JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 31-35

Clinical Application of Loop-Mediated Isothermal Amplification in the Rapid Detection of Methicillin-Resistant *Staphylococcus aureus*

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(Received: 02 June 2015; accepted: 10 August 2015)

To develop a detection assay for staphylococcal mecA and spa by using loop-mediated isothermal amplification (LAMP) method. Staphylococcus aureus and other related species were subjected to the detection of mecAand spa by both PCR and LAMP methods. The LAMP successfully amplified the genes under isothermal conditions at 64! within 60 min, and demonstrated identical results with the conventional PCR methods. The detection limits of the LAMP for mecA and spa, by gel electro-phoresis, were 10^2 and 10 cells per tube, respectively. The naked-eye inspec-tions were possible with 10^3 and 10 cells for detection of mecA and spa respectively. The LAMP method was then applied to sputum and dental plaque samples. The LAMP and PCR demonstrated identical results for the plaque samples, although frequency in detection of mecA and spa by the LAMP was relatively lower for the sputum samples when compared to the PCR methods. Application of the LAMP enabled a rapid detection assay for mecA and spa. The assay may be applicable to clinical plaque samples. The LAMP offers an alternative detection assay for mecA and spa with a great advantage of the rapidity.

Key words: loop-mediated isothermal amplification LAMP, Methicillin-resistant Staphylococcus aureus, mecA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial infective agent with one of the highest infection rates worldwide. Therefore, screening high-risk patients is a critical route to effectively control the infection and restrict the expansion. To this end, the correct and rapid detection of MRSA is the key to seek effective antibacterial drugs and contain MRSA infection, which is conducive for the prompt diagnosis of MRSA and reasonable treatment, especially during outbreaks. However, there are currently a variety of issues in MRSA detection, including the long duration, low detection rate, and sensitivity to the clinical sample quality, with the last being the most prominent challenge. In light of this, the development of a quick and effective MRSA detection method has been a focus of MRSA research.

Loop-mediated isothermal amplification (LAMP) boasts several advantages, including high specificity, high sensitivity, simple manipulation and suitability for high throughput sample examination[1]. The National Committee for Clinical Laboratory Standards (NCCLS) of United States proposed that MRSA can be defined as *S. aureus* isolates with positive detection of the *mecA* gene or penicillin-binding *protein* 2a (PBP2a) protein[2]. Staphylococcal protein A (spa) is a cell wall component of *S. aureus* and is species-specific [3]. This study employs LAMP to examine *mecA* and *spa* in clinical specimens and compares the results

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with those derived from the cefoxitin disk diffusion test, which is recommended by NCCLS. Our analyses indicate that the results are satisfactory, as reported in the following text.

MATERIALS AND METHODS

Materials, reagents and devices

Negative control group: *S. aureus* ATCC 25923 (Methicillin-sensitive) was purchased from the Institute of Microbiology, Heilongjiang Academy of Sciences. Positive control group: Methicillin-resistant *S. aureus* (MSA ATCC 43300) and quality control *E. coli* strain ATCC 25922 were purchased from Wenzhou Kont Biology & Technology., LTD. The test samples included 368 sputum specimen collected in the Jingmen Second People's Hospital between January 2012 and December 2013, including 89 samples from the Department of Neurosurgery of this hospital, 121 samples from the intensive care unit, 108 samples from the Department of Respiratory Medicine, 32 samples from the Department of Cardiology, and 32 samples from other departments. Bst DNA polymerase was purchased from New England Biolabs. DNA marker DL2000 and SYBR Green I were purchased from Tiangen Biotech (Beijing). The PCR amplifier (iCycler) and gel imaging system (DOS-2000) were purchased from Bio-Rad.

Culturing conditions

MRSA was cultured at 37°C on high-salt (6 mg/mL) mannitol agar plates supplemented with oxacillin for 48 h. Methicillin-sensitive *S. aureus* was cultured on mannitol salt agar plates.

