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Direct loop-mediated isothermal amplification assay for on-site detection of Staphylococcus aureus

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One sentence summary: The direct LAMP is a powerful tool for rapid and on-site detection of S. aureus in food samples.

ABSTRACT

Staphylococcus aureus (S. aureus) is a major human pathogen that may produce a variety of toxins and cause staphylococcal food poisoning. In the present study, a direct loop-mediated isothermal amplification (LAMP) assay was developed and validated to detect S. aureus in food samples. Without prior cultural enrichment and DNA extraction steps, bacterial DNA was released by heating at 100 °C for 5 min and directly subjected to LAMP assay. Using a set of LAMP primers recognizing six distinct regions of nuc gene, the developed direct LAMP assay was highly specific, and the analytical sensitivity was determined to be 7.6 × 10^2 CFU/mL. Moreover, with the pre-mixed LAMP reagents stored at –20 °C, the entire assay should be finished within 40 min. These features greatly simplified the operating procedure and made the direct LAMP a powerful tool for rapid and on-site detection of S. aureus in food samples.

Keywords: food-borne pathogen; Staphylococcus aureus; direct LAMP; on-site detection

INTRODUCTION

Staphylococcus aureus (S. aureus) is an extremely common pathogenic bacterium that can be found in a number of different food varieties, including mixed foods (pasta dishes, salads), meat and meat products, egg and egg products, vegetables, baked goods and cheeses (Zeleny et al. 2015). Due to its multidrug resistance and virulence, S. aureus has become a major concern to public health and food safety in many countries (Sheet et al. 2016; Shin et al. 2016; Jans et al. 2017; Lidiane et al. 2017; Miao et al. 2017; Miao et al. 2017). According to the reports from Chinese Center for Disease Control and Prevention, there are 240 000 illnesses annually in China with 1000 hospitalizations and six deaths induced by staphylococcal food poisoning (Scallan et al. 2011). Therefore, S. aureus detection is one of the routine programs for food safety testing in China (Chinese National food safety standard GB 4789.10-2010).

Nowadays, culture-based method is still used as a routine method for S. aureus detection. This conventional method involves enrichment and enumeration in liquid media, subsequent recovery and isolation of colonies on selective culture broth and further confirmation assays (Chinese National food safety standard GB 4789.10-2010). Generally, it requires a lengthy time span of 3–5 d. Molecular biology techniques such as polymerase chain reaction (PCR), real-time PCR and DNA hybridization have been developed and become popular for pathogen identification (Sudhaharan et al. 2015). Although these technologies can detect low number of bacterial cells, they usually need several hours. Moreover, such techniques required prior cultural enrichment and bacterial DNA isolation, preparation of enzyme reaction mixtures and expensive equipment (Kei et al. 2014). Therefore, most of these techniques are not suitable for on-site detection. Nevertheless, loop-mediated isothermal amplification (LAMP) is a simple, cost-effective and
rapid method that is often used as an alternative to PCR-based methodologies in pathogen detection (Hira et al. 2004; Kurosaki et al. 2007; Kanitkar et al. 2017; Lin et al. 2017; Yan et al. 2017). Nowadays, LAMP has been applied to detect many pathogens such as Salmonella, Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Listeria monocytogenes (Xu et al. 2010; Zhao et al. 2010a,b; Wang et al. 2011; Zhao et al. 2011; Wang et al. 2012; Liu et al. 2016, 2017). Besides these, LAMP assays were also demonstrated to be useful and powerful tools for examination of various Staphylococcus strains (Misawa et al. 2007; Xu et al. 2012; Wang et al. 2015). Most of these studies were based on detection the genetic elements of staphylococcal chromosomal cassette mec (such as mecA, orfX and femA), which has been reported to play a core role in the antimicrobial resistance characteristics, evolution and molecular epidemiology of staphylococcal diseases (Xu et al. 2011; Liu et al. 2016). Due to its simplicity and easiness of performance without any sophisticated instrumentation, LAMP can be easily adapted to any microbiology laboratory and on-site detection as well (Sudhaharan et al. 2015).

Like other kinds of molecular methods, bacterial DNA extraction and purification are the primary steps for LAMP assay, which have become a bottleneck for rapid detection (Yan et al. 2017). Actually, an easy, effective and low-cost DNA isolation protocol will greatly accelerate the detection process and broaden its practical application. Several studies have reported the successful production of DNA template (for PCR or LAMP assay) by simply boiling the specimens (Koizumi et al. 2012; Wang et al. 2012; Kei et al. 2014; Wozniakowski and Samorek-Salonowicz 2014; Wozniakowski et al. 2015; Imai et al. 2018). Therefore, we decided to take advantage of LAMP using boiled S. aureus cultures as DNA templates in this study. Moreover, the LAMP reagents were pre-mixed and stored at −20°C. With such preparation, the method can deliver a ‘sample-to-result’ time of approximately 30 min. Besides these, the results of LAMP reaction were visualized by naked eyes. These characters demonstrated the feasibility of direct LAMP to be used as a rapid and effective on-site method for S. aureus detection.

