

Fluorescent Detection of Methicillin Resistant *Staphylococcus aureus* by Loop-mediated Isothermal Amplification Assisted with Streptavidin-coated Quantum Dots

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Abstract

Background: Methicillin Resistance *Staphylococcus aureus* (MRSA) could be considered as a major concern in medicine can cause nosocomial infection and bacteremia, especially in patients using catheter and household medical devices.

Methods: Using molecular diagnostic methods are important for identification of MRSA from the Methicillin Sensitive *Staphylococcus aureus* (MSSA). Here we described a fluorescent assay using biotin-labelling Loop-mediated isothermal amplification (LAMP) method assisted with streptavidin-coated Quantum Dots (QDs) for detection of MRSA. For comparison, another fluorescent assay using LAMP assisted with Green Viewer (GV; a fluorescent dye) was applied for detection of MRSA. The *mecA* gene was selected as the target for amplification by LAMP and for biotin-labeling of the LAMP amplicons, biotin-11-dUTP was mixed with the dNTPs (deoxy Nucleotide Phosphates) in LAMP reaction. For determining the clinical performance of the developed assay, 30 blood samples with MRSA positive results were tested with QD-LAMP, the conventional LAMP, GV-LAMP, and Polymerase Chain Reaction (PCR).

Results: Obtained results indicated that % sensitivity of QD-LAMP was 86.66% for detection of *mecA* positive MRSA samples; however, the Limit of Detection (LoD) of QD-LAMP was 1.5×10^4 Colony Forming Unit (CFU).

Conclusion: The results suggested that the QD-LAMP assay was easy to operate and could be used for detection of MRSA in parallel to the blood culture with less sensitivity for detection of bacteremia and pediatric septicemia with low counts of MRSA.

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Introduction

Staphylococcus aureus (*S. aureus*) including Methicillin Resistance *S. aureus* (MRSA) is responsible for a wide range of serious diseases

such as skin infections osteomyelitis, endocarditis, pneumonia, toxic shock syndrome, scalded skin syndrome, and septicemia in humans^{1,2}.

MRSA, considered as a major concern in medicine, can cause nosocomial infection and bacteremia, especially in patients using catheter and household medical devices worldwide. Furthermore, it plays an important role in development of Multidrug Resistant (MDR) ^{1,3}.

The *mecA* gene is in charge of encoding an alternative Penicillin-Binding Protein, PBP 2a, and causing resistance to methicillin and all beta-lactam antibiotics by having low affinity to beta-lactam ring ^{1,4}. This gene along with its regulators are located on Staphylococcal Cassette Chromosome *mec* (SCC*mec*) ⁵.

Currently, *S. aureus* has commonly been diagnosed *via* routine standard procedures including colony morphology, Gram staining, differential tests (*e.g.* coagulase, catalase, DNase) and Polymerase Chain Reaction (PCR) ^{6,7}. These methods are time consuming and expensive; therefore, the tests cannot be used in the urgent and crucial situation ⁶. In addition, PCR method has been restricted by many extra problems, such as requiring special equipment, PCR thermocycler and expert personnel, operating space, complicated sample preparation procedure, cross-reaction of primers, self-inhibition because of dimers formation ^{8,9}. So, promotion of amplification and detection methods for diagnosing these bacterial agents is needed.

In the last decade, Loop-mediated isothermal amplification (LAMP) has been considered as a novel nucleic acid amplification method ¹⁰⁻¹⁴, and used to detect various micro-organisms, such as *Escherichia coli* ¹⁵, *Yersinia pseudotuberculosis* ¹⁶, *Salmonella* ¹⁷, *Vibrio parahaemolyticus* ^{18,19}, and MRSA ^{2,4,8,20,21}. This method is based on an auto-cycling strand displacement DNA synthesis performed by the Bst DNA polymerase large fragment which is extracted from *Bacillus stearothermophilus* ^{4,7}. The reaction happens under the isothermal conditions between 60-65°C in a single tube by a set of four to six specific designed primers *i.e.*, Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward outer Primer (F3), Backward outer Primer (B3), Loop primer F (Loop-F) and Loop primer B (Loop-B) ²²⁻²⁴. A dumbbell shape DNA is produced which acts as a template for cycle amplification with several inverted repeats of the target sequence and multi-

