

Research Article

Detection of *Staphylococcus aureus* in Milk Using Real-time Fluorescence Loop-mediated Isothermal Amplification

Ying Yu, Xiaoyan Ma and Wei Zhang

College of Food Science and Technology, Agricultural University of Hebei, 071001 Baoding, China

Abstract: *Staphylococcus aureus* is a kind of worldwide food-borne pathogen. Recently, *S. aureus* has gained considerable attention because of the increasing alimentary toxicosis incidence. In this study, a Real-Time fluorescence Loop-Mediated isothermal Amplification (RT-LAMP) was developed to detect *S. aureus* rapidly. The heat-stable nuclease (nuc) gene of *S. aureus*, the target sequence, was selected to design four special primers. A rapid detection method for *S. aureus* was initially established under optimum reaction conditions. The assay, performed for 40 min at 61°C, did not show cross reactivity with other bacterial species. The specificity and sensitivity of RT-LAMP for detecting *S. aureus* were 100% and 8.0 CFU/mL, respectively. Results indicated that RT-LAMP was a potential field-usable molecular tool for detecting *S. aureus*. This method can be an alternative to conventional LAMP in clinical applications and operational programs.

Keywords: Detection, nuclease gene, rapid, sensitivity, specificity

INTRODUCTION

Staphylococcus aureus is a Gram-positive spherical bacterium that occurs in the form of grape-like microscopic clusters. *S. aureus* is ubiquitous in nature. The bacterium exists in approximately 25% healthy people and is more common among individuals with skin, eye, nose or throat infections (Atanassova *et al.*, 2001; Martin *et al.*, 2004). *S. aureus* contamination can cause food poisoning. This contamination occurs as a result of improper refrigeration of food and poor sanitation used for food preparation. *S. aureus* can proliferate randomly at room temperature and produce *S. aureus* Enterotoxin (SE), a major contaminant that causes food poisoning (Chen, 2007). *S. aureus* can be killed by heat and pasteurization. However, SE can survive after heated at 100°C for 30 min without incurring damages (Tirado and Schimdt, 2001).

S. aureus is an important food-borne pathogen that causes many serious infections (Shriver-Lake *et al.*, 2003). Recently, *S. aureus* has gained considerable attention for the increasing incidences of alimentary toxicosis (Le Loir *et al.*, 2003). According to the U.S. Center for Disease Control, alimentary toxicosis caused by *S. aureus* ranked the second most prevalent bacterial alimentary toxicosis in the USA and in Canada, with prevalence rates of the second 33 and 45%, respectively of allbacterial alimentary toxicosis in the USA and 45% in Canada (Balaban and Rasooly, 2000; Tirado and Schimdt, 2001). Alimentary toxicosis cases are also reported in China every year (Luo *et al.*, 2012).

Currently, alimentary toxicosis caused by *S. aureus* has become worldwide public health concern because of its severe threat to human.

At present, detection of *Staphylococcus aureus* mainly uses the traditional isolation, culture and biochemical identification method (Moreira *et al.*, 2008), molecular biology methods have not been widely used. It can no longer meet the needs of the rapid detection of pathogens in food because of the complicated operation and time consuming. Thus, establishing a rapid, simple, convenient and effective technology to detect *S. aureus* is important to food safety.

Real-Time fluorescence Loop-Mediated isothermal Amplification (RT-LAMP) is a highly sensitive method for the detection of nucleic acids. RT-LAMP involves a portable equipment to amplify DNA. The principle of RT-LAMP is to enhance fluorescence after the fluorescent dye SYBR Green I intercalates to DNA (Lucchi *et al.*, 2010). Thus, the progressing reaction increases the amount of DNA in the system and enhances the fluorescence intensity, which then enables the instrument to automatically display the result. The reaction can be observed through this instrument within a short time (Uemura *et al.*, 2008).

RT-LAMP isothermal amplification system can be applied in real-time monitoring of nucleic acid isothermal amplification (Uemura *et al.*, 2008; Lucchi *et al.*, 2010). However, detecting food-borne pathogens using RT-LAMP has been rarely reported.

