Real-time recombinase-aided amplification with improved propidium monoazide for the rapid detection of viable *Escherichia coli* O157:H7 in milk

Dan Mu,¹ Donggen Zhou,² Guoyang Xie,¹ Ju Liu,¹ Zhengzheng Wang,¹ Qin Xiong,¹ and Hengyi Xu^{1*}

¹State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, 330047, PR China

²Ningbo International Travel Healthcare Center (Ningbo Customs Port Outpatient Department), Ningbo, 315010, PR China

ABSTRACT

Escherichia coli O157:H7, the causative agent of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome in humans, generates a effective harm to community health because of its high pathogenicity. A real-time recombinase-aided amplification (rRAA) is an emerging method for nucleic acid detection. However, genomic DNA of bacteria could exist in food and the environment for a long time after death and could be amplified by rRAA assay, resulting in false-positive signal; thus, developing a fast and sensitive method is necessary to detect viable foodborne pathogens in food products. In our research, rRAA assay coupled with an enhanced nucleic acid binding dye named improved propidium monoazide (PMAxx) was established and applied in viable *E. coli* O157:H7 identification in skim milk. The PMAxx could eliminate interference from dead bacteria by permeating impaired membranes and covalently linking to DNA to prevent DNA amplification. The PMAxx-rRAA assay was performed with high sensitivity and good specificity. The PMAxx-rRAA assay could detect as low as 5.4×10^0 cfu/mL of viable *E. coli* O157:H7 in pure culture, and 7.9×10^0 cfu/mL of viable *E. coli* O157:H7 in skim milk. In addition, the PMAxx-rRAA assay was performed in the presence of a high concentration of dead bacteria or nontarget bacteria in skim milk to verify the capacity to resist interference from dead bacteria and nontarget bacteria. Therefore, the established PMAxx-rRAA assay is a valuable tool for the identification of viable *E. coli* O157:H7 in complex food matrix.

Key words: *Escherichia coli* O157:H7, recombinase-aided amplification, improved propidium monoazide, viable foodborne pathogen, milk

INTRODUCTION

Escherichia coli O157:H7 generates a serious threat to community health because of its high pathogenicity (Rani et al., 2021a), resulting in serious symptoms such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Lin et al., 2019). *Escherichia coli* O157:H7 can invade human bodies through the ingestion of contaminated food (Chen et al., 2021a). Milk is easily contaminated by bacteria because of its rich nutrition and is regarded as a high-risk food (Han et al., 2018). More than 90% of the diseases related to dairy product consumption are caused by bacteria (Luo et al., 2017). Moreover, milk is a vital component of diets in many countries, which makes it more likely to lead to infection (Zhao et al., 2021). *Escherichia coli* O157:H7 is a common foodborne pathogen in milk. Reports have been made of *E. coli* O157:H7 infection outbreaks associated with unpasteurized milk (Honish et al., 2005) or cheese made with unpasteurized milk (Currie et al., 2018). Therefore, developing an effective approach to *E. coli* O157:H7 identification in milk is necessary.

The traditional culture-based methods are time consuming and labor intensive (Ma et al., 2021), and such methods cannot detect bacteria in the viable but nonculturable (VBNC) state because VBNC cells cannot grow on plates, but they retain virulence or can recover virulence after resuscitation (Li et al., 2014). In addressing the bottlenecks of the culture-based method, PCR has been developed in the foodborne pathogen detection field (Gaňová et al., 2021). This is a potential detection tool because of its high-speed detection process and high sensitivity compared with the culture-based method (Li et al., 2021). However, cumbersome equipment is required to control the temperature of denaturation and annealing, which is difficult for point-of-care testing (Mokhtarzadeh et al., 2017). Recently, isothermal nucleic acid amplification has been adopted to detect foodborne pathogens (Lee et al., 2020). Recombinase-aided amplification (RAA), a kind of isothermal nucleic acid amplification method,

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*Corresponding author: kidyxu@163.com or HengyiXu@ncu.edu.cn