### MATERIALS AND METHODS

#### Bacterial strains

Staphylococcus aureus ATCC 6538, Listeria monocytogenes ATCC 19112, Escherichia coli ATCC 25922, Sligella flexneri ATCC 12022 and Salmonella enteritidis ATCC 15611, Vibrio parahaemolyticus 17802, Bacillus cereus ATCC 11778 and Clostridium perfringens ATCC 13124 were purchased from the American Type Culture Collection (ATCC). All of the bacterial strains were cultured in tryptone soy broth (TSB) or lysogeny broth (LB) broth/agar plates at 37 °C, with media purchased from Beijing Land Bridge Technology Co. Ltd (Beijing, China). These bacteria were harvested from fresh cultures, and were decimal diluted by double-distilled H2O (ddH2O).

#### Preparation of S. aureus genomic DNA and template of LAMP assay

After S. aureus ATCC 6538 was cultivated at 37 °C for 24 h, its concentration was precisely determined by plate-counting technique and expressed as colony forming unit per mL of bacterial culture (CFU/mL). Then, bacterial DNA was isolated either by a ‘Bacterial Genomic DNA Extraction kit’ (Takara Biotecnology, Dalian, China), according to the manufacturer’s instructions, or by heating the cell at 100 °C for 5 min and immediately chilling on ice. One milliliter fresh cultured S. aureus (about 10^6–10^8 CFU/mL) was used for each extraction. All of the extractions were carried out in triplicate, and stored at −20°C until further used.

### Primer design

The nuc gene of S. aureus ATCC 6538 was downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/, accession number EF253320). Based on the sequence information and previous report (Sudhaharan et al. 2015), a set of LAMP primers targeting nuc gene was chosen. At the same time, the forward and reverse PCR primers of nuc gene were also designed by Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA). These primer sequences were tested against the Basic Local Alignment Search Tool (National Center of Biotechnology Information (NCBI), http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure specificity. All of the primers were synthesized by Sangon Biotech (Shanghai, China), and listed in Table 1.

### LAMP reaction

The LAMP reaction was carried out using the DNA thermo-statical amplification kit (Guangzhou Deaou Bio-technology Co., Ltd, Guangdong, China) following the manufacturer’s instructions. The visual LAMP detection was performed in a 10 μL reaction mixture containing 5 μL Reaction Buffer (2×), 0.2 μM each of F3/B3, 1.6 μM each of FIP/BIP, 0.4 μM each of FLP/BLP, 3.2 U Bst 2.0 WarmStart DNA polymerase, 0.5 μL S. aureus DNA template and 2.26 μL ddH2O. As described in our previous works, 10 μL mineral oil was added to the top of reaction mixture, and 0.4 μL dye (SYBR Green® I, Guangzhou Deaou) was added on the inner wall of tube cap of each reaction (Liu et al. 2016; Ye et al. 2016, 2017). Reaction tube was inoculated at 63 °C for 20–40 min, and thereafter the reaction solution was mixed with SYBR Green® I by shaking. All reactions were run in triplicate, and the negative controls were performed using sterile water instead of bacterial DNA template. The reaction was considered as positive if its color turned from orange to green under natural light, whereas for negative reactions the solution retained the original orange color.

The real-time fluorescence LAMP detection was carried out on the Mini Opticon Real-time PCR System (Bio-Rad Laboratories, Hercules, CA, USA), with reaction components similar to visual LAMP. However, instead of the SYBR Green® I and mineral oil, the SYTO® 9 Green (fluorescent nucleic acid stain,
Invitrogen Corp., Carlsbad, CA) was added into the reaction mixture. Like real-time PCR, fluorescence signals of the reaction were collected at 1-min intervals, and a typical sigmoid shaped fluorescence curve was produced. The threshold line of this fluorescence curve was set using the equation:

$$\text{Threshold} = 10 \times SD_{(1\text{st}-10\text{th})};$$

where $SD_{(1\text{st}-10\text{th})}$ is the standard deviation of fluorescence signals from first to tenth min. And the time point when fluorescence curve reached threshold line was called as ‘detection time.’