Table 1: Bacterial strains list

Species	Origin	Species	Origin
<i>Staphylococcus aureus</i>	CMCC26073	<i>Shigella boydii</i>	ATCC12022
<i>S. aureus</i>	CMCC26003	<i>Bacillus cereus</i>	CMCC63302
<i>S. aureus</i>	No. 12 (laboratory preserved)	<i>Yersinia enterocolitica</i>	CICC21669
<i>S. aureus</i>	No. 13 (laboratory preserved)	<i>Y. enterocolitica</i>	CMCC52302
<i>S. aureus</i>	No. 14 (laboratory preserved)	<i>S. aureus</i>	CICC21600
<i>S. aureus</i>	No. 15 (laboratory preserved)	<i>Clostridium perfringens</i>	ATCC13124
<i>S. aureus</i>	No. 16 (laboratory preserved)	<i>Proteusbacillus vulgaris</i>	CMCC49027
<i>S. aureus</i>	No. 17 (laboratory preserved)	<i>Salmonella typhimurium</i>	CMCC 50115
<i>Streptococcus hemolytic-β</i>	CMCC 32204	<i>C. perfringens</i>	ATCC13124
<i>Shigella dysenteriae</i>	CMCC51135	<i>P. vulgaris</i>	CMCC49027
<i>Shigella boydii</i>	CMCC51522	<i>Enterobacter sakazakii</i>	ATCC51024
<i>Shigella sonnei</i>	CMCC51334	<i>Vibrio parahaemolyticus</i>	ATCC 17802

CMCC: National center for medical culture collections; CICC: China center of industrial culture collection; ATCC: American type culture collection

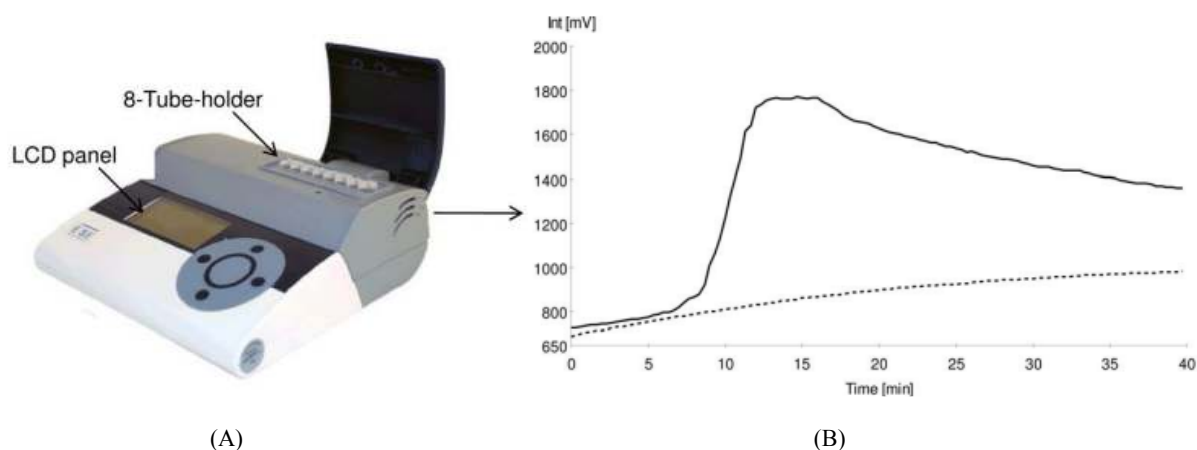


Fig. 1: Description of RT-LAMP

In this study, we used the RT-LAMP method to detect *S. aureus*. It only takes 40 min to display the results, which is the first time to use this method to detect *S. aureus* in the world.

MATERIALS AND METHODS

Materials:

Bacterial strains: The bacterial strains used in this study are listed in Table 1.