Table 1. The primers and probe sequences used in this study

Target gene	Oligonucleotides	Sequence (5'–3')
<i>fliC</i>	Forward primer	GTTAACCTTACCATTGCAAAGGTATATGTAC
	Reverse primer	GAAATATACTTATAACGCATCGACCAATGATT
	Probe	CCTTCAGAGTAGCGCCAAGATCTGTCG/i6FAMdT/TG(THF)AG/iBHQ1dT/GCCTGTCGCTAC3'C3
	Spacer	

can be performed at constant temperatures (37–42°C; Qin et al., 2021) and has a short detection time (less than 20 min) and high sensitivity (Shen et al., 2019). Recombinase-aided amplification does not require a complex instrument to provide cyclic temperature (Xue et al., 2020), which makes it more convenient in resource-poor settings than PCR. In this study, the specific fluorescence probe was added in the RAA system to achieve real-time fluorescence analysis.

Previous reports have illustrated that genomic DNA can stay in foods or the environment for a long time after bacterial death and can be normally amplified by the proposed real-time recombinase-aided amplification (rRAA; Gao et al., 2021), potentially overestimating the quantity of viable bacteria in samples and even resulting in false-positive signals (Lv et al., 2020). In other studies, some agents such as ethidium bromide monoazide (EMA) and propidium monoazide (PMA) have been applied in reducing interference from dead bacteria (Roumani et al., 2021). Improved propidium monoazide (PMAxx), a new and improved version of dye PMA, has the same spectral properties and better effects on distinguishing viable bacteria from dead bacteria compared with PMA (Lv et al., 2020). The PMAxx only penetrates the damaged membrane of dead bacteria and then reacts with bases of DNA to suppress DNA amplification (Nocker et al., 2006). However, viable bacteria with intact membranes keep PMAxx out (Dong et al., 2018). PMAxx, combined with quantitative PCR (Zhao et al., 2020), quantitative recombinase polymerase amplification (Lv et al., 2020), and real-time loop-mediated isothermal amplification, has been reported in detection for gram-positive bacteria and gram-negative bacteria (Nocker and Camper, 2009).

In this study, we developed a rRAA assay combined with DNA cross-linking dye named PMAxx for the identification of viable *E. coli* O157:H7. The rRAA is a one-step detection in a closed tube at 39°C and completed within 20 min. The PMAxx effectively distinguishes dead bacteria from viable bacteria in terms of membrane integrity. The proposed PMAxx-rRAA was performed simply, rapidly, and sensitively, and the

detection limit in *E. coli* O157:H7 culture was 5.4×10^0 cfu/mL. Moreover, PMAxx-RAA was applied in skim milk, and the limit of detection (LOD) was 7.9×10^0 cfu/mL and was satisfactorily performed in milk where a high concentration of dead bacteria or nontarget bacteria coexisted.

MATERIALS AND METHODS

Materials, Apparatus, and Reagent Preparation

Wizard Magnetic DNA Purification System for Food that used to extract genomic DNA was obtained from Promega Corporation; Protease K was obtained from Tiangen Biotech Co., Ltd. The primers and the specific probe based on the *fliC* gene were designed by Oligo 6.0 software (Table 1) and prepared by Sangon Biotech; the rRAA kit was supplied by Jiangsu Qitian Gene Technology Co. Ltd.

The fluorescence characterization analysis of PMAxx was photographed by confocal laser scanning microscope (CLSM; Leica TCS SP8); the QT-RAA-F1620 detection system was supplied by Jiangsu Qitian Gene Technology Co. Ltd.

The PMAxx (Bosunlife Co. Ltd.) was deliquesced in sterile water to prepare stock solution at the concentration of 20 mM and stored at –20°C in the dark until used; 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 137 mM sodium chloride, and 2 mM potassium dihydrogen phosphate were mixed to prepare PBS, and then diluted to prepare a 0.01 M PBS solution at pH of 7.4.

