Rapid Visualization and Detection of Staphylococcus Aureus Based on Loop-Mediated Isothermal Amplification

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Abstract

Staphylococcus aureus is a common clinical bacterial pathogen that can cause a diverse range of infections. The establishment of a rapid and reliable assay for the early diagnosis and detection of *S. aureus* is of great significance. In this study, we developed a closed-tube loop-mediated isothermal amplification (LAMP) assay for the visual detection of *S. aureus* using the colorimetric indicator hydroxy naphthol blue (HNB). The LAMP reaction was optimized by adjusting the amplification temperature, the concentrations of Mg^{2+} , dNTP, and HNB, and the incubation time. In the optimized reaction system, the specificity of LAMP for *S. aureus* was 100%. The results established that this method accurately identified *S. aureus*, with no cross-reactivity with 16 non-*S. aureus* strains. The limit of detection (LOD) of LAMP was 8 copies/reaction of purified plasmid DNA or 400 colony-forming units/reaction of *S. aureus*. Compared with conventional PCR, LAMP lowered the LOD by 10-fold. Finally, 220 clinically isolated strains of *S. aureus* and 149 non-*S. aureus* strains were used to evaluate the diagnostic efficacy of LAMP. The findings indicated that LAMP is a reliable test for *S. aureus* and could be a promising tool for the rapid diagnosis of *S. aureus* infections.

Introduction

Staphylococcus aureus is a common human pathogen that secretes a variety of virulence factors with significant pathogenicity (Otto 2014). *S. aureus* can cause a wide range of clinical infections, from localized skin and soft tissue infections to life-threatening necrotizing pneumonia (Oliveira et al. 2018). It is also the main cause of bacteremia and osteoarticular, pleuropulmonary, and device-related infections (Tong et al. 2015). *S. aureus* bacteremia is highly prevalent and extremely hard to treat, leading to increased mortality and the burden of additional medical costs (Jin et al. 2021; Naber 2009). Therefore, the development of a reliable and rapid test to accurately detect *S. aureus* presents an urgent clinical need.

The conventional detection of *S. aureus* is based on microbial culturing, including bacterial colony morphology, biochemical reactions, and antimicrobial susceptibility testing (Sheet et al. 2016). Although this method has good sensitivity and reliability, it has some obvious limitations. For example, some bacterial species grow only in specific nutrient matrices and their identification requires various selective media (Lazcka et al. 2007). Moreover, the culturing of strains is a labor-intensive and time-consuming process, taking approximately 2–5 days to produce the desired results (Zhou et al. 2021). Consequently, many patients may miss the optimal period of treatment. Moreover, the improper use of antibiotics may lead to bacterial resistance (Paule et al. 2005). In recent decades, various molecular diagnostic methods have been devised and applied for the classification and identification of pathogenic microorganisms (Mwaigwisya et al. 2015). Compared with microbiological culturing, molecular diagnostic technology has higher sensitivity, better reproducibility, and a shorter time required to produce results. For example, PCR technology as an alternative to microbiological culturing is popular in the field of pathogenic microbial diagnosis because of its great amplification performance (Florio et al. 2018). Several studies have reported on PCR assays for the detection of *S. aureus* in clinical samples with a high diagnostic accuracy

rate (Brakstad et al. 1992; Wang et al. 2019). However, a PCR assay requires expensive experimental instruments, reagents, and skilled personnel, which may not be readily available in many resource-poor areas. Therefore, a cost-effective, simple, and reliable assay for the identification of *S. aureus* should be developed as an alternative routine laboratory test.