ple loops ^{7,14}. The superiorities of LAMP method compared to other techniques are simplicity, rapidity, specificity and sensitivity ^{7,8,14}. Indeed, because of the large number of DNA copies (to 10⁹) produced in LAMP reaction, the product can be determined *via* simple methods such as colorimetry and photometry ^{21,24}.

Quantum Dots (QDs) are one of the well-known fluorescent materials that have several photophysical properties including long life, stability, high quantum yield, high extinction coefficient, and small size; these properties make it superior to other fluorescent materials ²⁵. QDs are excited with a wide range of wavelengths, but emulate only wavelengths at a specific wavelength ²⁶. We hypothesized that the biotin-labeled LAMP amplicons can be detected with the streptavidin-coated QDs to diminish time of assay and result in increased sensitivity and raising limit of MRSA detection by isothermal LAMP method (Figure 1). To the best of our knowledge, there are no diagnostic studies regarding the usage of QD-LAMP in detection of MRSA. In the current study, a LAMP protocol has been developed, optimized, evaluated and applied to detect *mecA* gene of MRSA, requiring about 60 min for the whole process.

Materials and Methods

Bacterial collection and culture

Thirty bacterial blood samples were obtained from positive cultures of MRSA at three hospitals. Then, we mixed one loop of colonized bacteria on trypticase soy agar with 10 ml normal saline. This dilution is equal to 1 McFarland, which read absorption at wavelength of 625 nm between 0.08 to 0.13 ^{27,28}. Subsequently, 500 µl of this dilution with 1.5×10¹⁶ Colony Forming Unit (CFU) cell density was mixed with 500 µl blood containing Ethylene Diamine Tetra Acetic acid (EDTA). Final dilution was 0.5 McFarland with 1.5×10⁸ CFU cell density that we utilized as bacterial sample in our study. The samples were confirmed by standard biochemical tests ²⁹. In brief, all of them were cultured on blood and mannitol salt agars and incubated overnight at 37°C. Strains that showed β-hemolysis in blood agar and changed the color of culture medium to yellow were subjected to Coagulase test and Oxacillin disk diffusion test.