Bacterial Strains, Culture Medium, and Growth Conditions

All strains used in this study are listed in Table 2. The bacterial pure culture was prepared by inoculating a single colony grown on Luria Bertani (LB) agar in 5 mL of LB broth, and then cultured at 37°C in a shake incubator with speed of 180 rpm for 12 h. The culture was washed by 5 min of centrifugation at $9,600 \times g$ at room temperature and redispersed in sterile PBS. All

the cells were inoculated on LB agar plates to count the number of viable bacteria.

Preparation of Viable and Dead Cells

A starting concentration of 10^9 cfu/mL of fresh cells was centrifuged and then washed twice with 1 mL of PBS. Ten-fold series dilutions of bacterial suspension were obtained by adding 50- μ L cultures of fresh cells into 450 μ L of PBS. The dead bacterial suspension was prepared by heating the bacterial suspension at 80°C for 15 min, and then transferred to refrigerator at 4°C for 5 min. All the samples were inoculated and incubated on LB agar plates at 37°C for 24 h to verify the viability of bacteria.

DNA Extraction

Genomic DNA was extracted by using the Wizard Magnetic DNA Purification System for Food according to the manufacturer's instructions. Bacterial cells were resuspended in 150 μ L of bacteria-free water. Then bacterial cells were lysed by shocking magnetic beads under the action of lysis buffer, and silicon-based magnetic beads can adsorb nucleic acid efficiently and specifically. Magnetic beads adsorbing nucleic acid were separated under the applied magnetic field, the supernatant was discarded. The magnetic beads were harvested with wash buffer to remove impurities. The DNA adsorbed on magnetic beads was desorbed and dissolved in elution buffer. The extracted DNA was stored in -20°C until use.

rRAA Assay Condition and Program

The rRAA assay was performed according to the manufacturer's instructions of the rRAA kit. Each reaction system included 10 μ M probe (0.6 μ L), 10 μ M forward primer and reverse primer (each 2.1 μ L), sterile water (12.7 μ L), and reaction buffer (25 μ L), and then the reagents were blended and transferred into the freeze-dried powder tubes including single-stranded DNA-binding protein, DNA polymerase, and recombinase. The reaction tubes were promptly centrifuged at a speed of $1,200 \times g$ for 30 s at room temperature, and genomic DNA (5 μ L) was added; then, 280 mM magnesium acetate solution (2.5 μ L) was pipetted to the tube cap. Then the samples were blended and centrifuged at once by the QT-RAA-B6100 pre-reaction system. (First, the reaction tubes were centrifuged with speed of $1,200 \times g$ for 10 s at 39°C, followed by keeping at a stationary temperature of 39°C for 4 min; then, the last procedure was centrifuged at $1,200 \times g$ for 10 s at 39°C). The tubes were immediately placed in the QT-RAA-F1620 detection system at a stationary temperature of 39°C for 20 min (Xie et al., 2021).

Fluorescence Characterization of the PMAx

Next, 10^8 cfu/mL of viable and heat-inactivated *E. coli* O157:H7 was prepared as described above. Subsequently, PMAx dye was added in samples at a final concentration of 60 μ M and incubated for 10 min in the dark, and then the samples were exposed for 10 min, after which all of the samples were washed twice with

Table 2. The bacterial strains used to evaluate specificity of the primer and probe, and the results of the recombinase-aided amplification (RAA) reaction

Bacterial strain	Strain ID	Source ¹	RAA results ²
<i>Escherichia coli</i> O157:H7	43888	ATCC	+
	44828	CMCC	+
<i>Escherichia coli</i>	23657	CICC	—
	44102	CMCC	—
<i>Staphylococcus aureus</i>	26001	CMCC	—
	26003	CMCC	—
<i>Bacillus cereus</i>	JDZ102Y	JX-CDC	—
<i>Listeria monocytogenes</i>	13932	ATCC	—
	19115	ATCC	—
<i>Listeria seeligeri</i>	35967	ATCC	—
<i>Salmonella</i> Enteritidis	13706	ATCC	—
<i>Salmonella</i> Typhimurium	50115	CMCC	—
<i>Pseudomonas aeruginosa</i>	10104	CMCC	—
<i>Bacillus subtilis</i>	20445	CICC	—
	63501	CMCC	—
<i>Monilia albicans</i>	98001	CMCC	—
<i>Cronobacter sakazakii</i>	29544	ATCC	—