Portable equipment: The portable fluorescence reader (ESE-Quant Tube Scanner) used for this study was developed by ESE GmbH (Stockach, Germany, Fig. 1A). The device has an eight-tube holder heating block with adjustable temperature setting and spectral devices to detect the amplified product using fluorescence spectra. The device is connected to a computer to generate real-time amplification plots as the reaction progresses and to display the results as positive or negative (Fig. 1B) (Lucchi *et al.*, 2010).

Main reagents: The reagents are as follows: Bst DNA polymerase, Taq DNA polymerase, dNTP, 10×loading buffer, 100 bp DNA Marker, LAMP primer (Takara Biotechnology (Dalian) Co., Ltd.), DNA Extraction Kit (EZ Spin Column Bacterial Genomic DNA Isolation

Kit UNIQ-10, Sango Biotech F (Shanghai) Co., Ltd.), Nutrient Broth, Nutrient Agar, 7.5% Sodium Chloride Broth, Baird-Parker Agar Base, among others (Beijing Land Bridge Technology Co., Ltd.).

Method:

Primer design: The specific LAMP primers of *S. aureus* were designed based on the conserved region of the heat-stable nuclease (*nuc*) gene of *S. aureus* (GenBank No: DQ507380.1) using an online primer explorer software (<http://primerexplorer.jpV4>) (Uemura *et al.*, 2008). The four primers are as follows: forward outer primer (F3), backward outer primer (B3), Forward Inner Primer (FIP) and Backward Inner Primer (BIP). FIP consists of an F1 complementary series and an F2 direction sequence. BIP contains a B1 complementary series and a B2 direction sequence. Details on LAMP are shown in Table 2 and Fig. 2.

Reaction conditions for RT-LAMP: All LAMP primers used in this study were purified using high-performance liquid chromatography.

Trial and error were used to obtain the appropriate reaction system, which comprised the following: 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 2 mM MgSO₄, 0.4 mM dNTPs, 10×Bst DNA polymerase buffer, 8 U Bst DNA polymerase, 0.5 μL of DNA template and 0.5 μL of SYBR Green I. Sterile

Table 2: LAMP primers

Primer name	Sequence (5'→3')
F3	GCCGAATTCGTTATGACAGAA
B3	CCACTTCTATTTACGCCGTTAT
FIP	CCTTTCGAAACATTAAGTATGATAGCCA-GTGCTGGCATATGTATGGCAATT
BIP	ACGCAAAGAGGTTTTTCTTTTTTCGC-TGCTGAGCTACTTAGACTTGAA

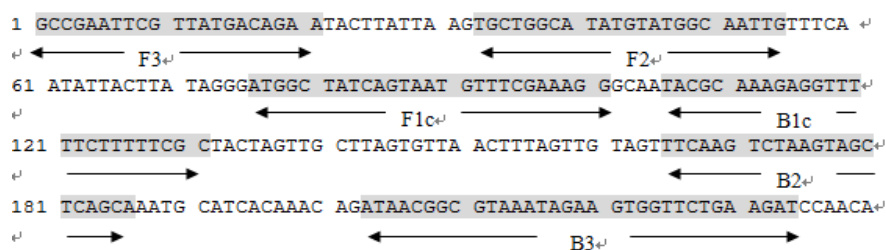


Fig. 2: Gene map of the target sequences

distilled water was added until the total volume of 25 μ L was reached.

The temperature of the device was set to 61°C and the reaction time to 90 min with the computer. The reaction can be terminated according to the reaction process.

Reaction conditions for LAMP: The reaction system for LAMP was similar to that for RT-LAMP, except that the absence of fluorescent dyes. The total volume was 25 μ L. The mixture was incubated at 61°C for 60 min and then heated at 80°C for 10 min to terminate the reaction. Subsequently, 5 μ L of the LAMP products were detected in 2% agarose gel electrophoresis. After the products were stained with ethidium bromide, DNA ladder was detected under UV light.