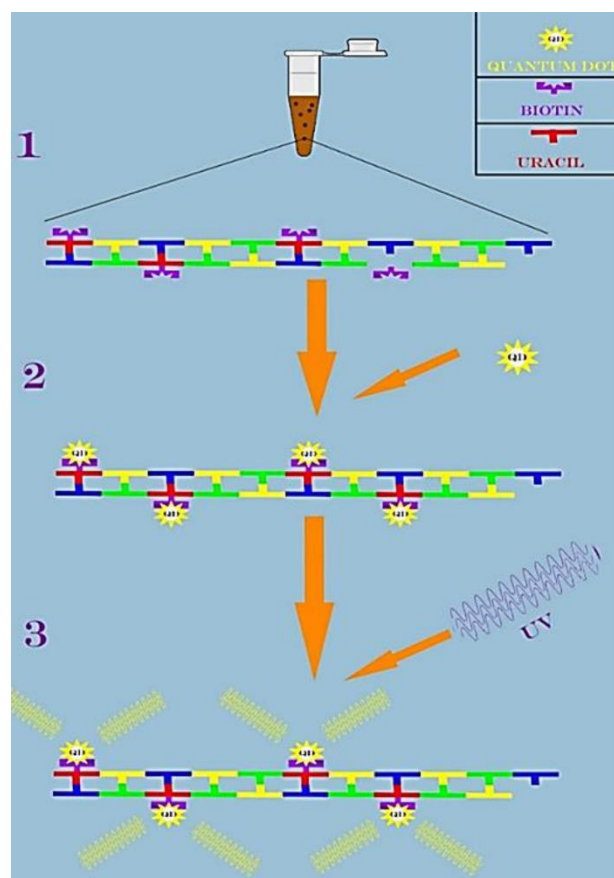


Figure 1. Schematic of QD-LAMP. 1) Entrance of biotin labeled nucleotides (biotin-11-dUTP) into DNA amplicons; 2) Binding streptavidin-coated quantum dots with the biotin-labeled LAMP amplicons; 3) UV radiation to LAMP products and the light-emission from QDs.

The sample was confirmed as *MRSA* strain if it was positive in the Coagulase test and had zone diameter of less 22 mm in Oxacillin disk diffusion test ²⁹.

Extraction of DNA template

DNA template from *MRSA* was obtained by QIAamp DNA mini kit produced by QIAGEN Company (Germany). Briefly 20 μ l proteinase K was pipetted into the 1.5 ml micro centrifuge tube. 200 μ l blood sample was added to the micro centrifuge tube. Then 200 μ l AL Buffer was added to the sample and mixed by pulse-vortexing for 15 s. For efficient lysis, the sample and Buffer AL were mixed completely to turn over a homogeneous solution. The tube was incubated at 56°C for 10 min. Afterward the tube was briefly centrifuged to remove drops from the inside of the lid. Next 200 μ l ethanol (96-100%) was added to the tube, and mixed again by pulse-vortexing for 15 s.

Then the mixture was applied from previous step to the QIAamp mini spin column (in a 2 ml collection tube) and centrifuged at 6000 \times g for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was removed. Next the QIAamp mini spin column was opened and add 500 μ l buffer AW1 and centrifuged again at 6000 \times g for 1 min. The QIAamp mini spin column was put in a clean tube, and the tube containing the filtrate was removed. Afterward, 500 μ l buffer AW2 was added and centrifuged at full speed (20,000 \times g) for 3 min. helping to remove buffer AW2 completely, the previous step was repeated. Again the QIAamp mini spin column was placed in a new 1.5 ml microcentrifuge tube and 200 μ l buffer AE was added and Incubated at room temperature (15-25°C) for 1 min, then centrifuged at 6000 \times g for 1 min. DNA was filtrated from the column and

placed in 1.5 ml tube³⁰.

Primer design of the *mecA* gene to LAMP assay

The *mecA* gene was selected for this study. Two pairs of primers, including F3 (5'- AGTTC TTTAGCGATTGCTTTA -3'), B3 (5'- TGGA AGTTAGATTGGGATCA -3'), FIP (5'- AATG TGGAAATTGGCCAATACAGGATTTAGATAC ATTCTTTGGAACGATG-3'), and BIP (5'- CG GTCTAAAATTTTACCACGTTCTGCGTCATT ATTCCAGGAATGCA-3'), were designed (Figure 2) according to the accession number NZ_CP010300.1 (i.e., NCBI Reference Sequence) by using online Primer Explorer V4 program (Primer Explorer, Eiken Chemical Co., Japan). The LAMP primers for amplification were synthesized by Bioneer Co. Ltd. (South Korea).

Optimization of the biotin-labelling LAMP

Optimization of the biotin-labelling LAMP was performed on a reference MRSA strain (ATCC 33591). A well-characterized strain of MRSA (ATCC 33591) and Methicillin Sensitive *Staphylococcus aureus* (MSSA) (ATCC 12600) was prepared from Iranian Biological Resource Center. Briefly, 20 μ l of the reaction mixture containing 1.6 μ M of each FIB and BIP primer, 0.2 μ M of each F3 and B3 primer, 2 μ l reaction buffer (10 \times), 0.8 M of betaine solution, 8 mM of MgSO₄, 1.4 mM of deoxy Nucleotide Phosphate (dNTP) mix content of dATP, dCTP, dGTP,

0.52 mM dTTP and 0.88 mM biotin-11-dUTP, 8 units of *Bst* DNA polymerase, 50 ng of DNA template up to 20 μ l dH₂O was used. The reaction mixture was incubated at 60 °C for 60 min, followed by the enzyme inactivation at 80 °C for 5 min. Negative control (with MSSA template DNA) and blank control (with no template DNA) were included in every reaction set.