¹CMCC = China Medical Culture Collection (Beijing, China); ATCC = American Type Culture Collection (Manassas, VA); JX-CDC = Jiang Xi Province Center for Disease Control and Prevention, China; CICC = Center of Industrial Culture Collection (Beijing, China).

²Results indicate positive or negative signals.

PBS. Furthermore, 100 μ L of the sample was pipetted to a microplate reader to measure with 3 replicates the fluorescence intensity of the dead and viable *E. coli* O157:H7 stained with PMAxx. Ten microliters of the sample was pipetted on the microslide and observed by the CLSM.

Optimization of PMAxx Concentration and Exposure Time

The target bacterial suspensions (approximately 10^6 cfu/mL) were resuspended in PBS and then viable cells and heat-inactivated cells were prepared. The PMAxx was added in target bacterial suspensions to a final concentration of 0, 40, 120, 200, and 280 μ M, followed by incubation for 10 min in the dark and exposure to a 500 W of halogen lamp source for 10 min. Then, the samples were washed 3 times with PBS and resuspended in sterile water. Subsequently, genomic DNA was extracted by a Wizard Magnetic DNA Purification System and then performed for rRAA reaction with 3 replicates.

Next, 10^6 cfu/mL of viable bacteria or 10^6 cfu/mL of dead bacteria were treated with PMAxx at optimized concentration. During PMAxx treatment, different exposure time (0, 5, 10, 15, 20 min) was performed. Then the genomic DNA were extracted for rRAA reaction. The fluorescent signals were measured with 3 replicates and collected using the QT-RAA-F1620 detection system.

Procedure of PMAxx Treatment

The bacterial suspensions were incubated with 40 μ M PMAxx in the dark for 10 min and shaken occasionally, allowing for permeating impaired membrane of dead bacteria. A 500-W halogen lamp source irradiated samples at a distance of 15 cm for 10 min to activate the dyes, and the samples were placed on ice to avoid additional inactivation by the heat derived from the halogen lamp. Then the samples were washed 3 times with PBS to remove excess PMAxx and then resuspended in sterile water (Zhou et al., 2019).

Specificity of the Primer and Probe Based on PMAxx-RAA

The specificity of the primers and the probe was evaluated by performing PMAxx-RAA for 15 nontarget bacteria. All the strains at 10^6 cfu/mL were treated with 40 μ M PMAxx and the DNA were extracted for RAA reaction.

Sensitivity of the PMAxx-RAA Assay in *E. Coli* O157:H7 Culture

To assess the sensitivity of the proposed method, 10-fold series dilutions of viable *E. coli* O157:H7 (10^0 to 10^6 cfu/mL) were prepared. Each sample was treated with PMAxx under the optimized conditions, then the genomic DNA were extracted for rRAA. Each DNA sample was detected individually and the fluorescent signals were measured with 3 replicates and collected using QT-RAA-F1620 detection system.

Sensitivity of the PMAxx-RAA Assay in Artificially Contaminated Milk

The PMAxx-RAA assay was further assess the possibility of application in skim milk. The skim milk was purchased from a local supermarket and autoclaved at 115°C/0.1 MPa for 20 min. Fifty microliters of 10-fold series dilutions of viable *E. coli* O157:H7 was pipetted into 450 μ L of sterilized skim milk to obtain milk samples including 10^0 to 10^6 cfu/mL of viable *E. coli* O157:H7. The PMAxx with 40 μ M was added into the milk samples and incubated in the dark for 10 min and exposed for 10 min. The samples were washed by 5 min of centrifugation with a speed of $9,600 \times g$ at room temperature and resuspended in PBS 3 times. Thirty microliters of protease K was added in samples at 56°C for 30 min to digest the milk proteins. Then the samples were washed twice with PBS, and redispersed in 150 μ L of sterile water. Genomic DNA was extracted and the fluorescent signals from samples were measured with 3 replicates and collected using the QT-RAA-F1620 detection system.