Restriction analysis of product: The reaction system comprised 2 μ L of 10 \times buffer, 1 μ L of Hpa I restriction enzyme and 5 μ L of the reaction product. Sterile distilled water was added until the total volume of 20 μ L was obtained. The mixture was incubated at 37°C for 3 h. Restricted DNA products were then separated using 2% agarose gel electrophoresis and visualized under UV light.

Specificity determination of RT-LAMP assay in pure cultures: The genomic DNA (listed in Table 1) was extracted using the boiling lysis method. DNA and water were used as the positive and negative controls, respectively. RT-LAMP assay was used to verify primer specificity.

Sensitivity determination of RT-LAMP assay in pure cultures: To determine the sensitivity of RT-LAMP for pure cultures, we prepared 10-fold serial dilutions from each exponential-phase culture in 0.8% normal saline. The plate count method showed that the pure culture contained 8.0 \times 10⁹ CFU/mL of *S. aureus*. Sensitivity tests for RT-LAMP assay were conducted in

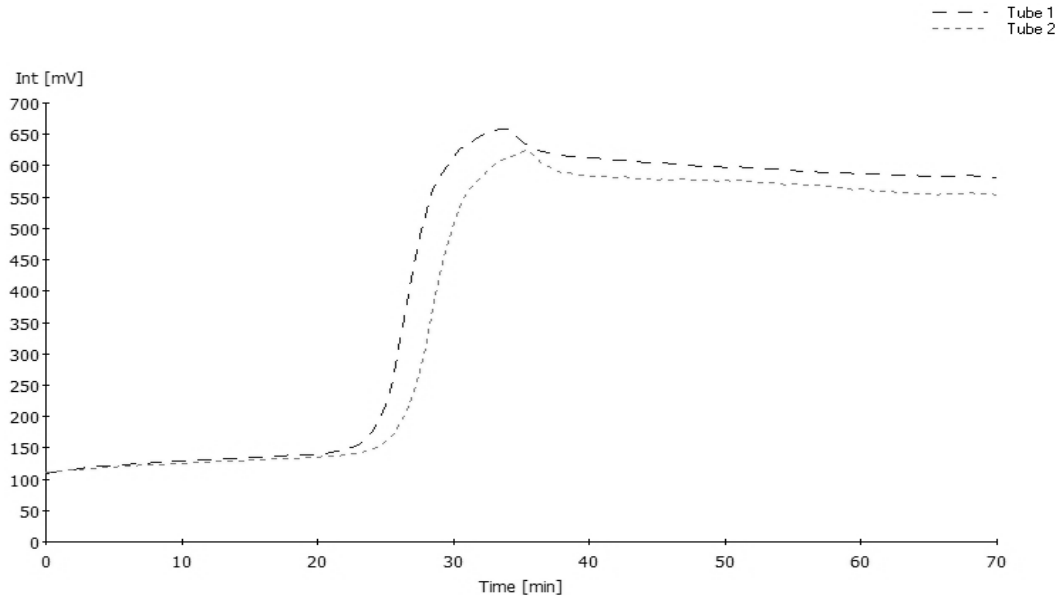
triplicate and detection limits were defined as the last positive dilutions.

Testing of food artificially contaminated with *S. aureus*: The FDA method was used to analyze and certify that the food samples were not infected by *S. aureus* before contamination. Initially, 25 mL of the sample was mixed with 225 mL of sterile normal saline and then oscillated. The different concentrations of the pure culture of *S. aureus* were used to artificially contaminate samples. The *S. aureus* count in the contaminated samples ranged from 2.3 \times 10⁹ CFU/mL to 2.3 \times 10⁰ CFU/mL. Approximately 1 mL of each dilution was used to extract the genome DNA of *S. aureus* and then stored.