Determining the LAMP products by quantum dot (QD) and green viewer (GV)

The biotin-labelled LAMP products were aliquot to two tubes (9 μ l in each tube), 1 μ l streptavidin-coated QD was added to the first tube and 1 μ l GV (a florescent dye purchased from Pars Tous Company, Mashhad, Iran) was added to the second tube. Afterwards, UV was brought to the tubes for 45 s in 25°C by the photo-optic system of Rotor-Gene-Q device (QIAGEN, Germany). In addition, the LAMP products were tested by 1% agarose gel electrophoresis.

PCR detection of *mecA* gene

In parallel, PCR targeting *mecA* gene was performed on 30 MRSA samples have been previously diagnosed by culture methods. PCR was carried out in a 20 μ l volume containing 10 μ l of 2 \times -PCR-Mix (Emerald Amp PCR Mix, Takara Bioscience Ltd., Japan), 1 μ l of F3 primer (0.2 μ M), 1 μ l of B3 primer (0.2 μ M), 7 μ l of deionized water and 50 ng of DNA template.



Figure 2. The sequences used for LAMP primers on the sequence with the accession number NZ_CP010300.1 from *Staphylococcus aureus* strain 27b_MRSA, complete genome; F3 primer, 1439-1459 nt; B3, 1622-1641 nt; FIP primer, 1468-1492; BIP primer, 1598-1619 nt.

Table 1. The results for PCR, conventional LAMP, GV-LAMP and QD-LAMP for 30 *MRSA*-positive blood cultures

Test	PCR		Conventional-LAMP		GV-LAMP		QD-LAMP	
Result	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	28	2	29	1	30	0	26	4
Numbers of samples	30		30		30		30	

The PCR program was 98°C for 10 s, 50°C for 30 s, 72°C for 1 min and repeated for 30 cycles. Following PCR, 10 μ l of each sample was subjected to electrophoresis on 1% agarose gel for 45 min.

Determination of the limit of detection (LoD) in LAMP and PCR

The sensitivity of LAMP and PCR assays was checked by using a serial dilution of *MRSA* DNA template. To determine LoD in LAMP and PCR assays, one loop of *MRSA* (ATCC 33591) cultured on tryptic soy agar media (specific media for *S. aureus*) was mixed with sterile normal saline (equivalent of 1 McFarland by reading the absorbance unit in 625 nm between 0.08-0.13) and serially diluted in 10 tubes with blood from 10^{-1} to 10^{-8} concentrations. 50 μ l of 1 McFarland *MRSA* concentration was spiked into 450 μ l of blood sample and diluted serially to 9 tubes contained 450 μ l of blood. The blood sample used here was obtained from a healthy donor with no infectious diseases. The blood samples were stored at 4 °C in K2-EDTA anti coagulated blood tube. Genomic DNA from each dilution was extracted by QIAamp mini kit and used as a DNA template for the LAMP and PCR.

Results

LAMP and PCR detections for *mecA* gene

All thirty *MRSA* samples were tested for *mecA* gene by PCR, conventional LAMP, GV-LAMP and QD-LAMP (Table 1). The GV-LAMP had higher sensitivity than other PCR-based tests, thus provided minimum false-negative predictions. The sensitivity of the performed PCRs, conventional LAMP, GV-LAMP and QD-LAMP on all positive *MRSA* samples were 93.33, 96.66, 100 and 86.66%, respectively.