Detection of *mecA* phenotype

Bacteria were streaked on blood agar three times to generate single colonies. Based on the NCCLS-recommended cefoxitin disk diffusion test, *S. aureus* can be identified as MRSA if the diameter of the inhibition zone is no greater than 19 mm. The minimum inhibitory concentration (MIC) of oxacillin was evaluated based on the agar dilution method.

DNA extraction

DNA was extracted from the cultured isolates using the pyrolysis method. Briefly, each isolate was inoculated into 10 mL of broth and incubated at 37°C overnight. Five microliters of bacterial broth was transferred into a 5-mL centrifuge tube and spun down at 10000 r/min for 5 min. The supernatant was discarded, and the pellet was supplemented with 5 mL of lysis buffer, which was incubated in a 100°C water bath for 10 min and centrifuged at 15000 r/min for 5 min. The resulting supernatant was used as a DNA template and frozen at -20°C. In addition, DNA was extracted the clinical sputum samples using the Sputum DNA Isolation Kit (ABgene, US) following the manufacturer's instructions.

PCR-based detection of *mecA* and *spa*

PCR amplification of *mecA* and *spa* was performed using previously published reaction conditions and primers[4,5]. The primer sequences were as follows: *mecA* forward primer (mA1): 5'-TGCTATCCACCCTCAAACAGG-3', *mecA* reverse primer (mA2): 5'-AACGTTGTAACCACCC C AA G A- 3', *spa* forward primer (1095F): 5'-GACGATCCTTCAGTGAGCAAAG- 3', and *spa* reverse primer (1517R): 5'-GCAGCAATTTTGTCAGCAGTA-3'. PCR products were electrophoresed on 2% agarose gel.

LAMP reaction

LAMP amplification of *mecA* and *spa* was performed using previously published reaction conditions and primers[6,7]. Each reaction contained 4 primers: a forward internal primer (FIP), a backward internal primer (BIP), and two external primers (F3 and B3) (Table 1). The LAMP reaction system had a volume of 25 µL and contained 40 pmol FIP, 40 pmol BIP, 5 pmol F3, 5 pmol B3, 2 µL template, 1 µL Bst DNA polymerase (8U), 1.6 mmol/L dNTPs, 0.8 mmol/L betaine, 4 mmol/L MgSO4, 20 mmol/L Tris-HCl (pH 8.8), 10 mmol/L KCl, and 10 mmol/L $(NH4)_2SO_4$, which were mixed well. The DNA was amplified for 60 min under 64°C isothermal conditions. Subsequently, the mixture was incubated at 80°C for 5 min to terminate the reaction.

Detection of LAMP products

0LAMP products were examined via visual examination or agarose gel electrophoresis. For the former approach, 1 µL of 10^{-1} SYBR Green I was added to the reaction mixture, and the color change was observed under natural light. For the latter, 2 µL of product was added and electrophoresed in a 2% agarose gel, which was incubated with 50 µg/mL ethidium bromide; the image was captured under a UV light (302 nm).

Examination of sputum specimens

0The LAMP protocol established by our group was employed to examine clinical sputum

samples, which were also subject to concurrent susceptibility test and PCR detection. The results of these approaches were compared, thereby determining the specificity and sensitivity of direct LAMP-based MRSA examination in sputum.

RESULTS

MRSA detection

The cefoxitin test revealed that the purchased *S. aureus* isolate had an average diameter of the inhibition zone of 14 mm (<19 mm), indicating that it was MRSA (Fig 1a). As a comparison, the average diameter of the quality control *E. coli* strain ATCC 25922 was 24 mm (Fig 1b) and that of MSSA was 29 mm (Fig 1c).

Specificity of the LAMP reaction

LAMP detection of *mecA* and *spa* was performed for the MRSA, MSSA and *E. coli* strains. DNA samples, prepared from 10^8 CFU/mL bacterial broth, were separated by agarose electrophoresis to reveal LAMP products. As shown in Fig 2, the LAMP assay successfully amplified *mecA* from the MRSA isolate but not from other strains. In addition, LAMP also successfully amplified *spa* from the MRSA and MSSA isolates. Moreover, these same DNA samples were also analyzed via conventional PCR to identify *mecA* and *spa*, and the results were consistent with that of LAMP (Fig 2).