Loop-mediated isothermal amplification (LAMP) is a viable alternative to PCR in the molecular diagnosis of bacterial pathogens. LAMP technology utilizes four primers to identify the target sequence with high selectivity and good amplification specificity (Notomi et al. 2000). It can amplify a limited initial number of DNA copies to one million copies in one hour at isothermal conditions and does not require pre-denaturation of the template (Notomi et al. 2015; Nagamine et al. 2001). Recently, LAMP technology has been applied to the detection of several pathogenic bacteria, parasites, viruses, and diseases (Wong et al. 2018; Wong et al. 2009; Fang et al. 2010). However, the conventional LAMP technique analyzes amplification results by agarose gel electrophoresis or turbidimetry, which often require extended workflow times or specialized equipment (Mori et al. 2001). Direct visual detection of turbidity to determine whether amplification has occurred is the simplest and most cost-effective method, but it is difficult to observe the precipitate even under ideal conditions, limiting the widespread use of LAMP in clinical laboratories (Mori et al. 2004; Miyamoto et al. 2015). Hydroxy naphthol blue (HNB) is a metal ion indicator, which can visualize DNA amplification by detecting the decrease of Mg^{2+} ion concentration during the LAMP reaction process (Goto et al. 2009). HNB is easy to use and the colors of negative and positive reactions are significantly different, which enables the LAMP reaction to achieve a clear and rapid visual detection.

Materials And Methods

Bacterial strains and genomic DNA template preparation

A total of 15 bacterial strains were used to analyze the specificity of the LAMP reaction, as listed in Table 1. A total of 369 bacterial strains were isolated from patients at the Sichuan Provincial Hospital of Traditional Chinese Medicine and Chengdu Fifth People's Hospital from January to May 2021 (Table S1). These strains were identified at the species level by the VITEK 2 compact system (Biomérieux, France) which was used as the gold standard for clinical diagnostic efficacy evaluation of the LAMP method. For enrichment culture, all strains were inoculated in tryptic soy broth (TSB) liquid medium and incubated at 37°C with shaking at 200 rpm for 12 hours. Ezup Column Bacteria Genomic DNA Purification Kit (Sangon, China) was used to extract the genome DNA following the manufacturer's protocols, and then stored at -20°C for subsequent experiments. For the 10-fold serial dilution of *S. aureus* (ATCC 25923), used for sensitivity assessment, we extracted its genomic DNA using 5× Hotshot + Tween reagent (Brewster et al. 2013).

Table 1
The strains used for LAMP specificity analysis

Species	Source	Number of strains	Results of LAMP
<i>S. aureus</i>	ATCC 25923	1	+
<i>Salmonella enterica</i>	ATCC 14028	1	-
<i>Serratia marcescens</i>	CMCC(B) 41002	1	-
<i>Enterobacter cloacae</i>	CMCC(B) 45301	1	-
<i>Klebsiella pneumoniae</i>	CMCC(B) 46117	1	-
<i>Alcaligenes faecalis</i>	ATCC 8750	1	-
<i>Enterococcus faecalis</i>	ATCC 29212	1	-
<i>Escherichia coli</i>	ATCC 25922	1	-
<i>Staphylococcus epidermidis</i>	ATCC 12228	1	-
<i>Shigella flexneri</i>	ATCC 12022	1	-
<i>Shigella dysenteriae</i>	CMCC(B) 51105	1	-
<i>Pseudomonas aeruginosa</i>	ATCC 27853	1	-
<i>Vibrio parahemolyticus</i>	ATCC 17802	1	-
<i>Vibrio alginolyticus</i>	CICC 21664	1	-
<i>Enterococcus faecium</i>	ATCC 19434	1	-
Notes: ATCC, American Type Culture Collection; CMCC, National Center for Medical Culture Collections; CICC, China Center of Industrial Culture Collection; +, positive result; -, negative result.			

Screening of target genes and primer design

We initially screened for genes specific to *S. aureus* by local BLAST and online BLAST was used to further identify the target gene, according to previously published procedures (Li et al. 2019). LAMP primers were designed using Primer Explorer V5 (<https://primerexplorer.jp/lampv5/index.html>) based on the target sequence. The primer design and screening process were carried out according to the primer designing manual (http://primerexplorer.jp/e/v5_manual/index.html). The specificity of primers was validated by BLASTN against the GenBank nucleotide database.