Interference Assessment of Dead Bacteria Coexisted in System

To determine effect of dead bacteria on detection of viable bacteria, the milk samples were performed for PMAxx-rRAA in the presence of a high concentration of dead bacteria. A total of 10^5 cfu/mL of dead *E. coli* O157:H7 was added into 10^3 to 10^6 cfu/mL of viable *E. coli* O157:H7 to verify the capacity of resisting interference from dead bacteria in skim milk. The skim milk samples only containing 10^5 cfu/mL of viable *E. coli* O157:H7 were used as the control. The samples were treated with PMAxx under the optimized condition. Genomic DNA were extracted and detected individually with 3 replicates and the fluorescent signals from samples were measured and collected using QT-RAA-F1620 detection system.

Assessment Interference of Nontarget Bacteria Coexisted in System

To assess effect of nontarget bacteria on detection of target bacteria, the skim milk contained high concentration of nontarget bacteria was performed for PMAxx-rRAA. The bacterial suspensions containing (1) only 10^6 cfu/mL of *E. coli* O157:H7 at a total volume 500 μ L of milk sample; (2) 10^6 cfu/mL of *E. coli* O157:H7 + 10^5 cfu/mL of *S. aureus* at a total volume 500 μ L of milk sample; (3) 10^6 cfu/mL of *E. coli* O157:H7 + 10^5 cfu/mL of *Bacillus cereus* at a total volume 500 μ L of milk sample. The DNA were extracted and performed with 3 replicates for the PMAxx-rRAA assay. The fluorescent signals from each sample were measured and collected using the QT-RAA-F1620 detection system.

Statistical Analysis

All of the detection procedures were repeated in triplicate. All figures were graphed with GraphPad Prism 8 (GraphPad Software Inc.). Error bars in the figures show the standard deviation from 3 measurements. Data were analyzed for significant differences by SPSS version 19 (SPSS Inc.). Results with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Principle of the PMAxx-rRAA

Based on the working principle of the method in our research, the PMAxx-rRAA assay for viable *E. coli* O157:H7 detection was established with 2 steps. The first step is PMAxx treatment. The PMAxx permeates impaired member of dead bacteria in the dark. In bright visible light, the azide group of PMAxx is turned to a nitrene with high reactivity. The nitrene reacts with DNA bases to form an irreversible covalent bond that can inhibit DNA amplification (Nocker et al., 2006). Viable bacteria with intact membrane keeps PMAxx out (Desneux et al., 2015). Therefore, the genomic DNA from viable bacteria is normally amplified, but the amplification of genomic DNA from dead bacteria is suppressed. The second step is the amplification of genomic DNA and read-out of fluorescence signal. The primers integrate with recombinase to form the primer-recombinase complex. Under the action of recombinase, the primers pair with homologous sequences of the genomic DNA, and the target double-stranded DNA is opened and stabilized by single-stranded DNA-binding protein to prevent re-formation of double-stranded DNA immediately (Wang et al., 2020); then, DNA polymerase with displacement activity catalyzes primer ex-

tension, and the original strand is displaced to generate amplicons (Zhang et al., 2017). The fluorescent probe paired with amplicon is cleaved, allowing real-time fluorescence screening on the accumulated amplified copies (Londoño et al., 2016). The fluorescence intensity were measured every 20 s. A procedure flowchart is shown in Figure 1.