LoD of LAMP assays (conventional LAMP, GV-LAMP and QD-LAMP) and PCR

The LoD for the PCR was 5×10^{-2} (equivalent of 1.5×10^7 number of bacteria). The LoD for the conventional LAMP (conventionally detected with gel electrophoresis) was 5×10^{-3} (equivalent of 1.5×10^6 number of bacteria) (Figure 3). The LoD for the GV-LAMP was 5×10^{-4} (equivalent of 1.5×10^5 number of bacteria) and the LoD for the QD-LAMP was 5×10^{-5} (equivalent of 1.5×10^4 number of bacteria) (Figure 4). LoDs obtained from conventional LAMP, GV-LAMP, QD-LAMP, and PCR assays showed that the PCR had the lowest LoD and QD-LAMP had the best LoD among the tests (Table 2).

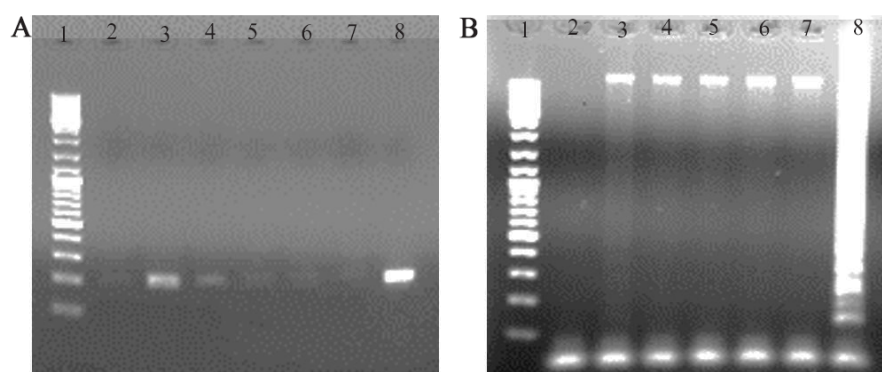


Figure 3. Gel electrophoreses of amplified *mecA* gene DNAs of 1.5×10^2 CFU (Lane 2), 1.5×10^3 CFU (Lane 3), 1.5×10^4 CFU (Lane 4), 1.5×10^5 CFU (Lane 5), 1.5×10^6 CFU (Lane 6), 1.5×10^7 CFU (Lane 7), and 1.5×10^8 CFU (lane 8) bacteria by PCR (A) and conventional LAMP (B); Lane 1, GeneRuler (100-10000 bp, Thermo Scientific, USA).