Construction of positive control plasmids

We constructed the positive control plasmids by inserting the target sequence into the pEASY®-T3 Cloning Vector (TransGen, China). Then, the plasmids were transferred to Trans1-T1 Phage Resistant Chemically Competent cells (TransGen, China) to yield positive clones. The transformed cells were then incubated in Luria-Bertani liquid medium for enrichment at 37°C with shaking at 200 rpm for 12 hours in

the presence of ampicillin. Positive plasmids were extracted from enriched clones with the TIANGEN pure Mini Plasmid Kit (Tiangen, China), and their sequences were verified by gene sequencing and stored at -20°C for subsequent experiments. All operating procedures were carried out according to the manufacturer's manuscript.

Optimizations for the LAMP assay

The optimization of the LAMP reaction was performed on a G1000 gene amplification instrument (Bioer Technology, China) with purified positive plasmids (8×10^5 copies/ μL) as templates. Nuclease-free water (TransGen, China) was used as a template for the LAMP blank control. The reaction parameters were sequentially optimized for temperatures in the range of 61–65 °C, Mg^{2+} concentrations between 2.0–10.0 mM, dNTP in the range of 0.6–1.4 mM, incubation times between 15–65 min, and HNB (Aladdin, China) between 60–180 μM . Effective amplification resulted in a color change from violet to sky blue. However, the color of the blank control reaction remained violet. Agarose gel electrophoresis was applied for the determination and verification of the amplification of fragments of the correct sizes. The positive amplification of LAMP showed typical ladder bands, while no bands were visible in the negative control. Images were captured by SmartGel 140 (Sage Creation Science, China). All experiments were repeated three times.

Specificity of the LAMP assay

The specificity of the LAMP assay was assessed by detecting genomic DNA extracted from 15 strains of bacterial based on the established optimal reaction system. In addition, a LAMP reaction mixture containing the genomic DNA of non-*S. aureus* strains was used as a negative control. For the blank control, nuclease-free water was used without any genomic DNA. The amplification results indicated by the color change of HNB dye were verified by agarose gel electrophoresis. All experiments were repeated three times.

Sensitivity of the LAMP assay

Two different templates, the positive control plasmid and genomic DNA of *S. aureus*, were used to evaluate the limit of detection (LOD) of LAMP reaction under optimized conditions. The extracted positive plasmids, at a concentration of 30.52 ng/ μL , were mixed with Tris-EDTA buffer (20 mmol/L Tris-HCl and 2 mmol/L EDTA) and diluted to 8×10^8 copies/ μL as a working solution, which was then serially diluted 10-fold to a working concentration (8×10^8 – 8×10^0 copies/ μL). To obtain a specific DNA copy number per μL from the DNA stock solution, we referred to the DNA Copy Number and Dilution Calculator tool (<https://www.thermofisher.cn/cn/zh/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>). The *S. aureus* bacterial suspensions (4×10^8 CFU/mL) was serially diluted 10-fold in TSB liquid medium to obtain a series of bacterial solution at various concentrations (4×10^8 – 4×10^0 CFU/mL). These bacterial suspensions were directly lysed by the 5× Hotshot + Tween reagent and the resulting solutions were used as the template for LAMP analysis. The LOD of LAMP was calculated based on the

minimum mean concentration of the positive plasmid and *S. aureus*. All experiments were repeated three times.

Application of LAMP in clinical strain samples

The clinical diagnostic efficacy of the LAMP assay was determined using the 369 clinical isolates. These samples were inoculated into TSB liquid medium for enrichment and the genomic DNA was extracted for LAMP experiments. PCR was also performed with specific primers F3 and B3. Finally, we compared the results of the LAMP assay with those of the PCR in terms of specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV). The calculation formula used were as follows: specificity = $TN / (TN + FP) \times 100\%$; sensitivity = $TP / (TP + FN) \times 100\%$; PPV = $TP / (TP + FP) \times 100\%$; NPV = $TN / (TN + FN) \times 100\%$.

Results

Selection of target gene and primers

Following the results of local BLAST and online BLAST screening, a highly conservative specific sequence in the *S. aureus* MJ163 chromosome was selected as the target gene in this study. Then, four LAMP primers for the target gene were designed as listed in Table 2.