Fluorescence Characterization of the PMAxx

The PMAxx could intercalate into genomic DNA and emit fluorescence (Liang et al., 2019), which could be measured by using a microplate reader. As shown in Figure 2(A), a characteristic peak appeared at 610 nm for heat-inactivated bacteria. However, peak value of the characteristic peak from the viable bacteria was relatively low compared with dead bacteria because PMAxx entered into dead bacteria but could not permeate into viable cells. The average fluorescence intensity at 610 nm reached 2.9340 for dead bacteria with PMAxx treatment [Figure 2(B)], whereas the average fluorescence intensity of the viable bacteria was only 0.8025. These results proved that PMAxx intercalated into dead bacterial DNA and generated a fluorescence at signal to a noise ratio of 3.6/1. In addition, PMAxx could intercalate into DNA and generate a characteristic fluorescence at an excitation wavelength of 475 nm, showed red color in the image of CLSM in Figure 2(C, D). These results proved that PMAxx was an efficient nucleic acid dye.

Optimization of PMAxx Concentration and Exposure Time

The concentration of PMAxx was a key factor that affected the suppression effect (Mendes Silva and Domingues, 2015). Excessive PMAxx exerted virulency effects on viable bacteria, which resulted in false-negative detection results, but low concentrations of PMAxx could not completely rule out the amplification signal from dead bacteria (Zhong and Zhao, 2018). Thus, an appropriate PMAxx concentration was required to optimize in order to ensuring that the presence of dead cells would not interfere with the results of viable bacteria detection. As shown in Figure 3(A), the higher the time threshold (TT) value, the higher the inhibitory effect of PMAxx. The TT value is defined as the time when the fluorescence intensity exceeds an arbitrary threshold (Chen et al., 2020). The TT value did not remarkably increase for viable cells, which indicated detection of viable bacteria was affected to a small extent. On the contrary, an evident increase in TT was observed from dead bacteria, which plateaued at 40 μ M PMAxx.

The significant ($P < 0.05$) differences in TT value of dead bacteria were 0 and 40 μM , but no significant difference between TT value of 40 and 120 μM ($P > 0.05$) for dead bacteria. The results indicated that the PMAxx could inhibit amplification of DNA from dead bacteria and achieve the best effect at 40 μM PMAxx. The optimal concentration of PMAxx is different for different species, cells in different growth states, and other biological parameters. Lv et al. (2020) reported that the optimal PMAxx final concentration was 10 μM for VBNC *E. coli* O157:H7 in real-time loop-mediated isothermal amplification. Rapid detection of VBNC *Cronobacter sakazakii* was achieved at 8 μM PMAxx when combined with droplet digital PCR (Lv et al., 2021). Therefore, the optimization is necessary.

The PMAxx intercalated into DNA bases after exposure to strong visible light; thus, exposure time was a critical parameter for PMAxx treatment. The exposure not only activated the dye to react with bases of nucleic acids but also inactivated any excess dye by forming hydroxyl amines (Yuan et al., 2018). Based on the results of optimization [Figure 3(B)], TT values of dead bacteria increased with increased exposure time and reached a plateau at 5 min, and the TT value decreased when the exposure time exceeded 10 min. The TT value at 10 min was similar to that at 5 min of exposure time. There were no significant difference between the TT value at 5 and 10 min ($P > 0.05$). To ensure the excess PMAxx dye was completely photolyzed without

affecting the detection results, 10 min of exposure time was selected (Lv et al., 2020). The TT value of viable cells did not increase remarkably, indicating that the detection of viable bacteria was not affected.

Therefore, in this study, the most optimum concentration was 40 μM PMAxx, and 10 min of exposure was selected as the optimum exposure time, which did not affect the detection signals of the viable bacteria and suppressed the signal amplification from the dead cells to the greatest extent.

Specificity of the Primer and Probe Based on PMAxx-rRAA

Seventeen strains were used to evaluate the specificity of the primers and the probe. The results are shown in Table 2; only the 2 *E. coli* O157:H7 showed a positive signal in the presence of *fliC* (Zhou et al., 2017). The 15 nontarget bacteria showed a negative signal. The primers and probe showed good specificity based on the PMAxx-rRAA.