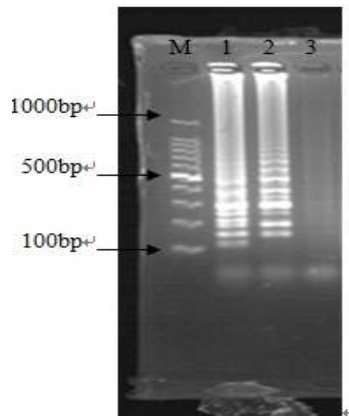
RESULT ANALYSIS

RT-LAMP for *S. aureus* detection: In this study, the nuc gene of *S. aureus* was selected as the target gene. The system was amplified using the method stated in "Reaction Conditions for RT-LAMP". The result was shown in Fig. 3A, in which tube 1, 2 and 3 represented the test tube, positive control and negative control, respectively. Fluorescence peaks were observed in tubes 1 and 2, whereas tube 3 had no amplification. After the reaction was terminated, the products were analyzed with agarose gel electrophoresis to confirm RT-LAMP amplification. Figure 3B demonstrated typical DNA ladders in tubes 1 and 2, whereas no DNA ladder was observed in tube 3. Visibility, the detection result of RT-LAMP was consistent with that of the common LAMP.

Restriction analysis of product: The amplified products were identified using agarose gel electrophoresis after digestion with the restriction endonuclease Hpa I. Figure 4 showed the two stripes with different sizes observed under UV lamp. The result of restriction endonuclease digestion was in accordance with the anticipated objective strap size.



(A)



(B)

Fig. 3: Detection of *S. aureus* using RT-LAMP (A) tube 1: test tube; tube 2: positive control; tube 3: negative control, (B) M: 100-bp marker; 1: tube 1; 2: tube 2; 3: tube 3

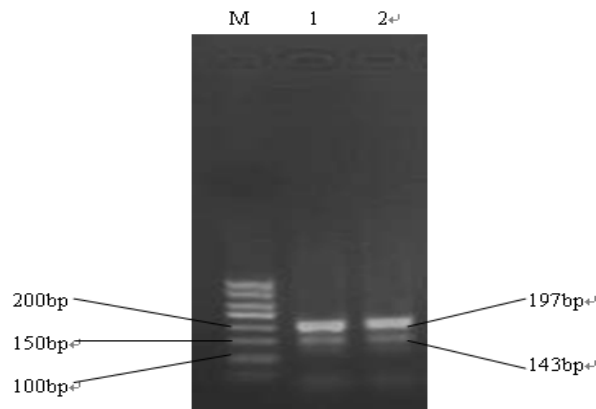
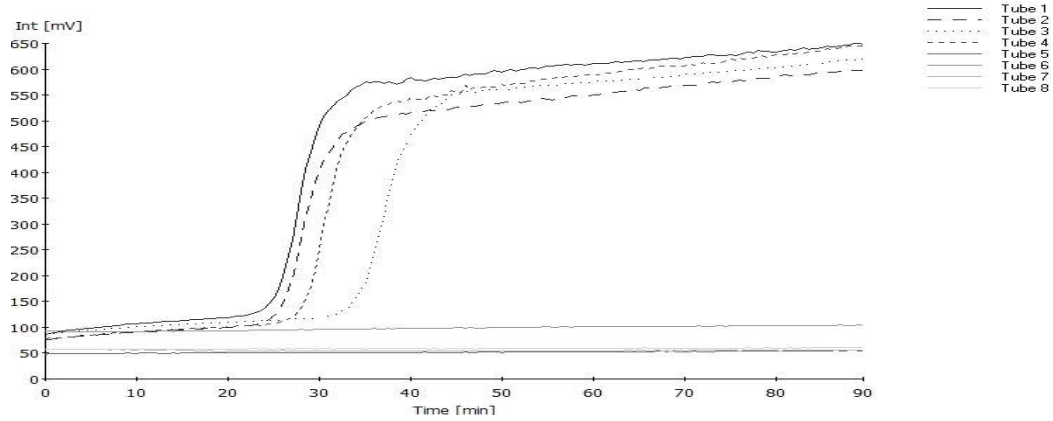
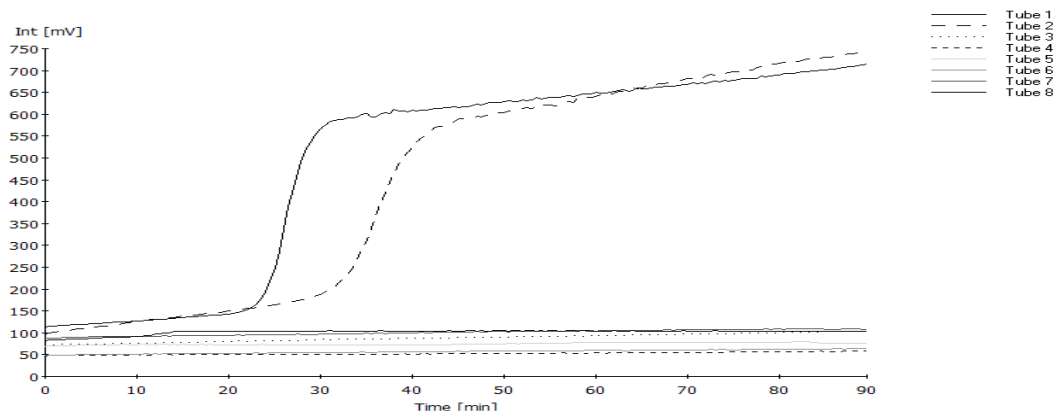