Quantum Dot-LAMP for Fluorescent Detection of MRSA

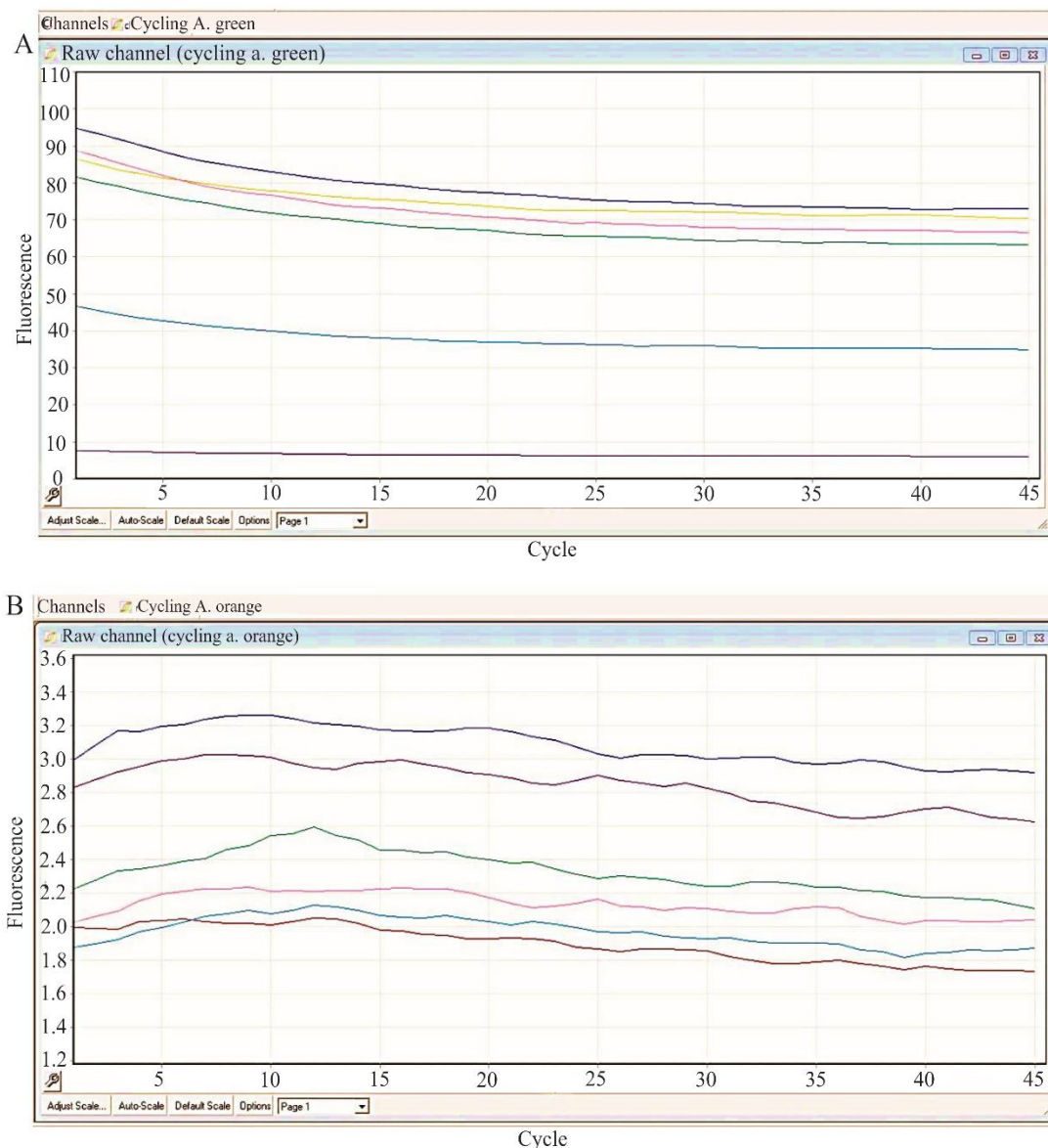


Figure 4. Fluorograms of amplified *mecA* gene DNAs (top to down series) of 1.5×10^8 CFU, 1.5×10^7 CFU, 1.5×10^6 CFU, 1.5×10^5 CFU, 1.5×10^4 CFU, and 1.5×10^3 CFU bacteria by GV-LAMP (A) and QD-LAMP (B).

Discussion

The results of our study showed that QD-LAMP had higher sensitivity for detection of MRSA. In this study, LAMP test was used for the simple and rapid detection of *S. aureus* directly from septicemia blood samples with no need for complicated devices such as thermocycler and gel documentation system. LAMP occurred in a single tube under isothermal condition by two pairs of designed primers. While the reactions was done, added GV and QDs to these products contained biotin-11-dUTP which could

give the ability to the products to distribute the light after UV radiation. In our study, the LAMP tests determined the better LoD when compared to the PCR. The LoD of the QD-LAMP test was respectively higher than PCR (5×10^{-5} vs. 5×10^{-2}). In the current study, utilizing biotin-11-dUTP caused higher affinity compared to QDs coated with streptavidin; hence, the LAMP amplicons produced a stronger light emission and thus allowed the reaction to be read in a real-time format.