Table 2
LAMP primers for *S. aureus*

Primer	Sequence (5'→ 3')
F	CGTTGGCTTAATAGATTAATTGTTG
B	ACGCACTCCTTTTCCAAAT
FIP	ACGGAATGTAAGCGTAAGAAACGCGTGCTAATGTTTTTAAATGGATTC
BIP	AGAAAGTCTAACGCTTAAACTGCTGAATTGCTGCTTGCATGAC

Optimal reaction system for LAMP

The critical parameters of the LAMP reaction, including amplification temperature, the concentration of Mg^{2+} and dNTP, and incubation time, were optimized to achieve optimal amplification efficiency. As shown in Fig. 1a, LAMP electrophoresis bands were brighter at 62°C than at other amplification temperatures, with no significant differences at 61, 63, 64, or 65°C. Therefore, 62°C was selected as the optimal reaction amplification temperature for subsequent experiments. For the optimization of Mg^{2+} concentrations at 62°C, the best amplification efficiency was observed at 8.0 mM Mg^{2+} (Fig. 1b). The maximum amount of amplification products was obtained at 1.2 mM dNTP (Fig. 1c). The amplification product was first detected when the reaction was performed for 35 minutes (Fig. 1d). For optimal amplification, an incubation time of 45 min was subsequently used as a LAMP reaction parameter. Based

on the above reaction system, 120 μM HNB was selected in subsequent experiments to achieve optimal visual detection (Fig. 1e).

In summary, the optimized LAMP system used in the present study contained 2.5 μL 10 $\times$ isothermal amplification buffer, 8.0 U Bst 2.0 DNA polymerase, 8.0 mM Mg^{2+} , 1.2 mM dNTP, 0.8 M betaine, 120 μM HNB, 0.2 μM each of primers F and B, 1.6 μM each of primers FIP (forward inner primer) and BIP (backward inner primer), DNA template and nucleasefree water. The reaction mixture was incubated at 62°C for 45 minutes and then heated at 80°C for 2 minutes to terminate the reaction.

Validation of the specificity of LAMP

A significant color change in the LAMP assay from violet to sky blue was only observed for the *S. aureus* strain, while the 14 non-*S. aureus* strains retained their original violet color (Fig. 2a). Agarose gel electrophoresis further confirmed that only the strain of *S. aureus* showed typical ladder-like pattern bands, while the others showed no bands (Fig. 2b). These results were consistent with the PCR assay using the primers F3 and B3 (Fig. 2c). These consistent results indicated that the LAMP assay that we developed specifically detects *S. aureus* and has no significant cross-reactivity with other species.

Limit of detection of LAMP

Significant color changes from violet to sky blue were observed in the 10-fold serial dilutions of the positive plasmid templates ranging from 8×10^8 to 8×10^0 copies/reaction when the LAMP assay was performed at 62°C for 45 min (Fig. 3a). The amplification results were further confirmed on 2% agarose gel electrophoresis (Fig. 3c). However, the LOD of conventional PCR was 8×10^1 copies/reaction for the positive plasmid (Fig. 3e). To further explore the sensitivity of the LAMP assay, a suspension of *S. aureus* was diluted to 4×10^8 , 4×10^7 , 4×10^6 , 4×10^5 , 4×10^4 , 4×10^3 , 4×10^2 , 4×10^1 , and 4×10^0 CFU/mL, respectively. Genomic DNA was extracted from these diluted samples and directly used for LAMP and PCR analysis. The results showed that at a dilution of 4×10^3 CFU/reaction *S. aureus* could be detected by PCR (Fig. 3f), while *S. aureus* could be detected by LAMP at a dilution of 4×10^2 CFU/reaction (Fig. 3b and 3d). Therefore, the value of LOD of the LAMP assay was 10-fold lower than that of PCR. Consequently, for the detection of *S. aureus* the LOD of the LAMP assay was superior to that of the PCR assay.