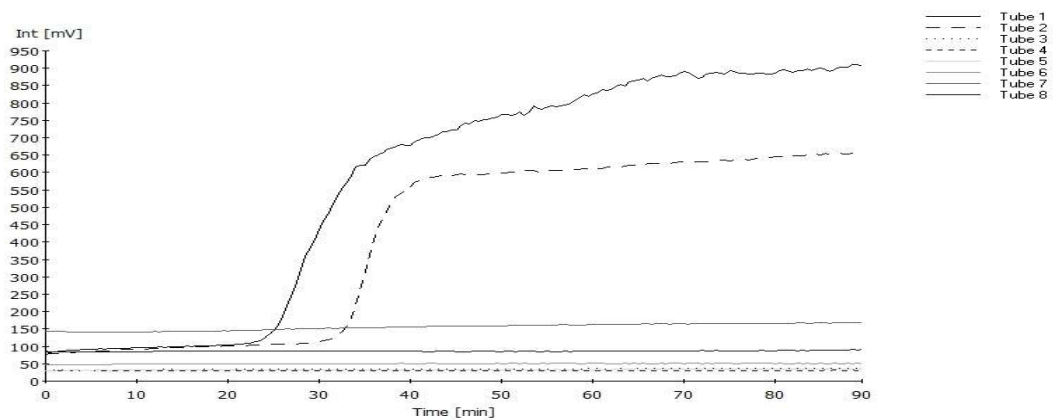
Fig. 4: Restriction analysis of the LAMP product
M: 50-bp marker; 1: Result of restriction analysis; 2: Result of restriction analysis



(A)

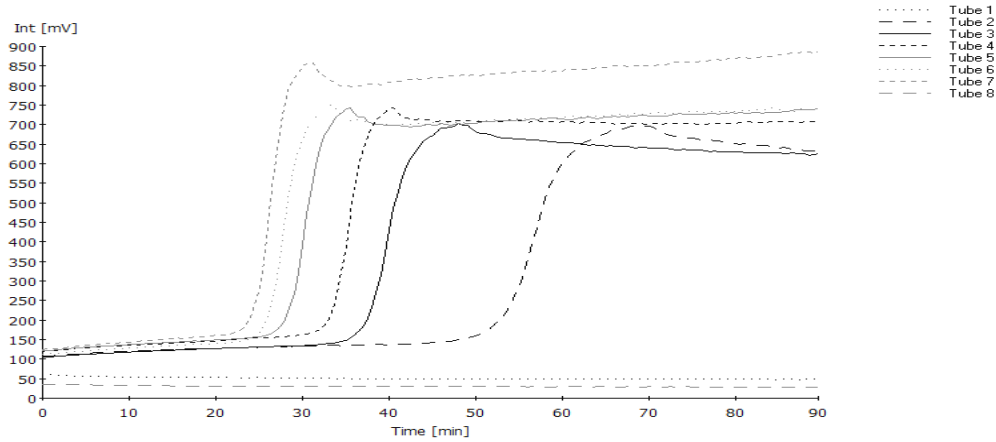


(B)

