvaGreen-dye based recombinase aided amplification (EvaGreen-RAA) assay with SAMRS primers effectively avoids the production of primer dimers, thus improving the detection sensitivity while eliminating the addition of fluorescent probes for RAA. The EvaGreen-RAA assay provides a new approach for allowing the detection of multiple pathogens in the milk.
Abbreviation: SAMRS=self-avoiding molecular recognition system.