Serial dilutions of the extracted MRSA (ATCC 43300) DNA were used as templates to determine the detection limit of LAMP, which was verified via electrophoresis and visual examination. As shown in Fig 3, the LAMP detection limit of *mecA* was below 10^2 CFU, and that of *spa* was below 10 CFU. The visual results are presented in Figure 4; when a DNA sample was higher than 10^3 CFU/mL, the LAMP reaction using SYBR Green I generated green products. On the contrary, when the bacterial number was below 10^2 CFU/mL, the initial orange color of the reaction mixture did not change after the amplification. A similar pattern was also found for the detection limit of *spa* when the bacterial concentration was 10 CFU/mL. Similar to the LAMP method, the detection limits of conventional PCR after pyrolytic extraction of bacterial DNA were 10^6 and10^7 CFU for *mecA* and *spa*, respectively.

LAMP detection of *S. aureus* in sputum samples

0The *mecA* and *spa* genes in the sputum samples were analyzed via LAMP. The results were also compared with those of conventional Kirby-Bauer testing as well as regular PCR. For the collected 368 clinical sputum samples, LAMP detected *spa* from 189 samples (51.4%) and *mecA* from 67 samples (18.2%). Moreover, LAMP and conventional PCR produced perfectly consistent results in the sputum examination .We also used one of the conventional methods, the Kirby-Bauer susceptibility test for MRSA, and compared the data with the LAMP detection data .

DISCUSSION

The rampant emergence of drug-resistant *S. aureus* isolates has become a global health concern. In particular, the rapid elevation of the MRSA proportion is generating significant challenges for clinical treatment. Hence, it is of vital importance to promptly diagnose, prevent and treat MRSA. Currently, the clinical detection methods for MRSA include cefoxitin disk diffusion and PCR. Traditional culturing of *S. aureus* requires a complicated protocol, is of long duration, and involves considerable operational experience that may skew the results. PCR and related methods, such as RT-PCR and quantitative PCR (qPCR), are short in duration and generate results rapidly, but they are rarely adopted in grass-root institutions due to the availability of the devices. In

Table 1. Primers of LAMP for mecA and spa

Target Gene	Primer	Sequence
mecA	F3	5'-aagatggcaaagatattcaact-3'
	B3	5'-aggttctttttatcttcggtta-3'
	FIP	5'-acctgtttgagggtggatagcatgatgcta aagttcaaaagagt-3'
	BIP	5'-gcacttgtaagcacaccttcacttcgttac tcatgccatac-3'
Spa	F3	5'-ggtgatacagtaaatgacattgc-3'
	B3	5'-acgctaatgataatccaccaa-3'
	FIP	5'-cttgaccaggtttgatcatgtttttactgct gacaaaattgctg-3'
	BIP	5'-aaccatgcagatgctaacaaagctacagtt gtaccgatgaatgg

comparison, the LAMP method employed in this study requires even simpler procedures than conventional PCR and does not require expensive equipment, as the reaction demands no temperature change; a simple water bath apparatus can accomplish the entire amplification process. Furthermore, the results are simple to interpret, which can be revealed by the generation of a magnesium pyrophosphate precipitate; alternatively, color change after the addition of the fluorescent dye SYBR green I can be directly observed with the naked eye. As a consequence, the temperature switch and electrophoresis observation, which are essential for conventional PCR, are eliminated to streamline the test time. In addition, the LAMP method has two internal primers as well as two external primers to recognize 6 different sequences, which results in higher specificity than PCR. Overall, LAMP can satisfy large-scale detection in a grass-roots scenario and overcomes the drawbacks of conventional PCR.