However, Su *et al* showed that the $25 \mu\text{l}$ reac-

Table 2. The comparison of LoDs obtained *via* conventional LAMP, GV-LAMP, QD-LAMP and PCR based on CFU unit

Assay	Concentration				
	5×10 ⁻¹ McFarland (1.5×10 ⁸ CFU)	5×10 ⁻² (1.5×10 ⁷ CFU)	5×10 ⁻³ (1.5×10 ⁶ CFU)	5×10 ⁻⁴ (1.5×10 ⁵ CFU)	5×10 ⁻⁵ (1.5×10 ⁴ CFU)
PCR	Positive	Positive	Negative	Negative	Negative
LAMP*	Positive	Positive	Positive	Negative	Negative
GV-LAMP	Positive	Positive	Positive	Positive	Negative
QD-LAMP	Positive	Positive	Positive	Positive	Positive

* Conventional LAMP

tant was found to be the most cost-efficient volume, and the detection limit was determined to be 10 DNA copies and 10 CFU/reaction. High specificity was observed when orfX-LAMP assay was subjected to 116 reference strains. For application, 557 (98.4%, 557/566) and 519 (91.7%, 519/566) tested strains had been detected positive by LAMP and PCR assays⁴. Also, Lim *et al*, showed that both LAMP and PCR assays had 100% specificity and 100% sensitivity, when tested on 30 spiked blood specimens (21 *MRSA*, eight non-*S. aureus* and one negative control). Their findings indicated that the LAMP assay was equally specific with a shorter detection time when compared to PCR in the identification of *S. aureus*⁸. In another study, out of 60 blood cultures positive for Gram-positive cocci in clusters, LAMP (*via* detection of the *FemA* and *MecA* genes) showed 100% sensitivity and specificity for identification of *MRSA/MSSA*. When coagulase negative staphylococci were tested, sensitivity for detection of methicillin resistance was 91.7% and specificity was 100%⁷. However, Goldmeyer *et al*, indicated that the LAMP amplicons could be detected using a disposable detection device. Moreover, the analytical sensitivity of the assay was 50 CFU per reaction, and the clinical sensitivity and specificity were both 100% for *S. aureus* detection and 100 and 98% for methicillin resistance determination, respectively³¹.

In this study, the whole process of GV-LAMP and QD-LAMP tests from the extraction of DNA samples to endpoint detection just needed 2 *hr* whereas the PCR assay needed 4 to 5 *hr* from DNA extraction to the visualization of the products using gel electrophoresis sys-

tem. However, Metwally *et al*, designed LAMP reaction for *MRSA* from the blood culture bottles in 120 *min* (60 *min* for preparation and 60 *min* for LAMP reaction), in addition to 50 *min* to extract DNA⁷. On the other hand, Sudharan *et al*, designed LAMP assay using phenotypic tests and conventional PCR for detection of *nuc* and *mecA* genes among clinical isolates of *Staphylococcus*.

In this study, after reaction, a DNA intercalating dye such as picogreen, SYBR green or propidium iodide made better visibility for results and detection was completely finished by naked eye². Also, Goldmeyer *et al*, designed a simple, rapid, and user-friendly procedure to identify *S. aureus* and determined its methicillin resistance directly from gram-positive cocci in cluster-containing blood culture medium. The samples were diluted and heated prior to amplification of the *nuc* and *mecA* genes with isothermal helicase-dependent amplification³¹.

Conclusion

In the past decade, LAMP has become the most valuable technology to identify microorganisms such as bacteria^{32,33}. LAMP reaction happens in a single tube to amplify the DNA under constant temperature³⁴. This characteristic of LAMP method in general eliminate the need for thermal cyclers^{13,35}. These rapid and simple features have the advantage of this method on PCR³⁶. Therefore, LAMP method has always been considered by clinicians for early diagnosis of infectious diseases and simple screening at the point of care^{33,37}.

This method can be very useful for low-income countries to diagnose infectious diseases.

es^{14,38}. Our results using GV-LAMP and QD-LAMP also demonstrated that LAMP in the real-time formats could be done with acceptable LoD for *MRSA*- positive blood samples particularly from the pediatrics with low quantity; however, we concluded that the QD-LAMP assay was very easy to operate and does not require complex devices when compared to PCR instrument.

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Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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