Sensitivity of the PMAxx-rRAA Assay in Pure Culture and in Spiked Milk Matrix

The PMAxx-rRAA assay with optimized parameters in 10-fold series dilutions of *E. coli* O157:H7 were performed to determine the sensitivity. Based on the fluorescent amplification curves and TT values shown

Figure 1. Overview of the detection process of *Escherichia coli* O157:H7 based on propidium monoazide (PMAxx)-real-time recombinase-aided amplification (rRAA) assay.

Figure 2. Characterization of *Escherichia coli* O157:H7 treated with propidium monoazide (PMAxx). (A) Fluorescence spectrum of the dead bacteria and viable bacteria stained with PMAxx; (B) average fluorescence intensity of the dead bacteria and viable bacteria stained with PMAxx; (C) confocal laser scanning microscope (CLSM) images of *E. coli* O157:H7 in a bright field; (D) CLSM images of dead *E. coli* O157:H7 stained with PMAxx and shown in red. Error bars represent SD from 3 independent experiments.

in Figure 4, 5.4×10^0 to 5.4×10^6 cfu/mL of *E. coli* O157:H7 could generate fluorescent signals, and the TT value decreased as the concentration of bacteria increased. In addition, the control without *E. coli*

O157:H7 could not generate a visible fluorescent signal and there was no TT value at 20 min (data not shown). Therefore, the PMAxx-rRAA could detect as low as 5.4×10^0 cfu/mL of viable *E. coli* O157:H7.

Figure 3. Optimization of the propidium monoazide (PMAxx) treatment condition. (A) Effect of PMAxx concentration, the result of “NO” is defined as no time threshold (TT) value within 20 min; and (B) effect of exposure time, the “NO” result is defined as no time threshold value within 20 min. Error bars represent SD from 3 independent experiments; some error bars are too short to be visible.

Figure 4. Sensitivity of the propidium monoazide (PMAxx)-recombinase-aided amplification (RAA) assay in pure culture. (A) Amplification curves from a series of bacterial dilutions ranging from 10^6 to 10^0 cfu/mL of *Escherichia coli* O157:H7 and (B) corresponding time threshold values. Error bars represent SD from 3 independent experiments; some error bars are too short to be visible.

Milk samples including 7.9×10^0 cfu/mL to 7.9×10^6 cfu/mL of *E. coli* O157:H7 were prepared to explore the application of PMAxx-rRAA. The milk protein might interfere with rRAA; therefore, protease K was used to digest proteins of milk. According to the results, the PMAxx-rRAA could be performed well in milk treated with protease K. As shown in Figure 5, the fluorescence amplification curves appeared from 7.9×10^0 cfu/mL to 7.9×10^6 cfu/mL, but the control without target bacteria did not show visible amplification signal. The TT values gradually decreased with the increase of bac-

terial concentration, and there was no TT value in the control (data not shown). These results confirmed that the PMAxx-rRAA could be applied in milk samples, and the LOD in milk samples was 7.9×10^0 cfu/mL.

Assessment Interference of Viable/Dead Bacteria Coexisted in the System

Mixtures containing viable *E. coli* O157:H7 (10^3 – 10^6 cfu/mL) and 10^5 cfu/mL of dead *E. coli* O157:H7 were prepared to assess the effect of high concentrations of

Figure 5. Sensitivity of the propidium monoazide (PMAxx)-recombinase-aided amplification (RAA) assay in skim milk. (A) Amplification curves from a series of bacterial dilutions ranging from 10^6 to 10^0 cfu/mL of *Escherichia coli* O157:H7 and (B) corresponding time threshold (TT) values. Error bars represent SD from 3 independent experiments; some error bars are too short to be visible.