**Determination the specificity and sensitivity of LAMP assay**

In order to determine the specificity of LAMP assay, the visual detection were carried out under the conditions described above, using DNA templates extracted from S. aureus, L. monocytogenes, E. coli, S. flexneri, S. enteritidis, V. parahaemolyticus, B. cereus and C. perfringens. The sensitivity of LAMP assay was assessed using DNA samples extracted from S. aureus of various concentrations. Briefly, the S. aureus cells were serially diluted with ddH$_2$O, to get final concentrations ranging from 7.6 to $7.6 \times 10^6$ CFU/mL. Then, 1 mL of each dilution was used for DNA extraction, and subsequently subjected to LAMP assay as described above. The limit of detection (LOD) was expressed as the lowest concentration of S. aureus (CFU/mL) that gave a positive result in all of three individual replicates.

**Stability of pre-mixed LAMP reagents and its application in real food samples**

In order to reduce the time and complexity of detection, the reagents for visual LAMP reaction were pre-mixed (including 1 × reaction buffer, 0.2 μM of each F3/B3, 1.6 μM of each FIP/BIP, 0.4 μM of each LF/LB, 0.32 U/μL of Bst 2.0 WarmStart DNA polymerase and mineral oil), and named as reagent A (Guangzhou Deaou). Reagent B was SYBR Green® I, which served as indicator (Guangzhou Deaou). Reagents A and B were stored at 4°C and −20°C, and tested periodically for up to 4-weeks. And the results were compared with that obtained by the conventional LAMP assay.

Moreover, the feasibility of developed LAMP with pre-mixed LAMP reagents was analyzed. For this purpose, 18 real food samples collected from local markets were subjected to S. aureus detection. The detection was performed as follows: 1 g of each sample was weight into a tube and mixed with 9 mL of sterilized water. The mixture was stand for 2 min, and 1 mL supernatant was used for DNA extraction by heating at 100°C for 5 min. Subsequently, these DNA samples were subjected to both LAMP and conventional PCR assays. The results of LAMP reaction were visualized as described earlier, and the PCR products were judged by gel electrophoresis. Each sample was tested twice to ensure reproducibility.

**RESULTS**

**Reaction time of the direct LAMP assay**

In the LAMP assay, the reaction temperature and optimum concentration of each component were determined based on pre-experiment and our previous works (as described in materials and methods; Liu et al. 2016; Ye et al. 2016, 2017). Under such conditions, the RealAmp reaction was performed to determine the optical amplification time, by S. aureus cells with concentrations ranged from 7.6–$7.6 \times 10^6$ CFU/mL. Using DNA extracted from these diluted Staphylococcus aureus by commercial kit, the LAMP reactions with S. aureus cells higher than $7.6 \times 10^5$ CFU/mL were successfully amplified within 25 min (Fig. 1). Thus, cut-off time of this method was set as 25 min as the vertical dash line shown in Fig. 1, and amplifications with detection time more than 25 min would be considered as negative reactions.

**Specificity of the S. aureus direct LAMP assay**

Specificity of the assay was evaluated by testing the amplification performance of the developed direct LAMP with DNA isolated from S. aureus and seven other bacterial species. As shown in Fig. 2, after incubation at 63°C for 25 min, only the solution color of reaction including S. aureus DNA turned from orange to green (line 1) in visual LAMP assay. No cross-reactivity was observed for other bacterial species. Thereafter, the LAMP assay was highly specific, which might result from the specific targeting of nuc gene by LAMP primers set.

**Sensitivity and Detection limit of S. aureus in different LAMP assays**

Sensitivity of the ‘direct LAMP’ was analyzed using fresh cultured S. aureus cells. The cells were 10-fold serially diluted,
and bacterial DNA was obtained by boiling the samples. As the results shown in Fig. 3a, the LOD was determined to be $7.6 \times 10^2$ CFU/mL of Staphylococcus aureus cells.

In parallel, the sensitivity of LAMP was also determined with serially diluted DNA samples of S. aureus. Using DNA samples extracted by commercial kit, the LOD was determined to be $7.6 \times 10^2$ CFU/mL in Visual-LAMP assays (Fig. 3b).

**Stability of the pre-mixed LAMP reagents**

After the reagent A and B were stored at 4°C and −20°C for 24 h, they were used to detect S. aureus by direct LAMP. Compared with LAMP assay using reagents stored at −20°C, the reaction color was obviously faded using reagents stored at 4°C (as shown in Fig. 4, Lane 2 vs Lane 3). Therefore, these results indicated that 4°C was unsuitable for the storage of the pre-mixed reagents.

Subsequently, the stability of pre-mixed LAMP reagents stored at −20°C was further analyzed. After stored for 7, 14, 21 and 28 days, the LAMP reactions were carried out and recorded. No color differences were observed among these reactions, which indicated that pre-mixed LAMP reagents were stable under −20°C.