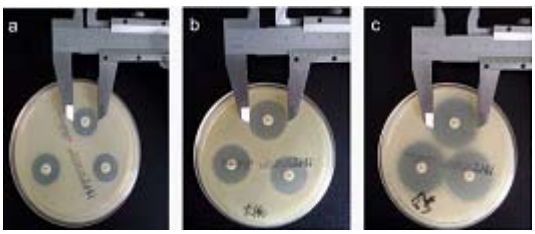

Fig. 1. Results of the cefoxitin test. a#, MRSA ATCC43300; b#, quality control *E. coli* AT CC25922; c#, ATCC25923

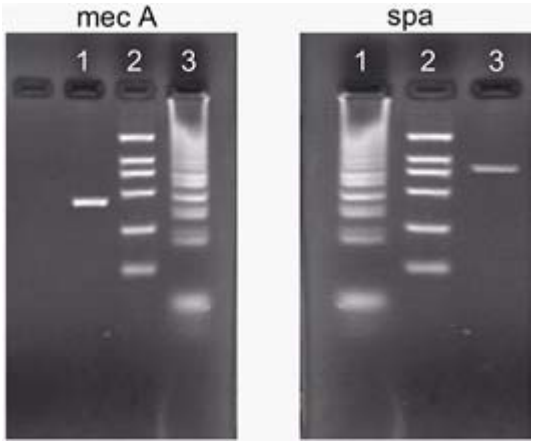

Fig. 2. Specificity of LAMP and PCR in the detection of *mecA* and *spa*. For the electrophoresis of *mecA:* 1#, PCR detection; 2#, MARK electrophoresis; and 3#, LAMP detection. For the electrophoresis of spa: 1#, LAMP detection; 2#, MARK electrophoresis; and 3#, PCR detection. From the top, the molecular weights of the bands in MARK were 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, and 600 bp, respectively

Using cultured bacteria and LAMP, the detection limits of *mecA* and *spa* are reported to be below 10^3 CFU and 10 CFU, respectively[8], as revealed by electrophoresis. In the same report, however, PCR was also employed to amplify bacterial DNA extracted via pyrolysis, which generated detection limits of 10^6 and 10^7 CFU for *mecA* and *spa*, respectively[8]. This clearly demonstrates that LAMP has a lower requirement for DNA purify, which is beneficial for clinical application. In this study, we used bacterial DNA extracted using a fast kit as the template when analyzing the clinical sputum samples. Although the quality of the DNA template was not as good as that used for PCR, LAMP nonetheless generated higher positive rates than PCR. Moreover, the LAMP reaction can be expedited by the addition of loop primers, which also boost sensitivity. As such, LAMP amplification exhibits tremendous advantages over conventional PCR.

In this study, we established a detection method to examine 368 clinical specimens and produced a positive detection rate of 18.2%, including three isolates of *mecA*-positive and drug-resistance negative samples. This phenomenon indicated that the drug resistance originating from the *mecA* expression of PBP2a is also regulated by other genes. In addition, one isolate was determined to be MRSA via conventional susceptibility test but shown to be *mecA* negative

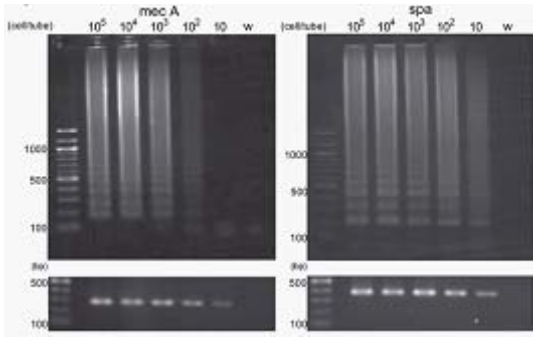

Fig. 3. The detection limits of LAMP for *mecA* and *spa*. From the left, the lanes correspond to bacterial concentrations of 10^5, 10^4, 10^3, 10^2, and 10 CFU/ml; the W lane represents the negative control

via LAMP. However, when the corresponding cultured broth was used, the isolate was found to be *mecA* positive. The discrepancy indicated that pyrolysis of *S. aureus* DNA may cause missed identification (false negative) for broth with low bacterial counts[9].

In summary, LAMP-based detection of *mecA* and *spa* can both be accomplished within one hour and boasts high specificity and sensitivity. In addition, because the results can be evaluated visually, LAMP exhibits the apparent advantages of being simple and fast. Hence, the LAMP detection method reported in this study has the potential be become a powerful tool to detect MRSA-associated *mecA* and *spa* genes.

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