Figure 6. Application of the propidium monoazide (PMAxx)-recombinase-aided amplification (RAA) method in the presence of 10^5 cfu/mL of dead *Escherichia coli* O157:H7 in milk. Error bars represent SD from 3 independent experiments; some error bars are too short to be visible.

dead *E. coli* O157:H7 on detection of viable bacteria. The samples were treated with 40 μ M PMAxx and exposed for 10 min and then the genomic DNA was extracted to perform rRAA. Based on the results in Figure 6, the recovery of the sample that only contained viable bacteria was defined as 100%, and the ratio of the corresponding TT value of the mixture to the average of the TT value of viable bacteria was defined as the recovery. The closer the recovery is to 100%, the smaller the effect of dead bacteria on detection of viable bacteria. The results in Figure 6 indicated that even a high concentration of dead bacteria will only affect the detection of viable bacteria slightly. Therefore, the PMAxx-rRAA can selectively detect viable bacteria in the presence of a high concentration of dead bacteria.

Interference of Nontarget Bacteria Based on the PMAxx-rRAA Assay

The mixtures were prepared by mixing *E. coli* O157:H7 with other species in skim milk to verify whether nontarget bacteria co-existing in the samples

could interfere with the detection results of target bacteria. The sample only included 10^5 cfu/mL of *E. coli* O157:H7, which served as the control. Based on the results in Figure 7, the similar reaction curve and satisfactory recovery indicated the nontarget bacteria only slightly interfered with the detection results of *E. coli* O157:H7. The reason for this is that the nontarget sequence interfered with the primer paired with the target sequence. In our research, we defined the average TT values of pure target bacteria as 100% of the recovery (group 1). The recovery of group 2 and group 3 was a ratio of the corresponding TT values to the average TT values of group 1 (Xie et al., 2021).

CONCLUSIONS

We established a rRAA assay coupled with PMAxx for identification of viable *E. coli* O157:H7 in skim milk. The proposed method showed outstanding advantages in sensitivity, detection time, and viable or dead determination compared with DNA-based isothermal methods (Table 3). The improved DNA cross-linking dye named PMAxx could intercalate into the genomic DNA and inhibit its duplication. The PMAxx only permeates into dead bacteria with impaired membranes and is kept out of viable bacteria. Therefore, the proposed PMAxx-rRAA could effectively detect viable *E. coli* O157:H7. The rRAA showed good specificity and efficiency. The detection could be completed within 20 min, and it did not require complicated equipment for the thermal cycle. The proposed PMAxx-rRAA assay showed a good LOD of 5.4×10^0 cfu/mL in pure culture and 7.9×10^0 cfu/mL in milk. Moreover, the method performed satisfactorily in milk in the presence of dead or nontarget bacteria.

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Table 3. Comparisons between different DNA-based isothermal methods for *Escherichia coli* O157:H7 detection

Detection method ¹	Detection time	Limit of detection (cfu/mL)	Vitality determination	Reference
Rti-LAMP	4 h	2.7	Yes	Chen et al., 2021b
RCA	30 min	4×10^3	No	Jiang et al., 2019
HDA	1 h	10^2	No	Rani et al., 2021b
HCR	90 min	7.2×10^1	No	Liang et al., 2020
SDA	50 min	10	No	Wu et al., 2015

¹Rti-LAMP = real-time loop-mediated isothermal amplification; RCA = rolling circle amplification; HDA = helicase-dependent amplification; HCR = hybridization chain reaction; SDA = strand displacement amplification.

Figure 7. Interference of nontarget bacteria (10^5 cfu/mL) based on the propidium monoazide (PMAxx)-recombinase-aided amplification (RAA) assay. (A) Amplification curves; (B) corresponding recovery, 1: only 10^6 cfu/mL of *Escherichia coli* O157:H7; 2: 10^6 cfu/mL of *E. coli* O157:H7 + 10^5 cfu/mL of *Staphylococcus aureus* at a; 3: 10^6 cfu/mL of *E. coli* O157:H7 + 10^5 cfu/mL of *Bacillus cereus*. Error bars represent SD from 3 independent experiments; some error bars are too short to be visible.

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ORCID

Ju Liu <https://orcid.org/0000-0002-5815-9482>

Hengyi Xu <https://orcid.org/0000-0003-0457-9665>