Clinical diagnostic efficacy of LAMP

Next, we analyzed 369 clinical isolates to determine the clinical diagnostic efficacy of the LAMP assay. Among 149 non-*S. aureus* strains one strain of *Klebsiella pneumoniae* exhibited non-specific amplification in the PCR reaction (Fig. 4c). Conversely, none of the non-*S. aureus* strains were effectively amplified in LAMP (Fig. 4a and 4b). Of the 220 *S. aureus*-positive samples, 2 strains were deemed negative by LAMP and PCR. Therefore, compared with the gold standard, the specificity of the LAMP assay was 100.00%, and the sensitivity was 99.09%. Meanwhile, the PPV of LAMP for the detection of *S.*

aureus was 100.00% and the NPV was 98.68%. The results of testing clinical strains revealed that LAMP is a reliable and accurate assay for the detection of *S. aureus*.

Discussion

S. aureus is a common clinical pathogen that causes a variety of serious hospital and community-acquired infections (Holland et al. 2014). Rapid and accurate diagnosis of *S. aureus* is a key component of treatment, prevention, and interruption of transmission. The currently existing diagnostic methods have limitations as they are either time-consuming (microbial culturing) or expensive (PCR). LAMP is a simple and cost-effective molecular diagnostic method with distinct advantages for the rapid diagnosis of pathogens.

In this study, we successfully developed, optimized, and validated a molecular biology diagnostic LAMP assay for the detection of *S. aureus*. The critical parameters of the LAMP reaction were optimized to achieve optimal amplification and performance by visual inspection. The optimized LAMP assay can accurately differentiate *S. aureus* from non-*S. aureus* strains, and sensitively detect trace amounts of *S. aureus*.

At present, a variety of colorimetric indicators have been developed for LAMP reaction endpoint detection. Although these indicators are reliable and convenient, they have some limitations. Calcein needs to bind the ionic form of manganese to work, but manganese may inhibit the LAMP reaction and reduce the sensitivity of the assay (Tanner et al. 2015). The fluorescent intercalating dye SYBR Green I is expensive, highly toxic, and requires additional testing equipment (Fischbach et al. 2015). Leuco crystal violet (LCV) requires the preparation of proprietary LCV immobilized tubes, which are complicated to operate (Miyamoto et al. 2015). In contrast, HNB is a cheap and stable synthetic dye. It indicates the result of the LAMP reaction by detecting the consumption of free magnesium ions in the reaction system, which is reliable and effective for LAMP. A multi-observer study showed that HNB as an indicator was superior to the other methods (Wastling et al. 2010). The use of the colorimetric indicator HNB makes the judgment of the LAMP amplification results easier, eliminating the need for tedious electrophoresis operations and greatly avoiding cross-contamination caused by aerosols (Nie 2005). Therefore, HNB was selected as the best dye for this study.

We compared the sensitivity of the LAMP assay with that of the PCR assay and found that the LOD of the LAMP was 10-fold lower than PCR, both for the positive plasmid and *S. aureus*. Several related studies have also confirmed that the sensitivity of LAMP assay was equivalent or higher compared to PCR (Choopara et al. 2021; Maeda et al. 2005; Prusty et al. 2016). In addition, LAMP technology is more convenient, time-saving, and cost-saving. The entire detection process of LAMP, including template preparation (approximately 40 min), isothermal amplification (45 min), and result determination (approximately 2 min), can be completed within 90 min and the cost of analysis is also lower than that of PCR (Zheng et al. 2018). More importantly, LAMP testing is easy to operate, which can reduce labor costs. Finally, LAMP is often described as a less demanding test than PCR in terms of DNA purification. For

example, bacterial colonies are added directly to the reaction mixture for the LAMP reaction, or centrifuged pellet samples are boiled in the presence of 1% Triton X-100 and the released DNA can be used directly for the LAMP reaction without purification (Sowmya et al. 2012; Yan et al. 2017).