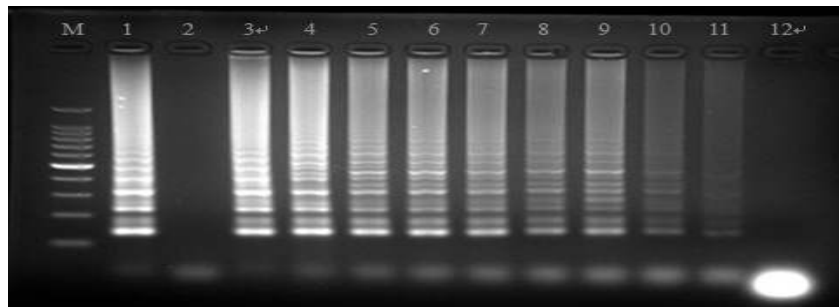


(C)

Fig. 5: Specificity test results of RT-LAMP, (A) tube 1: *Staphylococcus aureus* (CMCC26073); tube 2: *S. aureus* (laboratory preserved); tube 3: *S. aureus* (laboratory preserved); tube 4: *S. aureus* (laboratory preserved); tube 5: *Streptococcus hemolytic-β* (CMCC 32204); tube 6: *Shigella dysenteriae* (CMCC51135); tube 7: *Shigella boydii* (CMCC51522); tube 8: *Shigella sonnei* (CMCC51334), (B) tube 1: *Staphylococcus aureus* (CMCC26003); tube 2: *S. aureus* (laboratory preserved); tube 3: *Shigella boydii* (ATCC12022); tube 4: *Bacillus cereus* (CMCC63302); tube 5: *Yersinia enterocolitica* (CICC21699); tube 6: *Y. enterocolitica* (CMCC52302); tube 7: *S. aureus* (CICC21600); tube 8: *Clostridium perfringens* (ATCC13124), (C) tube 1: *Staphylococcus aureus* (laboratory preserved); tube 2: *S. aureus* (laboratory preserved); tube 3: *Proteusbacillus vulgaris* (CMCC49027); tube 4: *Salmonella typhimurium* (CMCC 50115); tube 5: *Clostridium perfringens* (ATCC13124); tube 6: *P. vulgaris* (CMCC49027); tube 7: *Enterobacter sakazakii* (ATCC51024); tube 8: *Vibrio parahaemolyticus* (ATCC 17802)



(A)



(B)

Fig. 6: Sensitivity test result of RT-LAMP, (A) tube 1: 8.0×10^{-1} CFU/mL; tube 2: 8.0×10^0 CFU/mL; tube 3: 8.0×10^1 CFU/mL; tube 4: 8.0×10^2 CFU/mL; tube 5: 8.0×10^3 CFU/mL; tube 6: 8.0×10^4 CFU/mL; tube 7: 8.0×10^5 CFU/mL; tube 8: negative control, (B) 1: positive control; 2: negative control; 3: 8.0×10^8 CFU/mL; 4: 8.0×10^7 CFU/mL; 5: 8.0×10^6 CFU/mL; 6: 8.0×10^5 CFU/mL; 7: 8.0×10^4 CFU/mL; 8: 8.0×10^3 CFU/mL; 9: 8.0×10^2 CFU/mL; 10: 8.0×10^1 CFU/mL; 11: 8.0×10^0 CFU/mL; 12: 8.0×10^{-1} CFU/mL; Physical quantities in italics, units in roman type