**DISCUSSION**

Staphylococcus aureus is one of the most frequent causative agents of food-borne disease. Many studies have reported the detection of S. aureus based on the nuc gene, as which is specific for S. aureus (Costa, Kay and Palladino 2005; Delibato et al. 2005; Pinto, Chenoll and Aznar 2005; Suhaili 2009; Cattoir et al. 2011; Montazeria et al. 2014; Panda et al. 2014; Montazeria et al. 2015). In this study, the LAMP primers set was also designed based on sequence of nuc gene. With inner and outer primers recognizing six distinct regions, the developed LAMP was highly specific (shown in Fig. 2).

In the case of molecular detection of pathogen, it takes at least 1 h for bacterial DNA extraction and purification. To reduce the cost and reaction time, a quick and simple template isolation

| Sample | Product description | Visual LAMP assays | PCR |
|--------|---------------------|--------------------|-----|
| 1      | Smoked chicken-1    | +                  | +   |
| 2      | Smoked chicken-2    | −                  | +   |
| 3      | Smoked chicken-3    | −                  | −   |
| 4      | Duck egg-1 (raw)    | +                  | +   |
| 5      | Duck egg-2 (raw)    | +                  | +   |
| 6      | Duck egg-3 (raw)    | +                  | +   |
| 7      | Minced pork-1       | +                  | +   |
| 8      | Minced pork-2       | +                  | +   |
| 9      | Pork slice-1 (cooked) | +               | +   |
| 10     | Pork slice-2 (cooked) | −              | −   |
| 11     | Cooked beef         | −                  | −   |
| 12     | Squid (raw)         | +                  | +   |
| 13     | Shrimp (raw)        | −                  | −   |
| 14     | Surimi              | +                  | +   |
| 15     | Roast fish          | −                  | −   |
| 16     | Smoked salmon       | −                  | −   |
| 17     | Dumpling-1 (raw)    | −                  | −   |
| 18     | Dumpling-2 (raw)    | −                  | −   |

+ , positive; −, negative.
procedure is in urgent need of LAMP reaction, especially for the on-site detection. It has been reported that plenty of bacterial cells would be leaked and a large amount of DNA would be released after heating at 94°C for 10 min (Lin et al. 2017). According to the results shown in Fig. 3, although the release of bacterial DNA due to cell breakage is 10-fold less efficient compared with DNA extraction using commercial kit, the established direct LAMP was sufficient to yield positive results for S. aureus of $7.6 \times 10^2$ CFU/mL. Moreover, omission of the DNA extraction procedure would obviously simplify the protocol and shorten the detection time. Based on the Chinese standard GB 29921-2013, the restriction limits of S. aureus load in food samples are $10^7$–$10^4$ CFU/g. Therefore, the developed direct LAMP has sufficiently sensitivity, and can be used as a fast and easy-to-perform diagnostic procedure for early S. aureus detection.

It should be highlighted that the detection time for S. aureus by the developed direct LAMP is greatly decreased. As eliminating the prior cultural enrichment and DNA isolation steps, the hands-on time per specimen can be finished within 40 min. The detection time was further reduced as the components of LAMP assay does not need to be pipetted one by one. And this could greatly avoid cross-contamination caused by aerosol as well (Liu et al. 2016; Ye et al. 2016, 2017). Compared with the analysis time of at least 3 h by conventional PCR and 2 h by conventional LAMP assays, the time-to-results of direct LAMP is reduced by 4.5 and 3 times, respectively. Although it is less sensitive, these characters makes the direct LAMP has promising potential as a valuable tool for rapid detection of S. aureus in food samples, and more appropriate for on-site testing.

The main limitation of this study was the small sample size collected for practical analysis. Further studies with more extensive food samples are needed to determine the feasibility of direct LAMP for S. aureus detection. However, the detection accuracy of developed direct LAMP assay was 100% when compared with the conventional PCR method, representing its high reliability for real food samples.

**CONCLUSION**

In conclusion, the developed direct LAMP assay is a rapid detection method for identifying *Staphylococcus aureus* with high specificity. In this assay, the DNA isolation procedure was eliminated by heating the specimens at 100°C for 5 min, and the LAMP components does not need to be pipetted one by one when the pre-mixed reagents were used. Under such conditions, the direct viral LAMP assay was reliable, and showed a sensitivity of $7.6 \times 10^2$ CFU/ml. The entire detection can be finished within 40 min. These characters make it time- and cost-effective, and more appropriate for rapid on-site detection. Therefore, the method developed in this study could be utilized as an efficient detection tool for early and on-site detection of S. aureus in food samples.

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**Conflict of interest.** None declared.

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