The clinical diagnostic efficacy of LAMP analysis is shown in Table 3. A *Klebsiella pneumoniae* strain, isolated from the sputum of a patient with chronic obstructive pulmonary disease, showed a non-specific electrophoresis band by PCR amplification. The electrophoresis results showed that the amplified band size was over 250 bp, while the target fragment band size for *S. aureus* was 219 bp (Fig. 4c). One possible explanation is that the genome of this strain has some base fragments homologous to the target gene in *S. aureus*. However, due to the specific amplification mechanism, the LAMP reaction did not produce a false-positive result for this sample. This indicates that the specificity of the LAMP assay (100%) is superior to that of the PCR (99.33%). In other related studies, the specificity of both detection methods for bacterial pathogens was consistent (Thakur et al. 2018; Zhao et al. 2013). Meanwhile, 2 strains of *S. aureus* were not identified by LAMP, one case isolated from a sputum sample of a patient with cerebral hemorrhage, and the other isolated from the blood of a shock patient. We speculate that this result may be related to a genetic mutation of the strains themselves. Another point worth mentioning is that although the LOD of the LAMP assay was lower than that of the PCR assay, the sensitivity of the PCR assay is equivalent to that of the LAMP assay in clinical strain detection, which may be related to the low number of clinical strains we analyzed. Our study provides a rapid detection protocol for *S. aureus*. However, for point-of-care testing, further research is still needed. If LAMP technology is integrated into a microfluidic system, DNA extraction, isothermal amplification, and colorimetric analysis could be completed in one step, which will have greater potential for clinical applications (Liu et al. 2020; Wan et al. 2019; Zhang et al. 2019).

Table 3
Diagnostic efficacy of LAMP and PCR in clinical bacteria strains

Method	Specificity	Sensitivity	NPV	PPV
LAMP	100%	99.09%	98.68%	100%
PCR	99.33%	99.09%	98.67%	99.54%
Notes: NPV, negative predictive value; PPV, positive predictive value.				

Conclusions

In summary, we successfully developed a LAMP assay for the rapid detection of *S. aureus*. In particular, the colorimetric indicator HNB was used to indicate the LAMP test result, and the positive LAMP reactions were easily identified by the naked eye under ambient light. Application of LAMP for rapid detection of clinical isolates gave a PPV of 100% and a NPV of 98.68%. LAMP is simpler, faster, and easier to perform than conventional PCR and culture microbiology. Therefore, the present research shows that LAMP can be an effective tool for the immediate diagnosis of *S. aureus* infection.

Declarations

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Conflict of interest The authors declare no competing interests.

Availability of data and material The datasets and materials generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval All the samples were residual specimens after diagnostic sampling therefore do not involve ethics approval.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

Figure 1

Optimization for LAMP assay (a) Optimization of amplification temperatures. (b) Optimization of Mg²⁺ concentrations at 62 °C. (c) Optimization of dNTP concentrations at 62 °C with 8.0 mM Mg²⁺. (d) Optimization of incubation time at 62 °C with 8.0 mM Mg²⁺ and 1.2 mM dNTP. (e) Optimization of HNB concentrations at 62 °C for 45 min with 8.0 mM Mg²⁺ and 1.2 mM dNTP. M, marker; +, positive result; -, negative result.

Figure 2

Specificity of the LAMP and PCR assays (a) The visualization results of LAMP. (b) Agarose gel electrophoresis results of LAMP. (c) Agarose gel electrophoresis results of PCR. BC, blank control; M, marker.

Figure 3

LOD of the LAMP and PCR assays (a) The visualization results of LAMP for the positive plasmid. (b) The visualization results of LAMP for *S. aureus*. (c) Agarose gel electrophoresis results of LAMP for the positive plasmid. (d) Agarose gel electrophoresis results of LAMP for *S. aureus*. (e) Agarose gel electrophoresis results of PCR for the positive plasmid. (f) Agarose gel electrophoresis results of PCR for *S. aureus*. BC, blank control; CFU, colony-forming unit; M, marker.

Figure 4

LAMP and PCR assays for detection of clinical isolates (a) The visualization results of LAMP. (b) Agarose gel electrophoresis results of LAMP. (c) Agarose gel electrophoresis results of PCR. BC, blank control; M, marker.

Supplementary Files

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