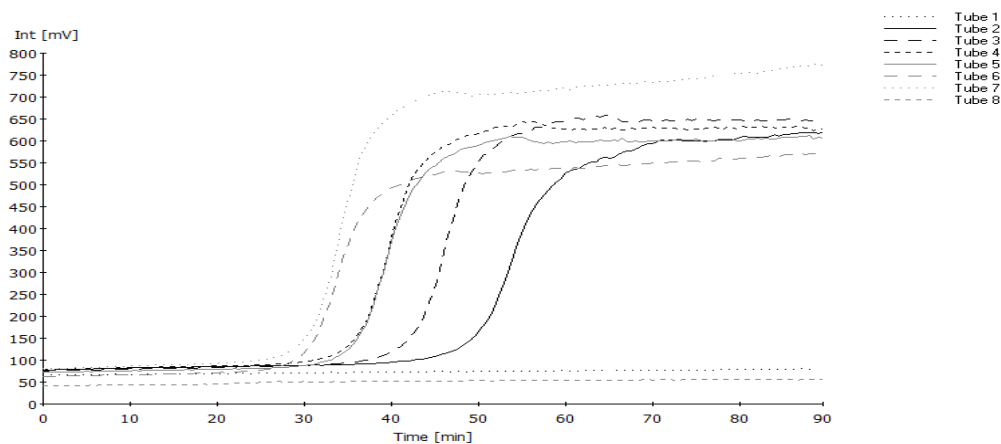


Fig.7: Results of food artificially contaminated with *S. aureus*
 Tube 1: 2.3×10^{-1} CFU/mL; Tube 2: 2.3×10^0 CFU/mL; Tube 3: 2.3×10^1 CFU/mL; Tube 4: 2.3×10^2 CFU/mL; Tube 5: 2.3×10^3 CFU/mL; Tube 6: 2.3×10^4 CFU/mL; Tube 7: 2.3×10^5 CFU/mL; Tube 8: negative control

Specificity of RT-LAMP assay in pure cultures: All tests between *S. aureus* and the other species shown in Table 1 were compared using RT-LAMP. As shown in

Fig. 5A to C, only the eight strains of *S. aureus* had fluorescence peaks. The results indicated that RT-LAMP had remarkable specificity.

Sensitivity of RT-LAMP assay in pure cultures: We diluted the *S. aureus* cultures serially to 10-fold and obtained cell concentrations within the range of 8.0×10^{-1} to 8.0×10^9 CFU/mL to determine the detection sensitivity of RT-LAMP for pure cultures. The extracted DNA from each dilution was used as templates and then analyzed using RT-LAMP and conventional LAMP. The sensitivity results were shown in Fig. 6. As shown in Fig. 6A, the last fluorescence peak occurred after 52 min with the cell concentration of 8.0×10^0 CFU/mL. As shown in Fig. 6B, the Lowest Detection limit (LOD) of RT-LAMP was 8.0 CFU/mL, whereas that of conventional LAMP was 80 CFU/mL. It was demonstrated that the sensitivity of RT-LAMP was 10-fold higher than that of conventional LAMP.

Testing of food artificially contaminated with *S. aureus*: The milk samples were artificially contaminated with *S. aureus* using serial dilutions ranged from 2.3×10^0 to 2.3×10^9 CFU/mL. No fluorescence peak was observed at 2.3×10^{-1} CFU/mL dilution and the instrument was considered negative. Therefore, the LOD of RT-LAMP for testing the milk samples artificially contaminated with *S. aureus* was 23 CFU/mL (Fig. 7).

CONCLUSION

The heat-stable nuc gene was selected as the target sequence and then tested using RT-LAMP. RT-LAMP was established by adding SYBR Green I to the reaction system and using a real-time monitoring instrument to detect *S. aureus*. Results showed that the sensitivity and LOD of RT-LAMP for detecting *S. aureus* are 8.0 and 23 CFU/mL, respectively. The sensitivity of RT-LAMP is 10-fold higher than that of conventional LAMP. The entire detection procedure was simple, convenient, rapid, specific and sensitive. The detection result was evident and accurate. Therefore, RT-LAMP is faster and easier than conventional LAMP and may be applied in general clinical laboratories.

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